

AMERICAN FERN JOURNAL

Volume 102

Number 1

January–March 2012

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

- The Structure of Petioles in *Pteris* (Pteridaceae)
Olga G. Martínez and Ignacio Vilte 1
- The Role of Aquaporins in Water Balance in *Cheilanthes lanosa* (Adiantaceae) Gametophytes
Hope L. Diamond, Heather R. Jones, and Lucinda J. Swatzell 11
- The Effects of Exogenous Cytokinin on the Morphology and Gender Expression of *Osmunda regalis* Gametophytes
Gary K. Greer, Margaret A. Dietrich, Joseph A. DeVol, and April Rebert 32
- Optimization of Protocol for Isolation of Genomic DNA from Leaves of *Selaginella* Species Suitable for RAPD Analysis and Study of their Genetic Variation
Sayantani Das, Maumita Bandyopadhyay, and Subir Bera 47
- Molecular Evidence on the Origin of *Osmunda ×mildei* (Osmundaceae)
C. Tsutsumi, Y. Hirayama, M. Kato, Y. Yatabe-Kakugawa, and S.-Z. Zhang 55
- The Tree Fern Highland Lace is a Cultivar of *Sphaeropteris cooperi*
Daniel G. Yansura and Barbara J. Hoshizaki 69
- Elaphoglossum montanum*, a New Species from Southern Brazil
Maria A. Kieling-Rubio and Paulo G. Windisch 78
- SHORTER NOTES
- First Record of *Pellaea ovata* (Pteridaceae) from Brazil
Leonardo Biral and Jefferson Prado 83
- Botrychium simplex* E. Hitchcock: a New Moonwort for the Indian Himalayan Mountains
B. S. Kholia 86

The American Fern Society

Council for 2012

MICHAEL WINDHAM, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President</i>
KATHLEEN PRYER, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President-Elect</i>
MARY STENSVOLD, USDA Forest Service, 204 Siginaka Way, Sitka, AK 99835.	<i>Secretary</i>
JAMES D. CAPONETTI, Div. of Biology, University of Tennessee, Knoxville, TN 37996-0830.	<i>Treasurer</i>
GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.	<i>Membership Secretary</i>
JAMES D. MONTGOMERY, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.	<i>Curator of Publications</i>
JENNIFER M. O. GEIGER, Dept. of Natural Sciences, Carroll College, Helena, MT 59625.	<i>Journal Editor</i>
R. JAMES HICKEY, Dept. of Botany, Miami University, Oxford, OH 45056.	<i>Memoir Editor</i>
JOAN N. E. HUDSON, Dept. of Biological Science, Sam Houston State University, Huntsville, TX 77341-2116.	
DAVID SCHWARTZ, 9715 Christey Way, Bakersfield, CA 93312-5617.	<i>Bulletin Editors</i>

American Fern Journal

EDITOR

JENNIFER M. O. GEIGER Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-4461, e-mail: jgeiger@carroll.edu

MANAGING EDITOR

JILL ANNE DILL Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-5176, e-mail: jdill@carroll.edu

ASSOCIATE EDITORS

NAN CRYSTAL ARENS Dept. of Geoscience, Hobart & William Smith Colleges, Geneva, NY 14456	
JOSE MARÍA GABRIEL Y GALÁN Dept. de Biología Vegetal I, Universidad Complutense de Madrid, Madrid, Spain 28040	
GERALD J. GASTONY Dept. of Biology, Indiana University, Bloomington, IN 47405-6801	
GARY K. GREER Biology Dept., Grand Valley State University, Allendale, MI 49401	
CHRISTOPHER H. HAUFLER Dept. of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045-2106	
R. JAMES HICKEY Dept. of Botany, Miami University, Oxford, OH 45056	
PAULO LABIAK Universidade Federal do Paraná, Dept. de Botânica, 81531-980, Curitiba-PR, Brasil	
ROBBIN C. MORAN New York Botanical Garden, Bronx, NY 10458-5126	
NATHALIE NAGALINGUM Royal Botanical Garden Sydney, Sydney, NSW, Australia	
CARL ROTHFELS Botany Dept., Duke University, Durham, NC 27708	
EMILY SESSA Botany Dept., University of Wisconsin-Madison, Madison, WI 53706	
MICHAEL SUNDUE Pringle Herbarium, The University of Vermont, Burlington, VT 05405	
JAMES E. WATKINS, JR. Harvard University, Cambridge, MA 02138	

The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, % Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

Claims for missing issues, made 6 months (domestic) to 12 months (foreign) after the date of issue, and orders for back issues should be addressed to Dr. James D. Montgomery, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.

Changes of address, dues, and applications for membership should be sent to the Membership Secretary.

General inquiries concerning ferns should be addressed to the Secretary.

Back volumes are available for most years as printed issues or on microfiche. Please contact the Back Issues Curator for prices and availability.

Subscriptions:

Society Membership – USA, Canada, Mexico (includes Journal and Fiddlehead Forum)	\$25
Society Membership – All other countries (includes Journal and Fiddlehead Forum)	\$32
Society Life Membership – \$300 (add \$140 mailing surcharge for outside USA, Canada, Mexico)	
Regular Membership – USA, Canada, Mexico (includes Fiddlehead Forum)	\$12
Regular Membership – All other countries (includes Fiddlehead Forum)	\$19
Institutional Membership – \$35 to USA, Canada, Mexico; \$45 elsewhere (-\$2 agency fee)	

POSTMASTER: Send address changes to American Fern Journal, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

The Structure of Petioles in *Pteris* (Pteridaceae)

OLGA G. MARTÍNEZ and IGNACIO VILTE

IBIGEO, Herbario MCNS, Fac. Cs. Naturales, Universidad Nacional de Salta, Av. Bolivia 5150, 4400-Salta, Argentina, e-mail: martinog@unsa.edu.ar

ABSTRACT.—We studied the petiole structure of twelve American species of *Pteris*: *P. ciliaris*, *P. cretica*, *P. deflexa*, *P. denticulata*, *P. ensiformis*, *P. exigua*, *P. inermis*, *P. multifida*, *P. mutilata*, *P. quadriaurita*, *P. tristicula* and *P. vittata*, using both light and scanning electron microscopy. We found that petioles are characterized externally by the presence of an adaxial groove and two dorsal-lateral ventilation areas. Internally, petioles are monostelic V-, U- or inverted- Ω -shaped axes. The vascular system is surrounded by one endodermis cell layer and 1–3 pericycle strata. We propose a classification of the vascular bundles into four types considering their shape, the shape of the xylem ends, the number of protoxylem zones and the presence of parenchyma bands in the xylem.

KEY WORDS.—*Pteris*, vascular bundles, petioles, ventilation areas

The paraphyletic genus *Pteris* (Schuettpelz and Pryer, 2007; Prado *et al.*, 2007) includes about 200 mainly pantropical species (Prado and Windisch, 2000). In the American continent there are about 60 species (Tryon and Tryon, 1982). In this paper we report the structure of vascular bundles of the petioles of twelve *Pteris* species, native or naturalized in the Americas: *P. ciliaris* D.C. Eaton, *P. cretica* L., *P. deflexa* Link, *P. denticulata* Sw., *P. ensiformis* Burm.f., *P. exigua* O.G. Martínez and J. Prado (Martínez and Prado, 2011), *P. inermis* (Rosenst.) de la Sota, *P. multifida* Poir., *P. mutilata* L., *P. quadriaurita* Retz., *P. tristicula* Raddi and *P. vittata* L.

Many authors consider the structure of vascular bundles significant in identifying different taxonomic groups, including Ogura (1972), White (1974), Lin and De Vol (1977, 1978), Graçano *et al.* (2001), Hernández *et al.* (2006), Hernández-Hernández *et al.* (2007), Srivastava (2008a, 2008b), among others. Ogura (1972) describe the structure of petioles in *Pteris* as being made only by one stele along their entire course, or several at the base that at some point meet. Lin and De Vol (1977) present a key to Taiwan ferns on the basis of petiole structure, and they provide diagnostic value to the number and shape of vascular bundles, sclerenchyma distribution, presence and number of adaxial grooves, ventilation areas and indument. Lin and De Vol (1978) describe the petiole structure of nine *Pteris* species (including *P. multifida*) and recognize “V”, “U” or “ Ω ”-shaped vascular bundles. Graçano *et al.* (2001) indicate that the petiole vascular bundles of *P. denticulata* and *P. leptophylla* Sw. are “U”-shaped, and assume an inverted Ω shape in *P. propinqua* J. Agardh. Bondada *et al.* (2006) report that the petiole in *P. vittata* has a “U”-shaped vascular bundle. In all cases, the xylem is described with the ends folded of the “hippocampus” type.

There have been no prior anatomical studies of most of the species treated in this work, so we expect our results will add to the anatomical and phylogenetic knowledge about this genus.

MATERIALS AND METHODS

The studies were conducted on herbarium material provided by the following institutions: BA, BM, CTES, G, K, LP, LPB, MCNS, NY, P, S, SI, SP, UNR, US and Z. Living specimens were collected in Argentina and were deposited in the MCNS Herbarium.

For anatomical studies, we considered three areas of the petiole: basal (next to the rhizome), middle (half of the petiole) and distal (next to the blade). Histological sections were made with a rotary microtome and freehand, stained with Safranin-Fast Green and mounted in Canada balsam. The histochemical tests used to detect cutin and suberin, lignin and tannins were Sudan III and fluoroglucine Fe_3Cl with CaCO_3 , respectively (D'Ambrogio Argüeso, 1986). To observe xylem we macerated using the Jeffrey technique (Jeffrey, 1917).

Observations, illustrations and photographs were done with a light Zeiss Standard 16 microscope and a scanning electron microscope of the JEOL JSM 6480LL model, belonging to the Universidad Nacional de Salta (Argentina). Samples for SEM observations were subjected to an increasing alcohol series, and then dried to critical point with CO_2 . The metallization was carried out with a thin gold-palladium coating. For the schemes, the conducting tissues are represented by Metcalfe and Chalk (1950), as suggested by Martínez (2003). Illustrations were made with the aid of a camera lucida.

MATERIAL STUDIED. *P. ciliaris*.—CUBA. East: without locality, 150 m, 9/VI/1915, Ekman 5364 (S); Santiago de Cuba: 1843-4, Linden 1924 (P). HAITI. South: Jérémie, Massif de la Hotte, western group, 800 m, 22/VII/1948, Ekman 10403 (G); Idem, 700 m, 25/XII/1926, Ekman 7415 (BM).

P. cretica.—ARGENTINA. **Salta**: Dpto. Capital, Quebrada de San Lorenzo, 1500 m, 10/III/2002, Martínez 913 (MCNS). MEXICO. **Distrito Federal**: Cañada de Contreras, 2800 m, 30/IV/1966, Rzedowski 22 217 (LP). PERU. **Amazonas**: Chachapoyas, 5/VI/1962, Wurdack 776 (K).

P. deflexa.—ARGENTINA. **Jujuy**: Dpto. Manuel Belgrano, camino a Tiraxi, 9 km of RN 9, 10/XII/1998, Morrone 3202 (CTES). **Salta**: Dpto. Santa Victoria, Parque Nacional Baritú, Abra de Minas, S 22° 27.4' W64°44.4', 1400 m, 10/VIII/2009, Martínez *et al.* 1831 (MCNS). **Tucumán**: Dpto. Monteros, Qda. del Portugués, 1550 m, Martínez 703, 30/XI/1999 (MCNS).

P. denticulata.—ARGENTINA. **Jujuy**: Dpto. Ledesma, Parque Nacional Calilegua, camino a Valle Grande, 20/IV/2002, Martínez 914 (MCNS). **Misiones**: Dpto. Candelaria, Candelaria, 8/IX/1993, Arbo *et al.* 6007 (LP). BOLIVIA. **Santa Cruz**: Valle Grande, S18°48' W63°49', 1000 m, 24/V/1996, Kessler *et al.* 6043 (LPB); CUBA. **Oriente**: Pinar de Yigüe, 500 m. 29/III/1915, Ekman 5138 (S).

P. ensiformis.—UNITED STATES OF AMERICA. **California**: Los Angeles, 20/XI/2002, Hoshizaky s.n. (MCNS 1814). JAPAN. **Hong Kong**: Tai Po Kau Forest Reserve, 21/XI/1980, Kramer *et al.* 8225 (Z). TAIWAN. **Satun**, E100°10' N 6°42', 100 m, 27/VIII/1995, Larsen *et al.* 46081 (NY).

P. exigua.—ARGENTINA. **Jujuy**: Dpto. Ledesma, camino Calilegua-San Francisco, 17/III/2009, Martínez y Chambi 1802 (MCNS), **Salta**: Dpto. Capital,

Quebrada Los Berros, 1500 m, 11/IV/2002, Martínez 881 (MCNS), **Tucumán:** Dpto. Burreyacu, Cerro Nogalito, 12/VI/1929, Venturi 8879 (SI).

P. inermis.—ARGENTINA. **Jujuy:** Dpto. Ledesma, camino a Valle Grande, 22/II/1972, Cabrera *et al.* 22384 (LP), Parque Nacional Calilegua, RP 83 S 23°41,9' W 64° 52,8', 1365 m, 17/III/2009, Martínez y Chambi 1778 (MCNS). **Salta:** Dpto. Capital, Quebrada de San Lorenzo, S 24°43.1 'W 65°30.5', 10/VII/2010, Martínez y Chambi 1919 (MCNS).

P. multifida.—ARGENTINA. **Chaco:** Dpto. Primero de Mayo: Colonia Benítez, IV/1983, Romero 1 (LP). BOLIVIA. **Santa Cruz de la Sierra:** Biocentro Gembé, 400 m, 02/IV/2010, Martínez 1872 (MCNS). CUBA. **La Habana:** Guanabacoa 7/XII/1921, Ekman 13543 (S).

P. mutilata.—CUBA. La Habana: Lomas de la Jaula, 11/VI/1914, Ekman 1313 (S). HAITI. West: Gonave Island, VIII/1927, Eyerdam 247 (CA). PUERTO RICO. Rio Abajo State Forest, N 18°20,3' W 66°42,0', 4/VI/1999. Acevedo-Rodriguez 10657 (US).

P. quadriaurita.—ARGENTINA. **Jujuy:** Dpto. Ledesma, Parque Nacional Calilegua, ruta provincial 83, Mesada Las Colmenas, Martínez y Guerra 1993, 24/VIII/2010 (MCNS). **Salta:** Dpto. Orán, Los Naranjos, a orillas del río San Andrés, 1150 m, Martínez y Prada 1746, 18/06/2008 (MCNS); Rio Pescado, 8 km de Arazayal, 25/X/1970, Vervoorst y Cuezco 7804 (BA).

P. tristicula.—ARGENTINA. **Corrientes:** Ituzaingó, Arroyo Garapé y río Paraná, 45 km E de Ituzaingó, Tressens *et al.* 408, 23–24/10/1974 (LP). **Salta:** Dpto. Orán, Río Blanquito a los Naranjos, S 23°41.7' W 64°51.9', 610 m, Martínez y Prada 1697, 17/VI/2008 (MCNS). BRAZIL. Rio de Janeiro, São Carlos, Fazenda Canchim, ca. 8 km. NE of São Carlos, 22/VI/1961, Eiten *et al.* 3182 (SP).

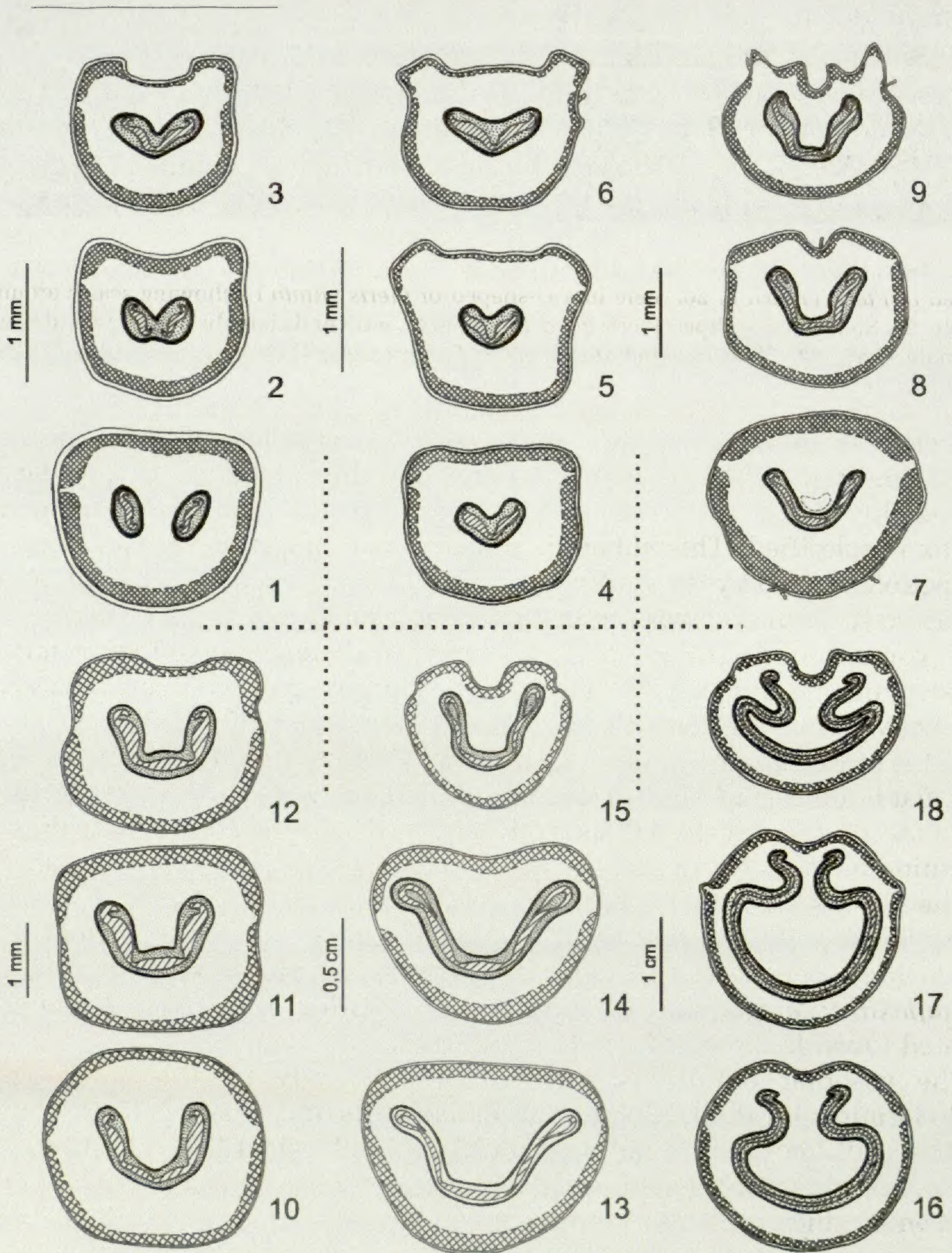
P. vittata.—ARGENTINA. **Entre Ríos:** Dpto. Rosario del Talar, Acebal, Sorarú 39, 31/III/1963 (LP). **Jujuy:** Dpto. Ledesma, Libertador General San Martín, 600 m, Martínez 1399, 13/V/2007 (MCNS). **Santa Fe:** Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Lewis 1585, 25/III/1983 (UNR 4624).

RESULTS

Externally, the petioles are green, yellow with macules or brown, always with a dark base. Their length varies between 1/2–2/3 of the total length of the fronds. The petioles of the fertile fronds are longer than those of the sterile fronds. The diameter in the middle ranges from 1 mm in *P. ensiformis* up to 20 mm in *P. deflexa*.

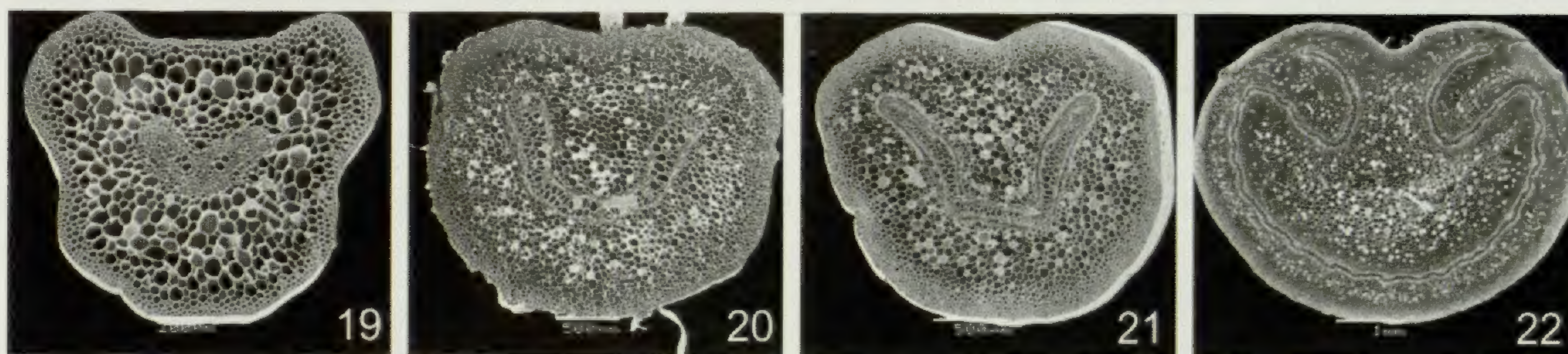
In cross section, petioles (Figs. 1–18) have bases with triangular or tetragonal boundaries (Figs. 1, 4, 7, 10, 13, 16), and subcircular middle (Figs. 2, 5, 8, 11, 14, 17) to upper sections (Figs. 3, 6, 9, 12, 15, 18) with a simple groove on the adaxial side. This groove runs lengthwise over the entire axis including the rachis, with a considerable depth from the lower basal third, and, as in the base, it is usually slightly concave. Two continuous white lines parallel to this groove, one on each side, are ventilation areas.

The indumentum of petioles consists of scales and trichomes. Scales are common in the base, similar to the rhizomatic structure; they are basifixed,



FIGS. 1-18. Petioles cross-section. 1-3. *Pteris cretica* L. 4-6. *Pteris ensiformis* Burm.f., 7-9. *Pteris denticulata* Sw. 10-12. *Pteris vittata* L., 13-15. *Pteris quadriaurita* Retz., 16-18. *Pteris deflexa* Link. 1, 4, 7, 10, 13, 16. Basal section, close to the rhizome. 2, 5, 8, 11, 14, 17. Middle section of petioles. 3, 6, 9, 12, 16, 18. Upper section, near the blade. grid = sclerenchyma, thick line = endodermis + pericycle, dotted = phloem, parallel lines = xylem.

brown, deltoid, subulate, linear-lanceolate, sub-opaque, colored in all taxa except *P. ciliaris*, which presents discolored scales and a sclerotic middle area of more intense color. Trichomes are simple, translucent to whitish, 2-4-cell, except *P. multifida* with 5-9-cell, and usually deciduous; thus the petioles of mature plants are glabrous except in the region of the longitudinal groove.



FIGS. 19–22. SEM photograph of monostelic petioles, in the middle of the shaft. **19.** Stele in V-shaped of *Pteris cretica* L. **20.** Stele in a U-shaped of *Pteris vittata* L. showing scales around the petiole. **21.** Stele in a U-shaped *Pteris quadriaurita* Retz. with ends largely elongated and bent over the main shaft. **22.** Stele inverted Ω -shaped of *Pteris exigua* O.G. Martínez and J. Prado with trichomes in the longitudinal groove.

In cross section (Fig. 19–22), petioles show a cortex formed -from the outside in- of a monostratified epidermis covered by a thick cuticle, and 2–18 layers of sclerenchyma and parenchyma (Fig. 23). In large plants as *P. deflexa* the whole cortex is sclerified. This subepidermal supporting tissue has two gaps along the petiole formed by the ventilation areas. The presence of tannins is common in the cortex, which gives the mature petioles a brown to dark color.

In young and fragile petioles, of 1 mm in diameter, ventilation areas are substomatal chambers, while in the robust petioles these areas are composed of parenchymatous tissue, with few intercellular spaces (Fig. 24).

In the medulla, depending on the cross section area, there are one or two vascular bundles. In *P. cretica* and *P. multifida*, there are two bundles on the base that fuse in the lower third of the petiole by the V-shaped abaxial ends (Fig. 1–3). The nine remaining species have monostelic petioles along the entire shaft.

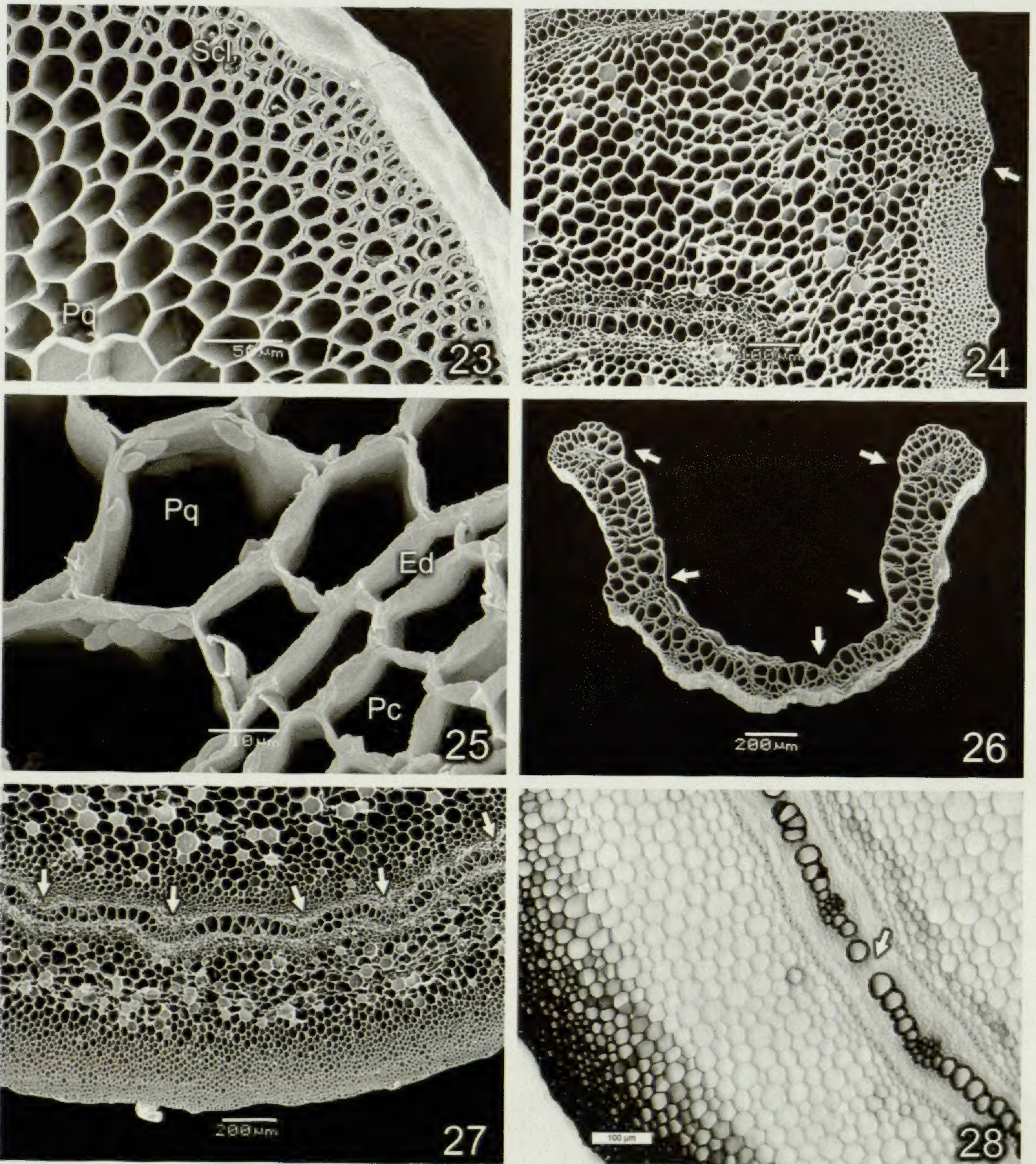
The stele is “V”-shaped (Fig. 19) in *P. ensiformis*, *P. ciliaris*, *P. cretica*, *P. multifida* and *P. mutilata*, “U”-shaped (Figs. 20, 21) in *P. vittata* and *P. denticulata*, *P. tristicula*, *P. quadriaurita*, and “inverted Ω -shaped” (Fig. 22) in *P. deflexa*, *P. exigua* and *P. inermis*. The opening of the vascular bundle is located towards the adaxial side in all cases.

The vascular bundle is surrounded externally by the monostratified endodermis with thickening on the radial walls and a pericycle consisting of (1–)2–3 cell layers surrounding the phloem and xylem (Fig. 25). Morphologically, the xylem is characterized by having curved ends in *P. vittata* or long bent ends which sometimes make contact with the main axis in *P. denticulata*, *P. tristicula* and *P. quadriaurita*. Around the vascular bundles, strands of fibers consist of 4–12 cells, which are isolated in small plants or together in a continuous or discontinuous ring depending on the age of plants.

Depending on the shape of the vascular strands and xylem structure, we propose a classification of the vascular bundles in four possible types:

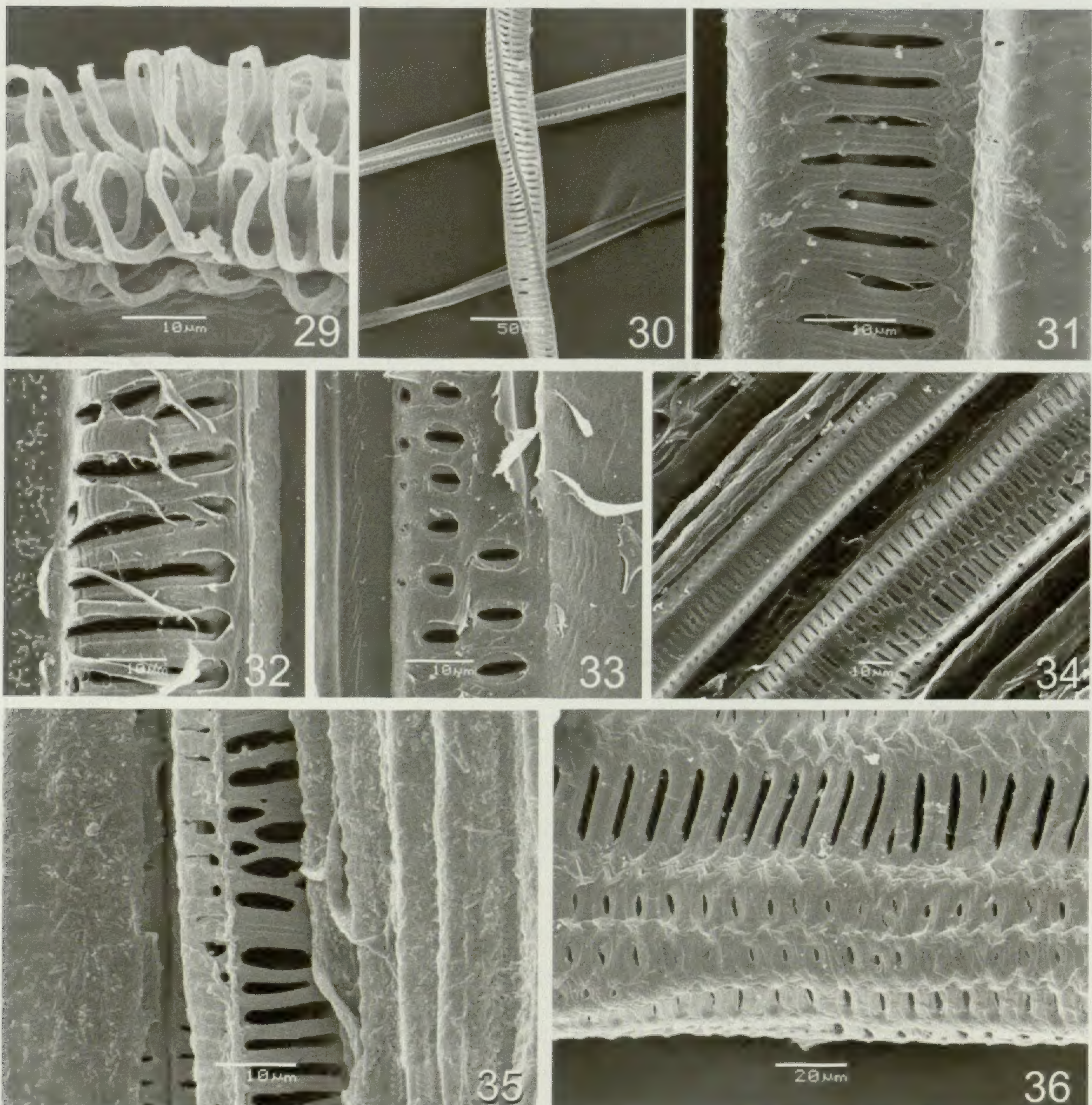
Type I. V-shaped (Fig. 19), with the ends of the xylem folded in a short hook, with three protoxylem areas: *P. ciliaris*, *P. cretica*, *P. ensiformis*, *P. multifida* and *P. mutilata*.

Type II. U-shaped, with two forms: type II-a (Figs. 20, 26) with shortly curved xylem ends and with five to six protoxylem areas observed in *P.*



FIGS. 23–28. Cross section of petioles photographed with SEM and light microscope. 23. Cortex of *Pteris cretica* L., showing epidermis, sclerenchyma (Scl) and parenchyma (Pq). 24. Cortex of *Pteris tristicola* Raddi, the arrow points to the ventilation area. 25. Middle zone of the petiole in *Pteris denticulata* Sw., pointing parenchyma with chloroplasts (Pq), endodermis composed of a layer of rectangular cells (Ed), and pericycle (Pc) with more or less isodiametric cells. 26. Xylem of *Pteris vittata* L., the arrows indicate protoxylem areas. 27. Part of the vascular bundle of *Pteris deflexa* Link, the arrows point to protoxylem areas. 28. Detail of xylem interrupted by a parenchyma band in *Pteris inermis* (Rosenst.) de la Sota.

vittata, and type II-b (Fig. 21) with the ends long extended, sometimes joined to the main axis, with four protoxylem areas: *Pteris denticulata*, *P. quadriaurita* and *P. tristicola*.



FIGS. 29–36. SEM photographs of tracheids. 29. Helical thickening in the protoxylem of *Pteris denticulate* Sw. 30. Tracheids with scalariform thickening in *Pteris mutilata* L. Detail of the pits 31. *Pteris mutilata* L. 32–33. *Pteris cretica* L. 34–35. *Pteris deflexa* Link. 36. *Pteris inermis* (Rosenst.) de la Sota.

Type III. Inverted- Ω -shaped (Figs. 22, 27), with more than ten protoxylem areas and the xylem interrupted by parenchyma bands (Fig. 28): *Pteris deflexa*, *P. exigua* and *P. inermis*.

The xylem is mesarch, with protoxylem of helical to ringed walls (Fig. 29). The metaxylem has elongated to lenticular pits (Figs. 30–36).

DISCUSSION

The external morphological characters of the *Pteris* petioles, such as a central channel and two lines covering the ventilation areas, have also been

observed in *Asplenium*, *Christella*, *Dennstaedtia*, *Microlepia*, *Pteridium*, and others by Lin and De Vol (1978). The brown to purple coloration of petioles, a color also frequent in *Adiantum*, *Cheilanthes* and *Pytirogramma*, among others, is attributed to the presence of tannins.

The number of steles is an important characteristic for rapid identification of different taxonomic groups. Lin and De Vol (1978) characterized Pteridaceae by the presence of two vascular bundles in the base of petioles that fuse upwards. This has been found in two species, *P. cretica* and *P. multifida*, whose xylem bundles join by the abaxial ends in the lower basal third of the shaft. Ogura (1972) finds such development of the vascular bundle in *Onoclea*, *Woodsia* and *Athyrium*, and calls it the "Onoclea form."

The twelve species studied have a monostelic axis from the middle petiole, although the stele shape varies. According to the classification proposed by Ogura (1972) for the monostelic axes, *P. ensiformis*, *P. ciliaris*, *P. cretica*, *P. multifida* and *P. mutilata* present Hymenophyllaceae type stele, whereas *P. denticulata*, *P. quadriaurita* and *P. tristricula* have Loxoma type vascular strand, and *P. deflexa*, *P. exigua* and *P. inermis* present the Pteris podophylla type. This author also describes two distinct types for the genus *Pteris*, the Loxoma type in *P. longifolia* Wall., *P. tremula* R. Br. and *P. flavellata* Sieb. et Zucc. and the Pteris podophylla type for the same species.

According to the classification suggested in this study, the type I, or V-shaped Hymenophyllaceae type (Ogura 1972), has been mentioned for *Adiantum* (Pteridaceae) and identified by Bidin and Walker (1985) as "saucer" shaped. Ogura (1972) considers this type to be a derivative of the Loxoma type. Lin and De Vol (1978) describe a petiole cross section of *P. multifida* that matches that described here for the species.

The type II, U-shaped or Loxoma type, has been reported for *P. denticulata*, *P. excelsa* Gaudich., *P. leptophylla* Sw., *P. semipinnata* L. and *Pteris vittata* (Lin and De Vol, 1977, 1978; Graçano *et al.*, 2001; Bondada *et al.*, 2006). Ogura (1972) mentioned the existence of modified forms within the Loxoma type, due to the presence of a varying number of protoxylem areas, i.e., five in *P. longifolia* L. and four to eight in *P. tremula* and *P. flavellata*. In this study, we found differences in the number of protoxylem bundles and in the ends of the xylem, so we proposed two different subtypes, II a and b. The first subtype is characteristic of *P. vittata* while the subtype II-b, is characterized by having ends fused to the main shaft, which gives the appearance of rounded ends, and this is likely why Ogura (1972) considered the species of *Pteris* mentioned above to have a xylem devoid of hooks.

The type III, inverted Ω -shaped, has been reported for *P. propinqua* (Graçano *et al.*, 2001) and *P. wallichiana* J. Agardh (Lin and De Vol, 1978). This monostelic type is considered by Ogura (1972) as a simple form derived from a polystelic type comprising several meristeles arranged in a horseshoe, called Saccoloma. Also Gifford and Foster (1996) qualify the monostelic condition of vascular bundles as primitive, and the polystelic condition as specialized. According to this criterion and considering that several interruptions caused by parenchyma bands have been found in the Ω form and the fact that the

numerous protoxylem groups are distributed regularly, we may consider this type to be a derivative form within the genus *Pteris*. The presence of parenchyma in the xylem has been cited in the rhizome of *Astrolepis sinuata* (Lag. ex Sw.) D.M. Benham & Windham (Scheiner and Carlquist, 1997), but no studies describing this feature in petioles have been found.

Potgieter and van Wyk (1992) mention the existence of intercellular pectic projections in the petioles of many ferns, including some *Pteris* species. These structures have not been recorded in the specimens studied, although specific future studies with more precise techniques could detect them. In the same way, the ultrastructural details of the xylem of these species could be studied.

The results of this work show that *Pteris vittata* has different characteristics in the vascular bundles from other species of *Pteris*. Martínez (2010) showed that spore characteristics of *P. vittata* are different from other species of *Pteris*. With the spore differences, these vascular bundle results reaffirm the results of molecular studies by Prado *et al.* (2007), which determined that to achieve monophyly of *Pteris P. vittata* should be segregated from other species of *Pteris*.

ACKNOWLEDGMENTS

We thank the herbarium curators who provided the material. We also thank Sherwin Carlquist for his valuable suggestions and review, one anonymous reviewer and the editor Jennifer Geiger who contributed to improve this manuscript; Pedro Villagrán for his help during the scanning electron microscopy process and María del C. Otero Cabada for the outline of the drawings. This project has been subsidized by the Consejo de Investigación de la Universidad Nacional de Salta.

LITERATURE CITED

- BIDIN, A. and T. WALKER. 1985. Comparative anatomy of the stipe of the fern genus *Adiantum* L. (Adiantaceae). *Gard. Bull. Sing.* 38(2):227–233.
- BONDADA, B., C. TU and L. Q. MA. 2006. Surface structure and anatomical aspects of Chinese brake fern (*Pteris vittata*; Pteridaceae). *Brittonia*. 58(3):217–228.
- CARLQUIST, S. and E. L. SCHNEIDER. 1997. SEM studies on vessels in Ferns. *Astrolepis*. *Amer. Fern J.* 87:43–50.
- D'AMBROGIO DE ARGÜESO, A. 1986. *Manual de Técnicas en Histología Vegetal*. Ed. Hemisferio Sur, Buenos Aires.
- GIFFORD, E. M. and A. S. FOSTER. 1996. *Morphology and evolution of vascular plants*. Freeman, New York.
- GRAÇANO, D., A. ALVES AZEVEDO and J. PRADO. 2001. Anatomia foliar das espécies de Pteridaceae do Parque estadual do Rio Doce (PERD-MG) *Rvta. brasil. Bot.*, São Paulo 24(3):333–347, <http://www.scielo.br/pdf/rbb/v24n3/6741.pdf>
- HERNÁNDEZ, V., T. TERRAZAS and G. ANGELES. 2006. Anatomía de seis especies de helechos del género *Dryopteris* (Dryopteridaceae) de México. *Rev. Biol. Trop.* 54(4):1157–1169. http://www.scielo.sa.cr/scielo.php?script=sci_arttextandpid=S0034-77442006000400014andlng=esandnrm=iso>. ISSN 0034-7744.
- HERNÁNDEZ-HERNÁNDEZ, V., T. TERRAZA and K. MEHLTRETER. 2007. Anatomía vegetativa de *Ctenitis melanosticta* (Dryopteris, Pteridophyta). *Bol. Soc. Bot. Mex.* 80:7–17.
- JEFFREY, E. C. 1917. *The anatomy of Wood plants*. University of Chicago Press, Chicago, USA.
- LIN, B. L. and C. D. DE VOL. 1977. The use of stipe characters in fern taxonomy I. *Taiwania* 22:91–99.
- LIN, B. L. and C. D. DE VOL. 1978. The use of stipe characters in fern taxonomy II. *Taiwania* 23:77–95.

- MARTÍNEZ, O. G. 2003. Morfología esporofítica y revisión sistemática del complejo *Pteris cretica* (Pteridaceae-Pteridophyta) en América. Tesis Doctoral. Universidad Nacional de Salta, Salta, Argentina.
- MARTÍNEZ, O. G. 2010. Gametófitos y esporófitos jóvenes de cuatro especies de helechos del género *Pteris* (Pteridaceae) naturalizadas en América. *Rev. Biol. Trop.* 58(1):89–102.
- MARTÍNEZ, O. G. and J. PRADO. 2011. *Pteris exigua* (Pteridaceae), a new endemic species from Tucumano-Boliviano forests in northwestern Argentina. *Brittonia* 63(2):295–299.
- METCALFE, C. R. and L. CHALK. 1950. Anatomy of the Dicotyledons. II. Clarendon Press, Oxford.
- OGURA, Y. 1972. Comparative anatomy of the vegetative organs of the Pteridophytes. *Handbuch der Pflanzenanatomie*. Borntraeger, Berlín, Alemania.
- POTGIETER, M. J. and A. E. VAN WYK. 1992. Intercellular pectic protuberances in plants: their structure and taxonomic significance. *Bot. Bull. Acad. Sinica* 33:295–316.
- PRADO, J. and P. G. WINDISCH. 2000. The genus *Pteris* L. (Pteridaceae) in Brazil. *Bol. Inst. Bot.* 13:103–199.
- PRADO, J., C. DEL N., RODRIGUES, A. SALATINO and M. L. F. SALATINO. 2007. Phylogenetic relationships among Pteridaceae, including Brazilian species, inferred from *rbcL* sequences. *Taxon* 56:355–364.
- SCHNEIDER, E. L. and S. CARLQUIST. 1997. SEM studies on vessels in Ferns. III. *Phlebodium* and *Polystichum*. *Int. J. Plant. Sci.* 158(3):343–349.
- SCHUETTPELZ, E. and K. M. PRYER. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. *Taxon* 56:1037–1050.
- SRIVASTAVA, K. 2008a. The Petiolar Structure of *Christella dentata* (Forssk.) Brownsey and Jermy. *Ethnobotanical Leaflets* 12:96–102. <http://opensiuc.lib.siu.edu/cgi/viewcontent.cgi?article=1045andcontext=ebl>
- SRIVASTAVA, K. 2008b. Epidermal Features and Petiolar Anatomy of *Angiopteris erecta* (Forst.) Hoffm. (Marattiaceae: Pteridophyta). *Ethnobotanical Leaflets* 12:139–149. <http://opensiuc.lib.siu.edu/cgi/viewcontent.cgi?article=1051andcontext=ebl>
- TRYON, R. M. and A. F. TRYON. 1982. Fern and allied plants with special reference to tropical America. Springer-Verlag, New York. Heidelberg. Berlin.
- WHITE, R. A. 1974. Comparative anatomical studies of the ferns. *Ann. Mo. Bot. Gard.* 61:379–387.

The Role of Aquaporins in Water Balance in *Cheilanthes lanosa* (Adiantaceae) Gametophytes

HOPE L. DIAMOND

University of North Texas, Risk Management Services, 1155 Union Circle #310950, Denton, Texas 76203-5017

HEATHER R. JONES and LUCINDA J. SWATZELL*

Southeast Missouri State University, Department of Biology, MS 6200, Cape Girardeau, Missouri 63701

ABSTRACT.—Aquaporins are transmembrane proteins that move water specifically and bidirectionally in response to internal cell signaling. With aquaporins, plant cells can control how, where, and when water moves across membranes. Thus, plants are in strong control of their environmental responses. Therefore, it seems likely that aquaporins would have a key role in water balance in xerophytic ferns, particularly in the gametophyte stage. To investigate the role of aquaporins in desiccation avoidance in xerophytic ferns gametophytes, *Cheilanthes lanosa* gametophytes were poisoned with micromolar mercury solutions, which block aquaporin channels, exposed to several osmolytes, and quantified the efflux of water from the cells was quantified. Results strongly suggest that aquaporins may very well play a role in water balance, but also pose some questions concerning the ability of the protonemal stage to fully manage water flow.

KEY WORDS.—aquaporin, *Cheilanthes lanosa*, fern, flux, gametophyte, mercury, NaCl, osmotic potential, sucrose, water

Water and osmoregulation has been a challenge since the beginning of cellular life. The first protocells would have had to constantly control the solutes and water content of the cytoplasm to maintain life. Therefore, it seems plausible that all bacterial, protozoan, animal, fungal, and plant cells would have and should still contain highly conserved mechanisms to move water in and out of cells (Chrispeels and Agre, 1994; Chrispeels and Maurel, 1994; Maurel, 1997).

Water-specific channels for water balance, aquaporins, exist in every kingdom and species. And, although common sense calls for the existence of such a practical mechanism, discovery of these channels was difficult. Indirect evidence demonstrated the rationale for such a simple mechanism at least twenty years before their discovery. Philip (1958) reviewed the data on water movement across lipid bilayers, and averred that the actual rate of water movement into cells is much more rapid than what should occur across a lipid bilayer. Yet, all future botanists were taught that water movement occurs through osmosis across a plant plasma membrane. Finally, in 1984, aquaporins were concurrently discovered in bovine eyes (Gorin *et al.*, 1984) and erythrocytes (Macey, 1984; Agre *et al.*, 1987). Very quickly, aquaporins were

* Author for communication

identified in rat kidney tubules, fungi, bacteria, and of course, plants (reviewed in Agre *et al.*, 1995; Chrispeels and Agre, 1994).

One impediment to the discovery of aquaporins was specificity. Any water-specific channel would have to exclude ions, which are smaller than water molecules. Confusion over potential structure helped delay the search for a water control mechanism. However, following the discovery of aquaporins, the elucidation of the structure revealed how an organism could effectively move water and exclude ions. The proteins are constructed from six membrane regions that form a spiral internal channel (Fotiadis *et al.*, 2001). Water, which is angled, flows through the spiraled channels but smaller molecules, such as sodium and potassium, cannot pass through the center (Fotiadis *et al.*, 2001). Aquaporins are controlled by a phosphorylation switch (Johnson and Chrispeels, 1991; Johansson *et al.*, 1996, 1998), and thus, cells are in control of water movement. The channels are also bidirectional; water may flow out of cells or into cells (Steudle, 1992; Tyerman *et al.*, 1999). For plants, this is a perfect control mechanism. Cytoplasm solute control and aquaporin gating combine to allow a functional cytoplasmic environment within a range of conditions (Kaldenhoff *et al.*, 1993, 1995; Maurel *et al.*, 1995; Maurel, 1997).

Studies on aquaporins have resolved many complicated questions regarding plant functions. Aquaporins exist in xylem and phloem and help prevent cavitation (Voicu *et al.*, 2009). Aquaporins exist in root cortical and endodermal cells and help generate or resolve pressure of water flow (Vandeleur *et al.*, 2005; Steudle and Peterson, 1998). Aquaporins in guard cell tonoplasts respond to changes in blue light and abscisic acid and allow water flow into and out of the vacuole (Kaldenhoff *et al.*, 1993). Aquaporins are also involved in water movement and management during water stress (Suga *et al.*, 2002).

Based on the above findings, we hypothesized that aquaporins may play a key role in the survival of fern gametophytes in xeric environments as mechanisms for water management. To test this hypothesis, we chose a common North American xerophytic fern, *Cheilanthes lanosa* (Michx.) D.C. Eat. for this study because it inhabits a variety of substrates (Mickel, 1979), though it is commonly found among other xerophytic species (Mohlenbrock, 1959), and possesses many of the features that other xerophytic ferns possess, such as microphylls (Pickett, 1931; Hevly, 1963; Quirk and Chambers, 1981), trichomes (Quirk and Chambers, 1981), mycorrhizal associations (Palmieri and Swatzell, 2004), cuticle on the gametophyte (Lingle *et al.*, 2004), and the ability to regenerate after desiccation (Diamond *et al.*, 2003). In addition, like many other xerophytic gametophytes, *C. lanosa* gametophytes are apogamous (Steil, 1933, 1939; Hevly, 1963). The gametophytes are also not heavily covered with wax, are one-cell in thickness, and appear generally unprotected from their surroundings. Thus, the *C. lanosa* gametophytes could represent the physiological state of numerous xerophytic ferns. We predicted that if it was possible to identify aquaporins in this fern gametophyte, and if we could poison these proteins with low levels of mercury, which blocks the protein channel (Preston *et al.*, 1993; Kuwahara *et al.*, 1997), we could effectively

control water movement out of desiccating cells. This would suggest at least a potential role for aquaporins in xerophytic gametophyte water management.

MATERIALS AND METHODS

To test our hypothesis, we examined different developmental stages and in different microclimates for the *Cheilanthes lanosa* gametophyte. Each development stage and microclimate condition will heretofore be designated as simply a "stage."

Plant Collection

Cheilanthes lanosa sporophylls were collected in the fall of 2003 after the first frost from Makanda, Illinois, placed in glass 9 cm 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.*, 1971) and thus spores were separated from the plant material using a 65 µm brass mesh sieve. Spores were stored at 4°C in the dark.

Culture Conditions

Wet grown (WG) gametophytes and protonemal callus (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 ddH₂O and sown on a modified tissue culture medium (TCM; 20 mM NH₄NO₃, 20 mM KNO₃, 1.5 mM MgSO₄·7H₂O, 1.0 mM MnSO₄·H₂O, 30.0 µM ZnSO₄·7H₂O, 0.1 µM CuSO₄·5H₂O, 3.0 mM CaCl₂·2H₂O, 5.0 µM KI, 0.1 µM CoCl₂·6H₂O, 0.8 mM KH₂PO₄, 0.9 mM H₃BO₃, 1.0 µM Na₂MoO₄·2H₂O, 0.1 mM FeSO₄·7H₂O, 0.1 mM Na₂EDTA, 0.23 µM kinetin, 0.86 µM 2,4-D, 0.4 µM nicotinic acid, 0.3 µM pyridoxine, 1.3 µM thiamine, 0.56 mM myo-inositol, pH 5.7; Smith, 1992) in 9 cm Petri dishes. Spores were incubated at 25°C in 0.175 µmol·m⁻²·s⁻¹ of continuous far red light (650–705 nm) for 10 d.

Following germination and protonemal development, the plates were separated. Some plates were left in the far red light to enhance callus growth and the remaining plates were then exposed to continuous white light and the protonema began planar growth into gametophytes.

Dry grown (DG) gametophytes.—Dry grown gametophytes were sown on fine grain white sand (Décor Sand, Activa Products Inc., Marshall, Texas, USA) wetted with 20 mL of TCM and incubated as the WG and callus cultures described above. Thereafter, upon drying, DG gametophytes wetted erratically with ddH₂O.

Antibody Production

To establish the molecular weight of the target protein, ensure specificity of binding of the anti-aquaporin antibody, and to detect the presence of

aquaporin-like proteins in gametophytes, an antibody against maize PIP1 aquaporin was raised using a homologous sequence from previously mapped aquaporins in maize (Chaumont *et al.*, 2000). Rabbit anti-aquaporin antibody was produced by Biosource (Invitrogen; Biosource, Camarillo, California, USA) against a conserved amino terminus sequence of PIP1 maize aquaporin, MEGKEEDVRVGANKFPERQPIGTSAQS as described by Chaumont *et al.* (2000).

ELISA (enzyme-linked immunosorbent assay)

In order to test for the presence of aquaporin-like proteins, an enzyme-linked immunosorbent assay was performed to detect a wide range of aquaporin concentrations in various gametophyte stages. Approximately 20 mg of WG gametophyte material was homogenized in 200 μ L of coating buffer (15 mM Na_2HCO_3 , 35 mM NaHCO_3 , pH 9.5) in a mortar and pestle. The sample was then centrifuged at 10,000 rpm for 10 min. The supernatant was retained and centrifuged again at 10,000 rpm for 10 min. The sample was then diluted in 1 ml of coating buffer. The concentration was approximately 3 μ g of material for every 50 μ L of coating buffer. The antigen-coating buffer solution was then pipetted in 50 μ L increments into a 96-well polystyrene microtiter plate. The plate was covered and allowed to incubate overnight at 4°C. The following morning, the antigen solution was removed by inverting the plate and washing three times with Tris-Tween washing buffer (TTW; 10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.2). In all the washing steps, the wells were completely filled (~350 μ L). Plates were blocked with 3% (w/v) low-fat milk powder in TTW for 30 min. The wells were then washed three times with TTW. Once the TTW was removed, 100 μ L of rabbit anti-aquaporin (1:500; Biosource, Camarillo, California, USA) in TTW, rabbit preimmune serum (1:500; Biosource, Camarillo, California, USA) in TTW, or TTW only (secondary antibody only control) was placed into the wells and allowed to incubate for 60 min. The wells were washed three times with TTW. The TTW was removed and 50 μ L of goat anti-rabbit horseradish peroxidase (1:500; Sigma-Aldrich, St. Louis, Missouri, USA) in TTW, or TTW only (primary antibody only control) was added and allowed to incubate for 60 min. The wells were again washed three times with TTW. The chromogenic enzyme reaction was then initiated by addition of freshly prepared solution of 1.5 mg *o*-phenylenediamine and 2 μ L of 30% (v/v) H_2O_2 dissolved in 2 mL of citrate-phosphate buffer (0.2 M Na_2HPO_4 , 0.1 M citric acid, pH 5.0) at 50 μ L per well. H_2O_2 was added immediately prior to use. The enzyme reaction was allowed to incubate for 15 min and the reaction was halted by the addition of 50 μ L of 0.5 M H_2SO_4 per well. The plate was then read at 492 nm on a Beckman DU640B spectrophotometer (Beckman Coulter, Fullerton, California, USA).

Immunoblotting

To establish the molecular weight of the target protein and to ensure specificity of binding of the anti-aquaporin antibody, immunoblotting was

performed. Approximately 100 μ L of *Cheilanthes lanosa* callus, WG, or DG plant material was ground in homogenization buffer [10 mM KCl, 1 mM MgCl₂, 50 mM HEPES, 300 mM sorbitol, 0.1% (w/v) BSA and 1 mM EDTA, pH to 7.2]. Samples were mixed 1:1 2X Laemmli sample buffer [Sambrook et al., 1989; 4% (w/v) SDS, 10% (v/v) mercaptoethanol, 0.002% (w/v) Bromphenol Blue, 20% (v/v) glycerol, 120 mM Tris, pH 6.8], incubated for 10 min at 90 °C, centrifuged at 10,000 rpm for 10 min. Total protein concentration was determined using the Bradford Assay (Stoschek, 1990). Samples were loaded onto a 12% (w/v) polyacrylamide gel at 35 μ l per well and 20 μ g/mL concentration. Samples were electrophoresed at 16 mA constant current for approximately 30 min. A broad range marker (Kaleidoscope; BioRad, Hercules, California, USA) was used for reference. Proteins were then transferred at 45 mA constant current for 20 min onto a nitrocellulose membrane (0.2 μ m pore size). Following transfer, the membranes were rinsed three times in TTBS [137 mM NaCl, 2.7 mM KCl, 24.8 mM Tris, 0.2% Tween (v/v)], and blocked in 3% (w/v) bovine serum albumin (BSA) in TTBS. Membranes were then rinsed three times for 5 min each in TTBS. Membranes were then incubated overnight at 4 °C in either preimmune serum (BioSource, Camarillo, California, USA; 1:500 in TTBS), rabbit anti-PIP1 aquaporin (BioSource, Camarillo, California, USA; 1:500 in TTBS), or TTBS only (secondary antibody only control). Membranes were again rinsed 3 times for 5 min in TTBS. Following the rinse, membranes were incubated in goat anti-rabbit alkaline phosphatase (1:500 in TTBS; Sigma-Aldrich, St. Louis, Missouri, USA), with the exception of the primary antibody only control, which was incubated in TTBS only, each for 1 h at 25 °C. Membranes were rinsed three times for 5 min each in TTBS. The membrane was then colorized with a fresh mixture of 20 mL of alkaline phosphate buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5), 132 μ L of nitro blue tetrazolium (NBT) stock, and 66 μ L of 5-Bromo-4-chloro-3'-indolyphosphate *P*-Toluidine (BCIP) stock (Sambrook *et al.*, 1989). After agitation for 5 min, the membrane was briefly rinsed with ddH₂O and the reaction was stopped with 20 mM ethylene diamine tetraacetic acid (EDTA).

Osmolality Stress

In order to test for aquaporin function and potential control of function in gametophyte stages, gametophytes were exposed to desiccating levels of osmolytes with and without mercury exposure. Although the preferred test for function of aquaporins is expression of mRNA in *Xenopus* oocytes (which do not express aquaporins), PIP1 mRNA does not express well in oocytes (Preston *et al.*, 1992). Therefore, another standard, the immersion of samples in mercuric chloride at low levels (1 mM; Macey, 1984) was used. Wet grown (WG) gametophytes, callus and dry grown (DG) gametophytes were exposed to 2 separate treatments. One treatment consisted of time increments of NaCl, CaCl₂, or sucrose and was used to show gametophyte response to long term increasing environmental stress. Gametophytes were pretreated in 200 μ L well slides in 100 μ L of ddH₂O or ddH₂O plus 1 mM HgCl₂ for 20 min. Gametophyte images were captured digitally. At t_0 , and subsequently every 5 min afterward,

50 mM increments of the respective solute were added until the solution in the well slide reached 500 mM osmolality. Images of gametophytes were captured after 1 h. The second treatment was used to show gametophyte response to immediate stress. This treatment began with a 20 min pretreatment in a 200 μ L well slide in 100 mM isotonic solute solution (NaCl, CaCl₂, or sucrose) or 100 mM isotonic solution plus 1 mM HgCl₂. Gametophyte images were captured digitally. At t_0 , 400 mM of solute in 100 μ L of ddH₂O was added to the well so that the solution in the well reached 500 mM osmolality. Images of the gametophytes were captured after 1 h.

Cell volume was determined using six diameter measurements. Average radii were used to extrapolate the sphere volume and surface area. Only hourly rates of efflux were calculated. This is because diffusion out of these cells was slow enough to allow the necessary resolution to demonstrate loss of function of water channels under mercury poisoning. Well slides were incubated in a moisture chamber in the interim times between increments to prevent water loss from the wells. The efflux of water out of each of 100 cells from each treatment was measured using Fick's Law:

$$\text{Flux} = -PS[(\text{Osm}_o - \text{Osm}_i)/D]$$

Where -P = permeability (negative because against the concentration gradient), S = surface area, Osm_o = osmolality outside of cell, Osm_i = osmolality inside of cell, and D = distance (modified from Qui et al., 2000).

Data Capture and Image Analysis

For cell volume and trichome position, gametophyte images were captured digitally on an Olympus SZ40 (Olympus America, Center Valley, Pennsylvania, USA) with SPOT Advanced 3.2 software (Diagnostic Instruments, Sterling Heights, Michigan, USA). Measurements were made using SPOT Advanced 3.2 software. Measurements of 100 cells were conducted for each treatment. To assess the influence of treatment, medium, fern stage and timing, a three-way Analysis of Variance (ANOVA; $P = 0.05$; $n = 3600$) with interaction was undertaken using the SAS General Linear Model Procedure (SAS 1999–2000; SAS, Cary, North Carolina, USA). A Tukey's Studentized test was then performed to determine significant differences between media, with a primary focus on the comparison of HgCl₂ and ddH₂O pretreatments. To graph individual treatments, box plots were developed using JMP Statistical Discovery Software (SAS Programming, Serial No. GV0KZ9JJ07, © 2007; SAS, Cary, North Carolina, USA) and modified using PaintShopPro 6.02 (Jasc Software, Ottawa, Ontario, Canada).

RESULTS

ELISA and Immunoblotting

To establish the potential presence of an aquaporin-like protein in germinating spores and gametophytes, ELISA was performed on callus and

immunoblotting was performed on DG and WG gametophytes. In ELISA, primary antibody only, secondary antibody only, and the preimmune serum controls were negative (Fig. 1A). The positive control, application of anti-aquaporin antibody applied to radish root homogenate, resulted in a strong cross-reaction of root total protein with the anti-aquaporin antibody. The immunoblotting procedure produced a single band when the anti-aquaporin antibody was applied to the mature gametophyte sample (Fig. 1B). Primary antibody only, secondary antibody only, and preimmune serum controls were negative. The positive control, application of anti-aquaporin antibody applied to radish root homogenate, resulted in a strong cross-reaction of root total protein with the anti-aquaporin antibody.

Increment Solute Treatments

NaCl and CaCl₂.—To measure the response of different stages of gametophytes to hyperpolarizing solutes, which destabilize plasma membranes and often alter membrane protein function, samples were exposed to incremental increases of NaCl and CaCl₂. When preincubated in ddH₂O and then exposed to NaCl increments, callus quickly plasmolyzed to the extent that protoplasm fluid was almost entirely extracted and chloroplasts clumped tightly with nuclei and other cell contents (Fig 2A). Callus cells visibly plasmolyzed when exposed to slow increments in NaCl concentrations. Mean of cell volume loss in treatments in which NaCl was added in slow increments was $6.65 \cdot 10^{-4} \text{ cm}^{-3} \pm 10.85 \cdot 10^{-4}$. Following an incubation in HgCl₂, callus flux levels significantly dropped (Fig. 2B). Cells appear fully intact up to 500 mM NaCl. In addition, variation in the response was greatly reduced. Mean of cell volume flux in NaCl treatments with a HgCl₂ incubation prior to the incremented NaCl treatment was $0.21 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.47 \cdot 10^{-4}$. Plasmolysis also occurred when WG gametophytes were exposed to slow increases in NaCl (Fig. 2C). Mean of cell volume loss in treatments in which NaCl was added in slow increments was $4.86 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.93 \cdot 10^{-4}$. WG flux levels significantly dropped after the introduction of HgCl₂ (Fig. 2D). Cells appear uncompromised. Standard deviations and variation was similar for both WG treatments. Mean of cell volume flux in NaCl treatments with HgCl₂ incubation prior to the incremented NaCl treatment was $0.55 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.23 \cdot 10^{-4}$. DG gametophytes appeared to maintain cell viability in NaCl increments up to 500 NaCl (Figs. available from author). Some cell volume loss was visible in older cells toward the center of the gametophyte. Mean of cell volume loss following a slow increase in NaCl was $3.96 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.40 \cdot 10^{-4}$. Mean of cell volume loss in NaCl increment treatment following a pre-incubation in HgCl₂ was $0.80 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.15 \cdot 10^{-4}$. DG flux levels significantly dropped after the introduction of HgCl₂.

These results were mirrored in the CaCl₂ increment treatments (Fig. 3). Callus cells visibly plasmolyze when exposed to slow increments in CaCl₂ concentrations (Fig 3A). Mean of cell volume loss in treatments in which CaCl₂ was added in slow increments was $12.18 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.59 \cdot 10^{-4}$. Flux in callus cell volume levels significantly dropped following pre-incubation in HgCl₂ (Fig 3B). Mean of cell

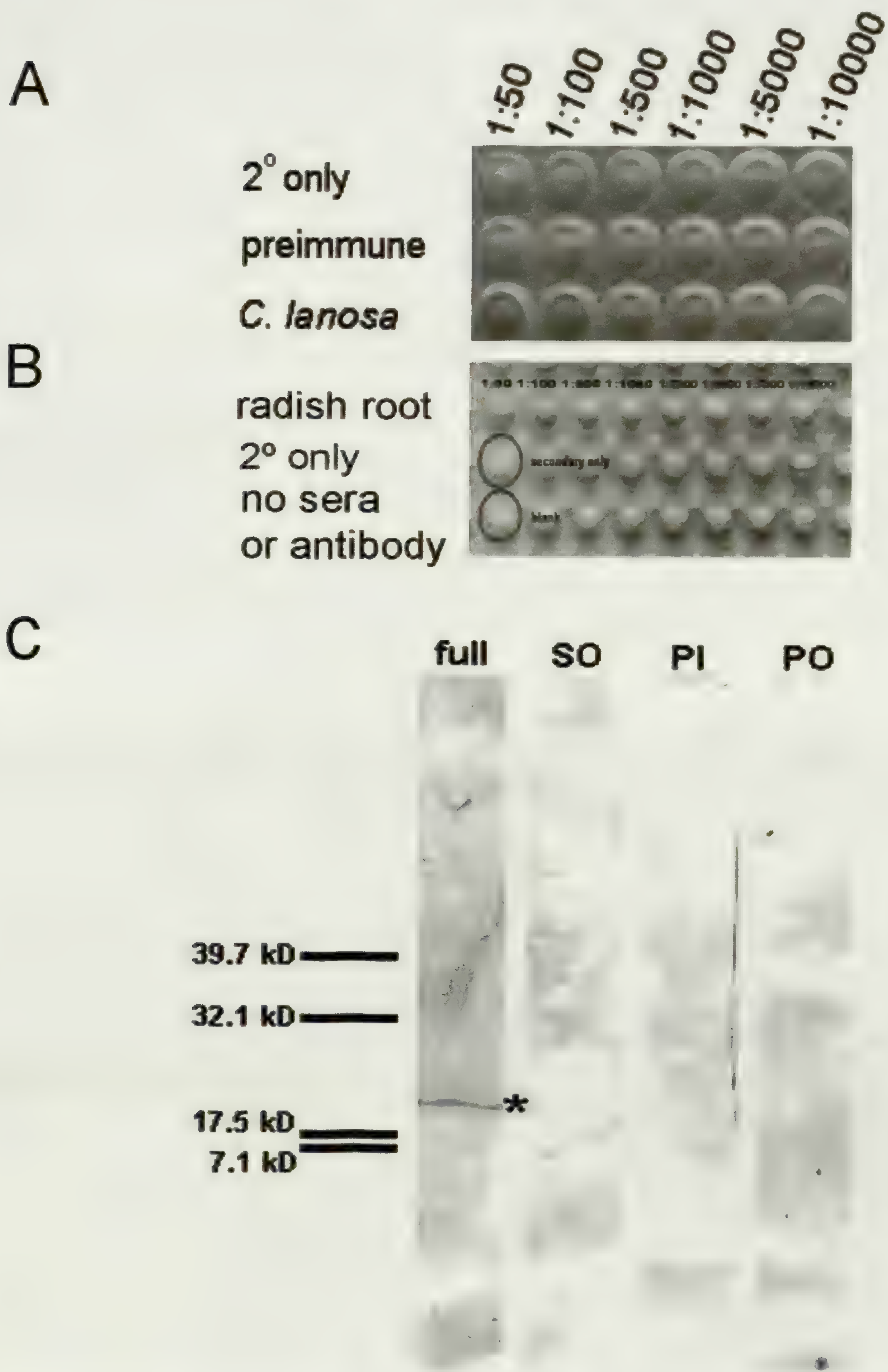


FIG. 1. Immunolabeling of Aquaporin-like Protein. (A) ELISA signal from cross-reaction of anti-aquaporin antibody with callus total protein is yellow. Stronger cross-reaction due to greater concentrations of antibody (antibody dilutions of 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000) results in more intense coloration and absorbance. Primary antibody only (not shown), secondary antibody only, and preimmune serum controls were negative. The positive control, anti-aquaporin antibody cross-reaction with radish root, is not shown in this image, but is located on the same

volume loss following CaCl_2 treatment with a pre-incubation in HgCl_2 was $2.20 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.80 \cdot 10^{-4}$. The treatment with pre-incubation in HgCl_2 also exhibited a much smaller amount of variation than the treatment without HgCl_2 . Heavy plasmolysis occurred when WG gametophytes were exposed to slow increments of CaCl_2 up to 500 mM (Fig. 3C). Mean of cell volume loss in treatments in which CaCl_2 was added in slow increments was $6.95 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.59 \cdot 10^{-4}$. WG flux levels significantly dropped after the introduction of HgCl_2 (Fig. 3D). Mean of cell volume loss in CaCl_2 increment treatments were preceded by pre-incubation in HgCl_2 was $1.95 \cdot 10^{-4} \text{ cm}^{-3} \pm 1.23 \cdot 10^{-4}$. DG gametophytes (Figs. available from author) plasmolyzed at higher concentrations of CaCl_2 (up to 500 mM). Mean of cell volume loss after increasing increments of CaCl_2 was $5.75 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.38 \cdot 10^{-4}$. DG cell volume flux levels were significantly lower when gametophytes were pre-incubated in HgCl_2 . There was also a substantial increase in variation in the response with pre-incubation in HgCl_2 . Mean of cell volume loss in gametophytes pretreated with HgCl_2 was $2.65 \cdot 10^{-4} \text{ cm}^{-3} \pm 1.05 \cdot 10^{-4}$.

Sucrose.—Sucrose was used to test gametophyte response to a pure osmolyte that does not depolarize or hyperpolarize membranes (Fig. 4). Callus cells were desiccated in a treatment of slow sucrose increments up to a total of 500 mM sucrose. Mean of cell volume loss was $9.45 \cdot 10^{-4} \text{ cm}^3 \pm 8.86 \cdot 10^{-4}$. Callus flux levels significantly dropped in treatments that included a pre-incubation in HgCl_2 (Fig. 4B). Mean of callus cell volume loss was $0.09 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.51 \cdot 10^{-4}$. In addition, variation was greatly reduced when protonemal callus was pre-incubated in HgCl_2 . WG gametophytes in a treatment in which sucrose is increased in increments up to 500 mM exhibited some cell volume loss in older cells (Fig. 4C). Mean of cell loss in sucrose increment treatment was $9.62 \cdot 10^{-4} \text{ cm}^{-3} \pm 1.64 \cdot 10^{-4}$. WG flux levels significantly dropped in sucrose increment treatments that were pre-incubated in HgCl_2 (Fig. 4D). Mean of cell volume loss in these treatments with HgCl_2 pre-incubation was $0.48 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.20 \cdot 10^{-4}$. Variation in response decreased markedly when gametophytes were pre-incubated in HgCl_2 . DG gametophytes (Figs. available from author) experienced some cell volume loss in treatments involving increments in sucrose molarity (up to 500 mM). Mean of DG cell volume loss in sucrose increment treatments was $10.80 \cdot 10^{-4} \text{ cm}^{-3} \pm 54.15 \cdot 10^{-4}$. DG cell volume loss significantly dropped in sucrose increment treatments that included a pre-incubation in HgCl_2 . Variation in gametophyte response was also less in treatments that included HgCl_2 pre-incubation. Mean of cell volume loss in treatments with HgCl_2 pre-incubation was $0.95 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.45 \cdot 10^{-4}$.

←

plate. (B) ELISA Positive Radish Root Control. Each immunoblot and ELISA contained a radish root control. PIP1 aquaporins express in germinating seeds and new plant roots and freshly germinated radish root reliably produced a strong signal. (C) Immunoblotting procedures resulted in a single band of total protein that cross-reacted with anti-aquaporin antibody. * = approximately 22.4 kD. Secondary only (SO), preimmune serum (PI), and primary only (PO) controls did not result in any cross reaction with the primary or secondary antibodies alone, or with the pre-inoculation serum.

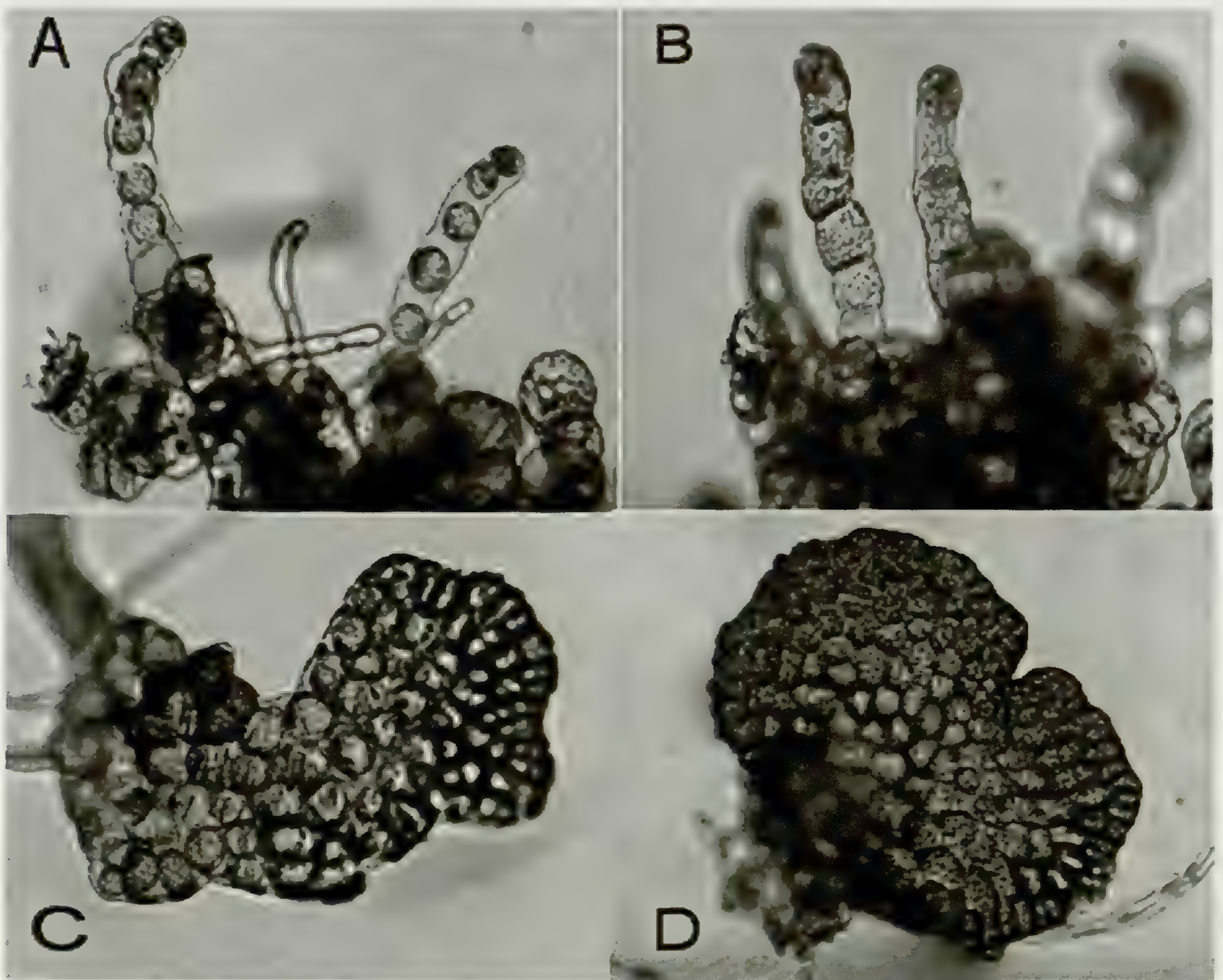


FIG. 2. Flux in Cell Volume of Gametophytes exposed to NaCl Increments. (A) Callus cells visibly plasmolyze when exposed to slow increments in NaCl concentrations. Mean of cell volume loss in treatments in which NaCl was added in slow increments = $6.65 \cdot 10^{-4} \text{ cm}^{-3} \pm 10.85 \cdot 10^{-4}$. (B) Following an incubation in HgCl_2 , callus flux levels significantly dropped. Cells appear fully intact up to 500 mM NaCl. In addition, variation in the response was greatly reduced. Mean of cell volume flux in NaCl treatments with a HgCl_2 incubation prior to the incremented NaCl treatment was $0.21 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.47 \cdot 10^{-4}$. (C) Plasmolysis occurred when WG gametophytes were exposed to slow increases in NaCl. Mean of cell volume loss in treatments in which NaCl was added in slow increments = $4.86 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.93 \cdot 10^{-4}$. (D) WG flux levels significantly dropped after the introduction of HgCl_2 . Cells appear uncompromised. Standard deviations and variation was similar for both WG treatments. Mean of cell volume flux in NaCl treatments with HgCl_2 incubation prior to the incremented NaCl treatment = $0.55 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.23 \cdot 10^{-4}$.

Immediate Immersion Treatments

NaCl₂ and CaCl₂.—To test for differences in flux that could be based on diffusion or aquaporin deactivation, stages were introduced to the same solute treatments but the timing of introduction was changed to immediate immersion in 500 mM solute. Following HgCl_2 pretreatment, all stages exhibited a significant reduction in flux. Callus cells lost cell volume very quickly when immersed in 500 mM NaCl (Fig. 5A). Mean of cell volume loss was $6.30 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.82 \cdot 10^{-4}$. Callus pre-incubated in HgCl_2 before immersion remains intact (Fig. 5B). Cell volume was significantly different than those cells treated only with NaCl. Mean cell volume loss in treatment

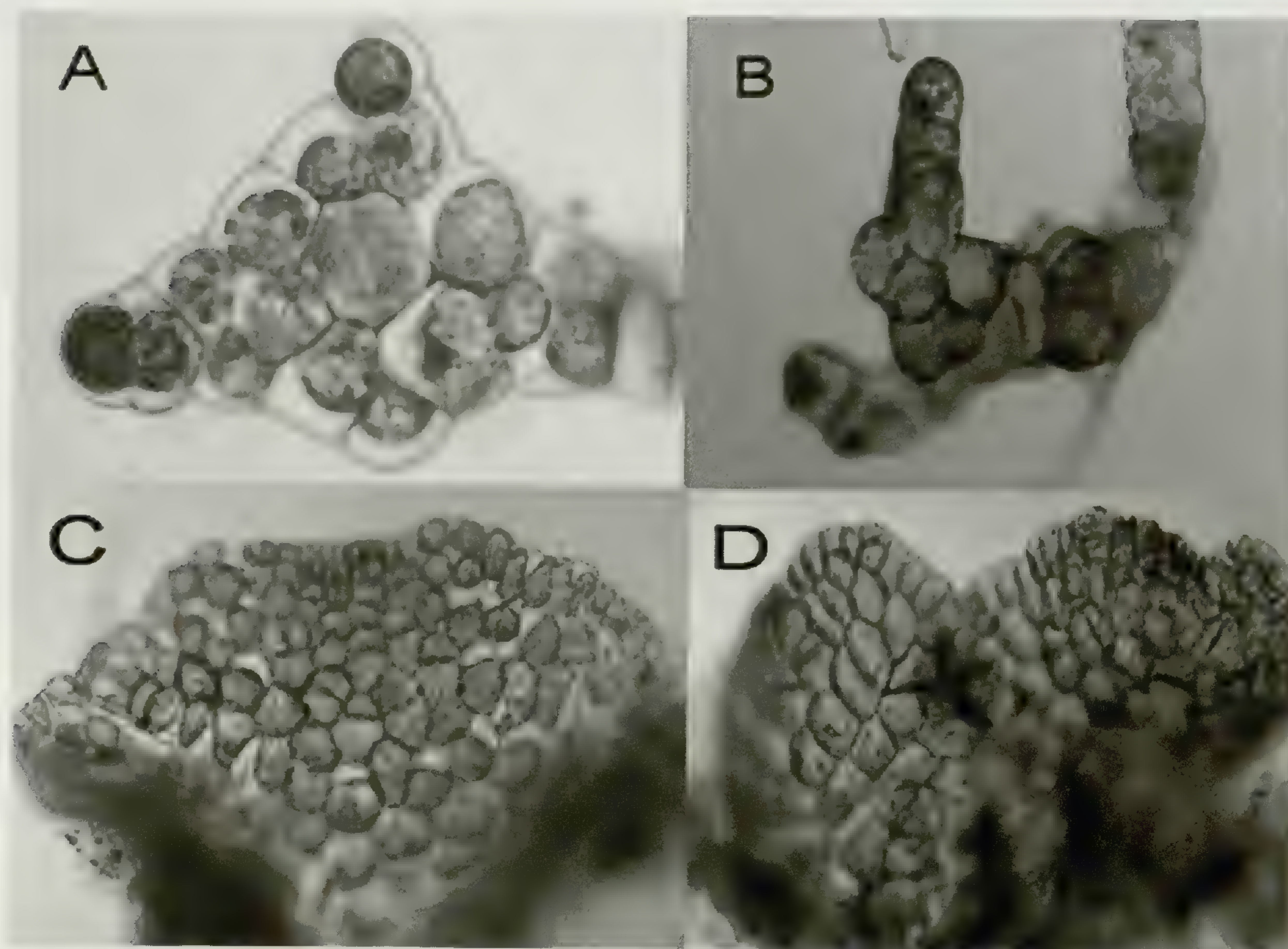


FIG. 3. Flux in Cell Volume of Gametophytes exposed to CaCl_2 Increments. (A) Callus cells visibly plasmolyze when exposed to slow increments in CaCl_2 concentrations. Mean of cell volume loss in treatments in which CaCl_2 was added in slow increments = $12.18 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.59 \cdot 10^{-4}$. (B) Flux in callus cell volume levels significantly dropped following pre-incubation in HgCl_2 . Mean of cell volume loss following CaCl_2 treatment with a pre-incubation in HgCl_2 = $2.20 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.80 \cdot 10^{-4}$. The treatment with pre-incubation in HgCl_2 also exhibited a much smaller amount of variation than the treatment without HgCl_2 . (C) Heavy plasmolysis occurred when WG gametophytes were exposed to slow increments of CaCl_2 up to 500 mM. Mean of cell volume loss in treatments in which CaCl_2 was added in slow increments = $6.95 \cdot 10^{-4} \text{ cm}^{-3} \pm 6.59 \cdot 10^{-4}$. (D) WG flux levels significantly dropped after the introduction of HgCl_2 . Mean of cell volume loss in CaCl_2 increment treatments were preceded by pre-incubation in HgCl_2 = $1.95 \cdot 10^{-4} \text{ cm}^{-3} \pm 1.23 \cdot 10^{-4}$.

with pre-incubation in HgCl_2 was $0.26 \cdot 10^{-4} \text{ cm}^{-3} \pm 14.29 \cdot 10^{-4}$. Variation in responses was also reduced when callus was exposed to HgCl_2 pre-incubation. WG gametophytes also plasmolyzed immediately in 500 mM NaCl (Fig. 5C). Mean of cell volume flux in NaCl immersion was $5.08 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.72 \cdot 10^{-4}$. Conversely, change in WG cell volume was minimal following treatment that included pre-incubation in HgCl_2 (Fig. 5D). Mean of cell volume flux in treatments that included HgCl_2 pretreatment was $1.85 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.37 \cdot 10^{-4}$. Variation in flux amounts also significantly decreased in the HgCl_2 pre-incubation treatment. DG gametophytes (Figs. available from author) were visibly affected by immediate immersion in 500 mM NaCl . Mean of cell volume loss in NaCl immediate immersion was $3.10 \cdot 10^{-4} \text{ cm}^{-3} \pm 1.00 \cdot 10^{-4}$. However, when DG gametophytes were pre-incubated in HgCl_2 prior to the NaCl immersion, there was a decrease in cell volume loss. Mean of cell volume

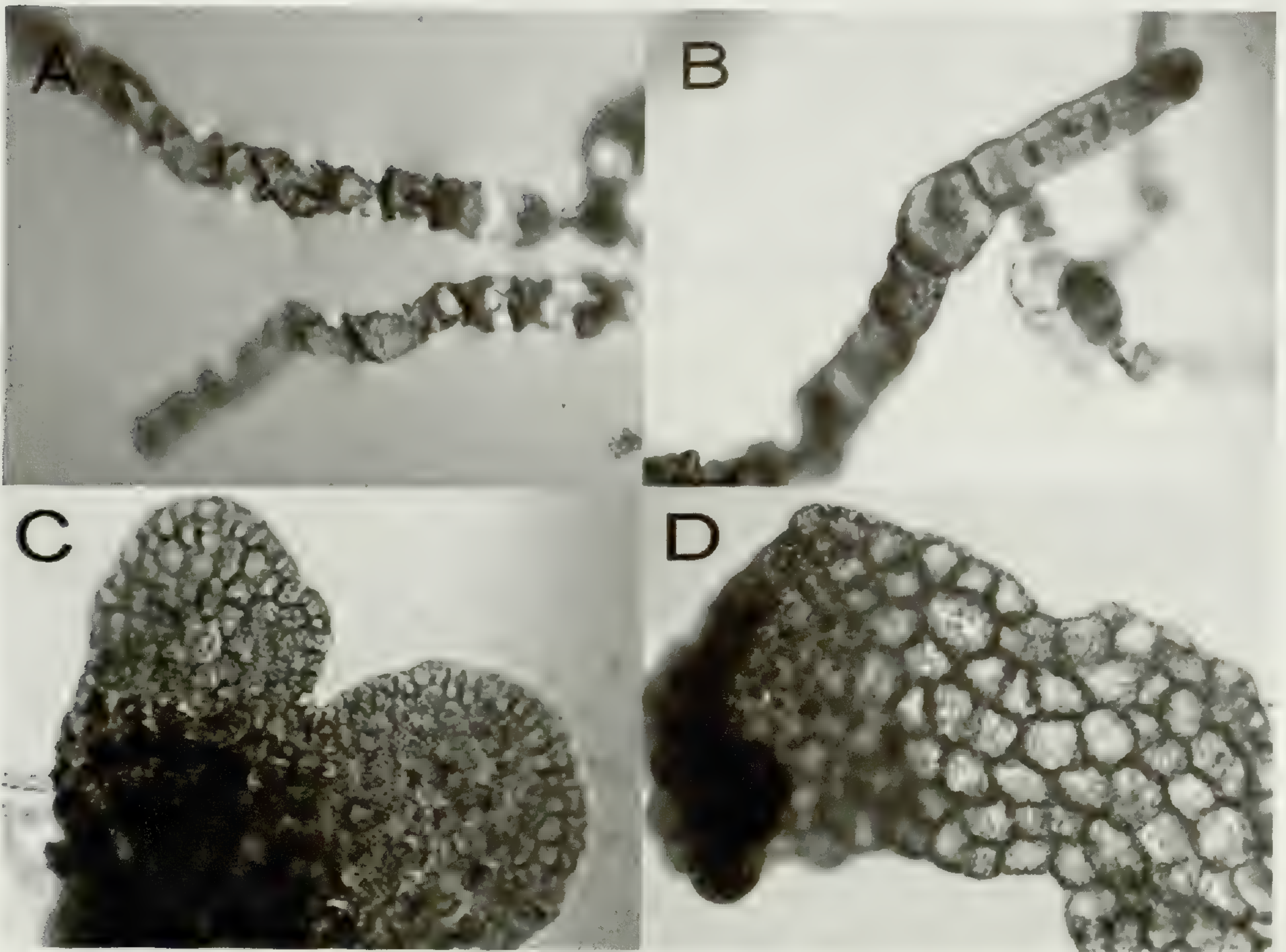


FIG. 4. Gametophytes and Sucrose Increments. (A) Callus cells were desiccated in a treatment of slow sucrose increments up to a total of 500 mM sucrose. Mean of cell volume loss = $9.45 \cdot 10^{-4} \text{ cm}^3 \pm 8.86 \cdot 10^{-4}$. (B) Callus flux levels significantly dropped in treatments that included a pre-incubation in HgCl_2 . Mean of callus cell volume loss = $0.09 \cdot 10^{-4} \text{ cm}^3 \pm 0.51 \cdot 10^{-4}$. In addition, variation was greatly reduced when protonemal callus was pre-incubated in HgCl_2 . (C) WG gametophytes in a treatment in which sucrose is increased in increments up to 500 mM exhibited some cell volume loss in older cells. Mean of cell loss in sucrose increment treatment = $9.62 \cdot 10^{-4} \text{ cm}^3 \pm 1.64 \cdot 10^{-4}$. (D) WG flux levels significantly dropped in sucrose increment treatments that were pre-incubated in HgCl_2 . Mean of cell volume loss in these treatments with HgCl_2 pre-incubation = $0.48 \cdot 10^{-4} \text{ cm}^3 \pm 3.20 \cdot 10^{-4}$. Variation in response decreased markedly when gametophytes were pre-incubated in HgCl_2 .

loss in HgCl_2 pretreated gametophytes was $0.80 \cdot 10^{-4} \text{ cm}^3 \pm 1.55 \cdot 10^{-4}$. There was also a substantial increase in variation in the response with pre-incubation in HgCl_2 .

CaCl_2 immediate immersion (Fig. 6) results were only slightly different than NaCl results. Callus plasmolyzed heavily upon immediate immersion in 500 mM CaCl_2 (Fig. 6A). Mean of callus cell volume loss in CaCl_2 was $6.73 \cdot 10^{-4} \text{ cm}^3 \pm 2.98 \cdot 10^{-4}$. However, callus gametophytes that were pretreated in HgCl_2 before immersion in CaCl_2 exhibited little volume flux (Fig. 6B). Mean of callus cell volume loss in treatments with HgCl_2 preincubation was $0.55 \cdot 10^{-4} \text{ cm}^3 \pm 0.50 \cdot 10^{-4}$. Variation in the responses also decreased in those cells preincubated in HgCl_2 . WG gametophyte cells also plasmolyzed heavily in CaCl_2 immediate immersion treatment (Fig. 6C). Mean

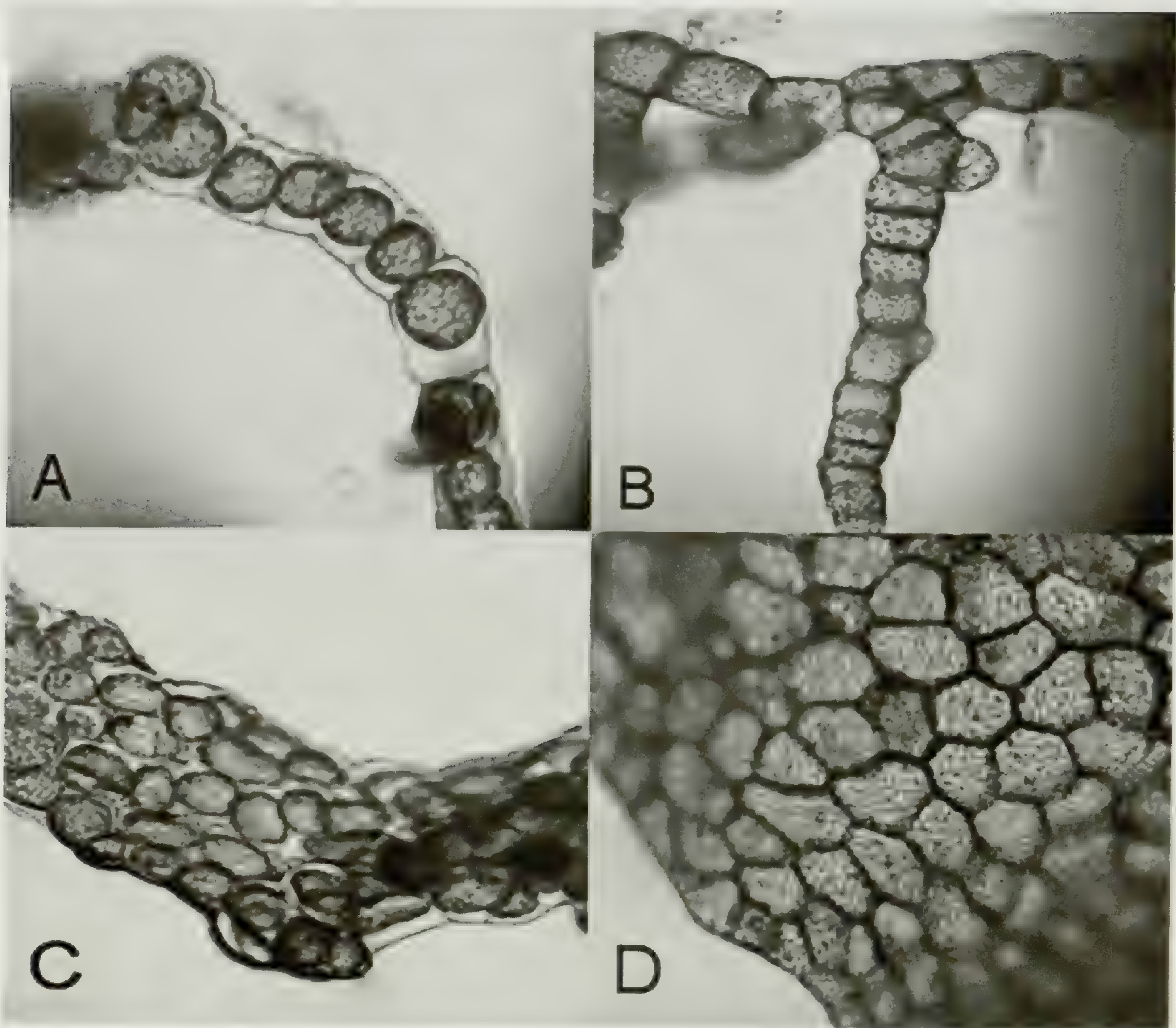


FIG. 5. Gametophytes and NaCl Immediate Immersion. (A) Callus cells lost cell volume very quickly when immersed in 500 mM NaCl. Mean of cell volume loss = $6.30 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.82 \cdot 10^{-4}$. (B) Callus pre-incubated in HgCl_2 before immersion remains intact. Cell volume was significantly different than those cells treated only with NaCl. Mean cell volume loss in treatment with pre-incubation in HgCl_2 = $0.26 \cdot 10^{-4} \text{ cm}^{-3} \pm 14.29 \cdot 10^{-4}$. Callus cell volume loss significantly dropped after the introduction of HgCl_2 . Variation in responses was also reduced when callus was exposed to HgCl_2 pre-incubation. (C) WG gametophytes also plasmolyzed immediately in 500 mM NaCl. Mean of cell volume flux in NaCl immersion = $5.08 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.72 \cdot 10^{-4}$. (D) Conversely, change in WG cell volume was minimal following treatment that included pre-incubation in HgCl_2 . Mean of cell volume flux in treatments that included HgCl_2 pretreatment = $1.85 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.37 \cdot 10^{-4}$. Variation in flux amounts also significantly decreased in the HgCl_2 pre-incubation treatment.

of cell volume loss in CaCl_2 immediate immersion was $6.77 \cdot 10^{-4} \text{ cm}^3 \pm 2.76 \cdot 10^{-4}$. WG gametophytes that were incubated in HgCl_2 prior to immediate immersion in CaCl_2 showed no visible volume loss (Fig. 6D). Mean of measured volume loss in this treatment was $0.44 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.08 \cdot 10^{-4}$. DG gametophytes (Figs. available from author) also underwent volume loss following immediate immersion in 500 CaCl_2 . Mean of DG volume loss was $3.04 \cdot 10^{-4} \text{ cm}^3 \pm 1.14 \cdot 10^{-4}$. Mean of DG treatments that included pretreatment

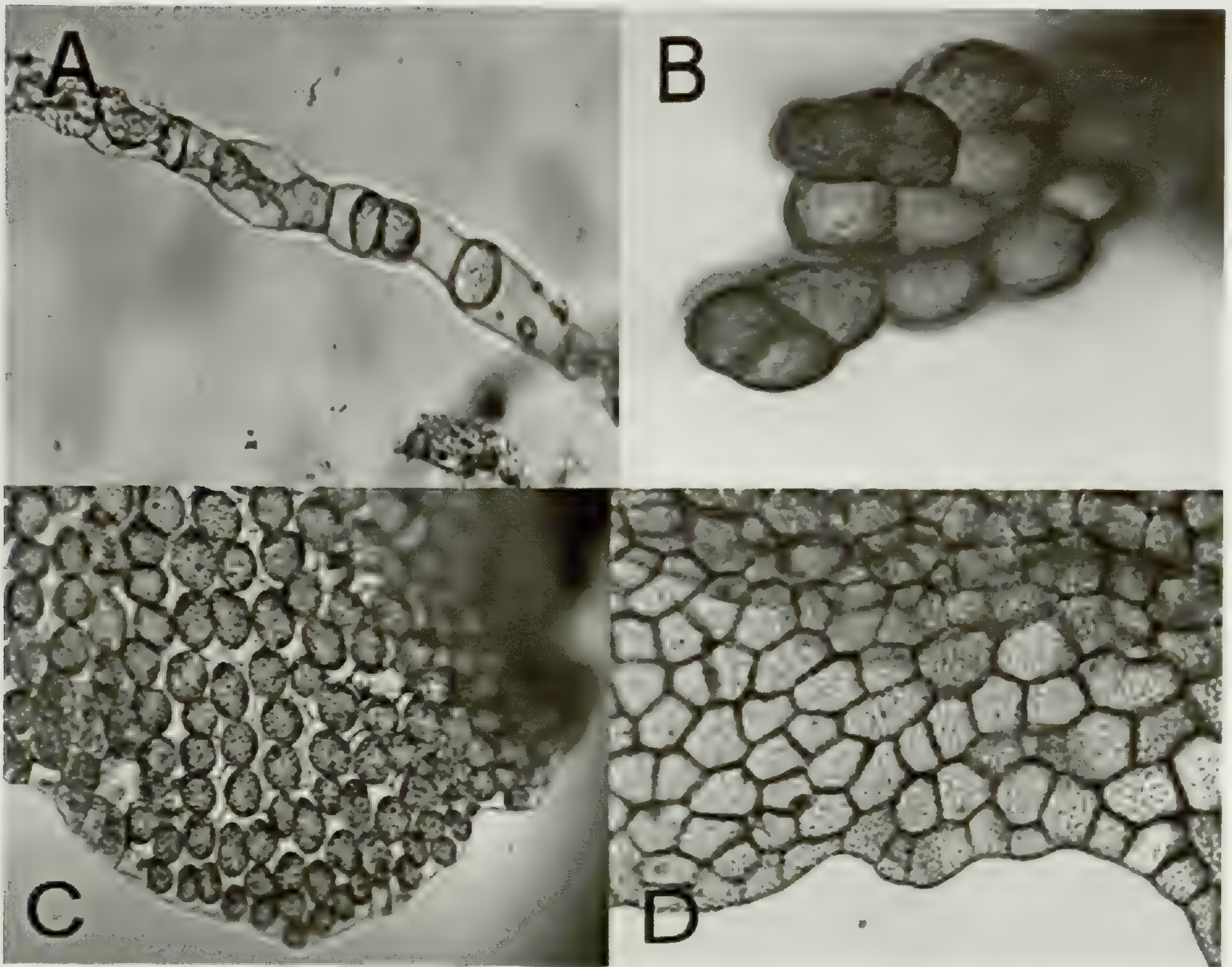


FIG. 6. Gametophytes in CaCl_2 Immediate Immersion (A) Callus plasmolyzed heavily upon immediate immersion in 500 mM CaCl_2 . Mean of callus cell volume loss in $\text{CaCl}_2 = 6.73 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.98 \cdot 10^{-4}$. (B) Callus gametophytes that were pretreated in HgCl_2 before immersion in CaCl_2 exhibited little volume flux. Mean of callus cell volume loss in treatments with HgCl_2 preincubation = $0.55 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.50 \cdot 10^{-4}$. Variation in the responses also decreased in those cells preincubated in HgCl_2 . (C) WG gametophyte cells also plasmolyzed heavily in CaCl_2 immediate immersion treatment. Mean of cell volume loss in CaCl_2 immediate immersion = $6.77 \cdot 10^{-4} \text{ cm}^{-3} \pm 2.76 \cdot 10^{-4}$. (D) WG gametophytes that were incubated in HgCl_2 prior to immediate immersion in CaCl_2 showed no visible volume loss. Mean of measured volume loss in this treatment = $0.44 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.08 \cdot 10^{-4}$.

in HgCl_2 was $0.49 \cdot 10^{-4} \text{ cm}^3 \pm 0.16 \cdot 10^{-4}$. There was also much less variation in the response with respect to volume loss. Overall, callus was the most vulnerable to desiccation from NaCl and CaCl_2 introduction. DG gametophytes were the least affected and visually seemed impervious to NaCl and CaCl_2 perturbation.

Sucrose.—Sucrose introduction following pretreatment with ddH_2O resulted in a mode of very little or no water loss in callus (Fig. 7) and WG stages (Fig. 7). Mean of callus cell volume loss was $0.24 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.98 \cdot 10^{-4}$ (Fig. 7A). Callus cells that were preincubated in HgCl_2 experienced a gain in cell volume (Fig. 7B). Mean change in cell volume gain was $0.10 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.48 \cdot 10^{-4}$. Variation in cell volume was less in treatments that included a

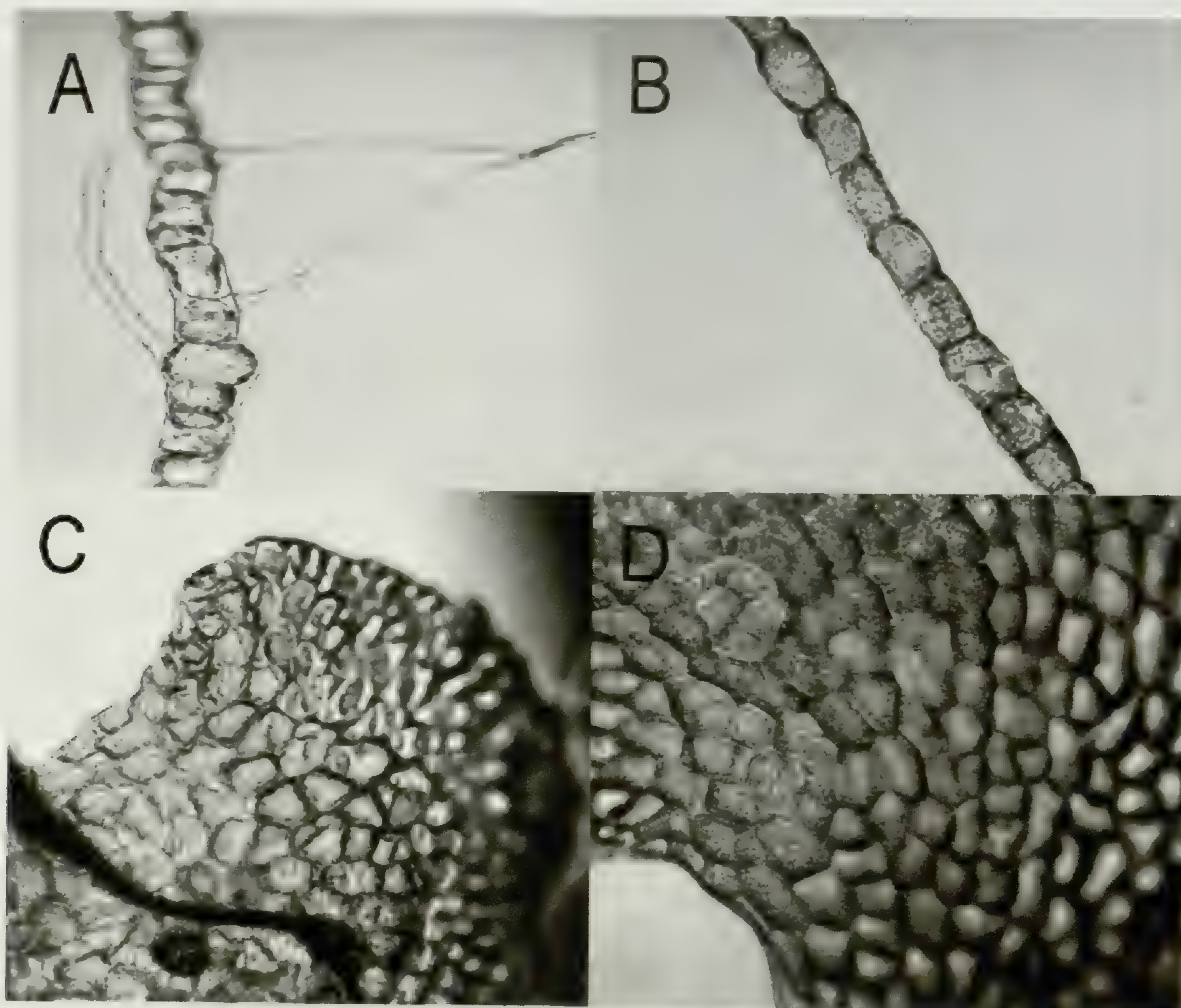


FIG. 7. Gametophytes in Sucrose Immediate Immersion (A) Callus cell moderately plasmolyzed upon immediate immersion in 500 mM sucrose. Mean of callus cell volume loss = $0.24 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.98 \cdot 10^{-4}$. (B) Callus cells immersed in 500 mM sucrose took on cell volume during treatment that included a preincubation in HgCl_2 . Mean change in cell volume = $0.10 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.48 \cdot 10^{-4}$. Variation in cell volume decreased in treatment that included a preincubation in HgCl_2 . (C) WG gametophyte cells statistically lost no cell volume in 500 mM sucrose, but that result was highly variable. Mean of cell loss when immediately immersed in sucrose = $0.91 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.00 \cdot 10^{-4}$. (D) WG gametophytes that were pretreated with HgCl_2 also showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone. Mean volume loss in this treatment = $0.93 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.24 \cdot 10^{-4}$.

preincubation in HgCl_2 than those preincubated in ddH_2O . WG gametophyte cells statistically lost no cell volume in 500 mM sucrose (Fig. 7C), but that result was highly variable. Mean of WG cell loss when immediately immersed in sucrose was $0.91 \cdot 10^{-4} \text{ cm}^{-3} \pm 3.00 \cdot 10^{-4}$. WG gametophytes that were pretreated with HgCl_2 also showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone (Fig. 7D). Mean volume loss in this treatment was $0.93 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.24 \cdot 10^{-4}$. Likewise, when WG gametophytes were pretreated with HgCl_2 , cells showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone. Mean volume loss in this treatment was $0.93 \cdot 10^{-4} \text{ cm}^{-3} \pm 0.24 \cdot 10^{-4}$. The

response to 500 mM sucrose in DG gametophyte cells (Figs. available from author) was similar to that of the WG gametophytes. There was some cell volume loss and the results were highly variable. Mean DG cell volume loss was $0.55 \cdot 10^{-4} \text{ cm}^3 \pm 5.04 \cdot 10^{-4}$. When DG gametophyte cells were preincubated with HgCl_2 , volume loss dropped dramatically with much less variation. Mean of cell volume loss in this treatment was $0.24 \cdot 10^{-4} \text{ cm}^3 \pm 0.76 \cdot 10^{-4}$.

DISCUSSION

Aquaporin-like proteins were present in all stages of gametophyte growth and play a role in water balance. Overall, HgCl_2 pretreatment inhibited water loss in all stages and even resulted in water uptake in some treatments of thinly walled protonemal cells.

Presence of an Aquaporin-like Protein

Results from ELISA controls, the preimmune serum, primary antibody only control, and secondary antibody only control, were negative. Preimmune serum, which is serum from rabbits prior to inoculation with the polypeptide that correlates with the N-terminus of aquaporins (Chaumont *et al.*, 2000), did not show any aquaporins from plants. The primary antibody bound to a single band only when followed by the secondary antibody and neither antibody bound alone. In addition, the antibody bound strongly (not shown) in ELISA and immunoblotting treatments to radish root homogenate (total protein fraction) as a positive control for the procedure. Germinating radish seeds were used as a positive control since PIP1 aquaporins are found primarily in germinating seeds and young shoots (Chaumont *et al.*, 2000). Cross reaction of anti-PIP1 antibodies and radish antigen produced a strong signal. In all gametophyte samples, signal increased linearly with the concentration of the antibody. Significant levels of signal (2–3 times control) were detected in all stages. Immunoblotting resulted in no detectable bands with the preimmune serum control, primary antibody only control, and secondary antibody only control. Only one band in the range of 28 kD, (Agre *et al.*, 1987) was observed, thereby excluding cross-reactivity with other proteins. Taken together, these data suggest that the antibody bound to only one antigen and the procedure was reasonably free of background signal. They also suggest that a PIP1 aquaporin-like protein is found in all stages of gametophyte growth.

Mercury and Aquaporin Function

PIP1 aquaporin-like protein presence and function in *Cheilanthes lanosa* gametophytes is partially responsible for water balance. Because the primary risk for gametophytes in an arid environment is desiccation (Boullard, 1979; Raven *et al.*, 2003), a test of efflux of water from three types of gametophytes was performed: the first, presumably most vulnerable stage, the protonemata [maintained on agar as callus under red light (Raghavan, 1980; Raghavan *et al.*, 1989)], the more mature gametophyte [maintained on agar and prompted to mature by blue light (Raghavan, 1980; Raghavan *et al.*, 1989)], and mature

gametophytes grown in drier conditions [on sand, erratically watered (Diamond and Swatzell, 2003)]. These were exposed to different types of solutes. NaCl and CaCl₂ were used to hyperpolarize plasma membranes and sucrose was used as an osmolyte (Lodish *et al.*, 2008). In all solutes, the gametophytes were introduced to up to 500 mM of solutes, either in one large increment or through gradual increments in molarity. Mercury pretreatments in all solutes were adequate to slow or stop water efflux for up to 1 h, even in 500 mM NaCl. Mercury is a direct aquaporin poison (Maggio and Joly, 1995; Heymann *et al.*, 1998). It binds to a cysteine within the water channel and blocks water movement (Preston *et al.*, 1993; Zhang *et al.*, 1993).

Although greater concentrations of HgCl₂ could block water movement more aggressively, mercury can also affect other physiological functions in plants (Macey, 1984). The preferred method to show aquaporin function involves the introduction of potential aquaporin mRNA into *Xenopus* oocytes. These oocytes are among the few types of cells that do not express aquaporins. Subsequent production of aquaporins in oocytes prompts water influx and cell disruption (Preston *et al.*, 1992). However, PIP1 aquaporins do not express well in *Xenopus* oocytes (Chaumont *et al.*, 2000). Therefore, a minimum of mercury was used, 1 mM HgCl₂ (Carvajal *et al.*, 1996; Maggio and Joly, 1995; Kaldenhoff *et al.*, 1995), which effectively blocked water flow for the duration of the test. Results suggest that aquaporins are responsible for the majority of water flow across *C. lanosa* plasma membranes, since without HgCl₂ in the same respective salt or sucrose solutions, cells immediately desiccate.

Cellular Control of Aquaporin-like Protein Function

Mercury treatment, however, can only show the basic function of aquaporins in gametophytes. Beyond basic function, control mechanisms can alter the way aquaporins function (Luu and Maurel, 2005). Changes in plant cell response to environmental perturbation suggest changes in control methods or activity (Luu and Maurel, 2005). For this reason, gametophytes were challenged with desiccation risk in two different ways. In the first set of tests, gametophytes were exposed to slowly increasing osmolalities. Cells were isotonic at 100 mM of NaCl. For perspective, this concentration would completely plasmolyze a tomato plant cell (Maggio and Joly, 1995). In ddH₂O, callus cells were rounded with substantial turgor. As the solute concentration increased, however, to what would be considered by most plant cells to be a devastating concentration, cells often lost water. Mercury pretreatment was able to block this loss, suggesting flow through aquaporin-like proteins. Most cells were able to avoid desiccation until they reached approximately 350 mM solute concentration. For example, after ddH₂O pretreatment, most callus cells were completely plasmolyzed by the time they reached 500 mM of solute and almost entirely desiccated, with roughly only enough volume for organelles. Desiccation occurred in the final 10–15 min as the environment solution increased beyond 350 mM of solute. However, preincubation in HgCl₂ apparently poisoned the major route of water loss. This suggests the presence

of proteins that are aquaporin-like in nature and function but does not test cellular control.

In the second set of tests, the immediate immersion tests, gametophytes were exposed to immediate and violent increases in solutes (Figs. 5–7). Gametophyte response to hyperpolarizing NaCl and CaCl₂ (Figs. 5, 6) were appropriately dramatic. Gametophytes desiccated. Due to the hyperpolarizing nature of the solutes, there should have been no control over an aquaporin-like protein (Luu and Maurel, 2005; Qui *et al.*, 2000). The channels would have opened and cells would have predictably desiccated just as the results showed. However, when presented with a true osmolyte, sucrose, the gametophytes with intact and controllable aquaporin-like proteins should have been able to alter aquaporin-like protein function and shut off water efflux (Luu and Maurel, 2005; Chaumont *et al.*, 2000). Predictably, with immediate immersion in sucrose (Fig. 7), gametophytes maintained water balance. Variation may be due to differences in the ability to defer diffusion or differences in development and physiology induced by their respective microenvironments. Therefore, the immediate immersion tests were able to demonstrate control of aquaporin-like protein function. [Higher plants control aquaporin function through phosphorylation (Maurel, 1997). Blue light also stimulates PIP function and inhibits TIP, which suggests signal transduction and cellular control (Ma *et al.*, 2001).

One exception to the differences between increments and immediate immersion experiments was the treatment of the DG gametophytes with sucrose. The treatment means were generally the same (0.0005) in preincubation with ddH₂O. However, the immediate immersion treatment is revealing. In the immediate immersion treatment, preincubation with ddH₂O, there is a huge variation in response. Yet, with preincubation of HgCl₂, there is little or no efflux. Clearly, the one difference between these two treatments is the flow of water across the membrane. The rapid influx of water may destabilize the cell and force the cell to rapidly adjust internally (Fitter and Hay, 1987; Taiz and Zeiger, 2006). The large amount of variation possibly suggests the occurrence of numerous signal transduction events or vacuolar adjustments that would characterize a stress response (Fitter and Hay, 1987; Taiz and Zeiger, 2006).

Conclusion

In the first stage of the fern life cycle, protonemal cells express aquaporins, but have thin cell walls and no cuticle. These cells are vulnerable to desiccation. They are also at risk in hypotonic situations. The one thing that protonema can control is aquaporin function. When perturbed, protonema can prevent immediate desiccation by disrupting water flow. However, the much slower osmotic diffusion would eventually result in desiccation. It is not surprising, then, that the protonemal stage thrives in sedimentary rock outcrops, which provide a small and continuous amount of water in the optimal amounts for the gametophytes (Dooley and Swatzell, 2002). WG gametophytes are somewhat

better at desiccation resistance in the face of sucrose, but membrane hyperpolarization in NaCl and CaCl₂ still promote rapid desiccation. Admittedly, a Petri dish and agar medium is atypical habitat. The effects on development and function may not be meaningful by themselves. What WG growth does show in conjunction with the DG, more typical growth, is that natural development confers resistance to desiccation. Under long term stress, regardless of the nature of the season, the mature gametophyte in its natural habitat is undeniably impervious to desiccation. This may be the fate of the *C. lanosa* gametophyte generation. The fern's ability to survive desiccation appears to be limited by that brief but vulnerable protonemal stage and by its very need for aquaporins to manage water balance and uptake. Its mechanism for water uptake can become the pathway of its water loss.

ACKNOWLEDGMENTS

Funding for this study was provided by the Grants and Research Funding Committee of Southeast Missouri State University. The authors thank Dr. Hite, Truman University, and Janese Jones for many helpful discussions.

SUPPLEMENTAL DATA WEBSITE

<http://cstl-csm.semo.edu/swatzell/AFJ/diamond2011.htm>

LITERATURE CITED

- AGRE, P., D. BROWN and S. NIELSEN. 1995. Aquaporin channels: Unanswered questions and unresolved controversies. *Current Opinion in Cell Biology* 7:472–483.
- AGRE, P., A. M. SABOORI, A. ASIMOS and B. L. SMITH. 1987. Purification and partial characterization of the M_r 30,000 integral membrane protein associated with the erythrocyte Rh(D) antigen. *Journal of Biological Chemistry* 262:17497–17503.
- BOULLARD, B. 1979. Considerations sur la Symbiose Fongique chez les Pteridophytes. *Syllogeus* 19. National Museum of Natural Science, Ottawa. pp. 1–61.
- CARVAJAL, M., D. T. COOKE and D. T. CLARKSON. 1996. Responses of wheat plants to nutrient deprivation may involve regulation of water-channel function. *Planta* 199:372–381.
- CHAUMONT, F., F. BARRIEU, R. JUNG and M. J. CHRISPEELS. 2000. Plasma membrane MIP proteins from maize cluster in two sequence subgroups with differential aquaporin activity. *Plant Physiology* 122:1025–1034.
- CHRISPEELS, M. J. and P. AGRE. 1994. Aquaporins: water channel proteins of plant and animal cells. *TIBS* 14:421–425.
- CHRISPEELS, M. J. and C. MAUREL. 1994. Aquaporins: The Molecular Basis of Facilitated Water Movement through Living Plant Cells? *Plant Physiology* 105:9–13.
- 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 L. J. SWATZELL. 2003. Cultivation of *Cheilanthes* species. http://www.american-fernsoc.org/pages/swatzell/cultivation_of_Cheilanthes_speci.htm. American Fern Society.
- 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.
- 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.
- FOTIADIS, D., P. JENOS, T. MINI, S. WIRTZ, S. MULLER, L. FRAYSSE, P. KJELLBOM and A. ENGEL. 2001. Structural characterization of two aquaporins isolated from native spinach leaf plasma membranes. *The Journal of Biological Chemistry* 19:1707–1714.
- GORIN, M. B., S. B. YANCEY, J. CLINE, J. P. REVEL and J. HORWITZ. 1984. The major intrinsic protein (MIP) of the bovine lens fiber membrane: Characterization and structure based on cDNA cloning. *Cell* 39:49–59.
- HEVLY, R. H. 1963. Adaptations of cheilanthoid ferns to desert environments. *Journal of the Arizona Academy of Science* 2:164–175.
- HEYMANN, J. B., P. AGRE and A. ENGEL. 1998. Progress on the structure and function of aquaporin 1. *Journal of Structural Biology* 121:191–206.
- 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. *Plant Cell* 8(7):1181–1191.
- JOHANSSON, I., M. KARLSSON, V. K. SHUKLA, M. J. CHRISPEELS, C. LARSSON and P. KJELLBOM. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. *The Plant Cell* 10:451–459.
- JOHNSON, K. and M. CHRISPEELS. 1991. Tonoplast-bound protein kinase phosphorylates tonoplast intrinsic protein. *Plant Physiology* 100:1787–1795.
- KALDENHOFF, R., A. KŠLLING and G. RICHTER. 1993. A novel blue light- and abscisic acid- inducible gene of *Arabidopsis thaliana* encoding an intrinsic membrane protein. *Plant Molecular Biology* 23:1187–1198.
- 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.
- KUWAHARA, M., Y. GU, K. ISHIBASHI, F. MARUMO and S. SASAKI. 1997. Mercury-sensitive residues and pore site in AQP3 water channel. *Biochemistry* 36(46):13975–13978.
- 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, Missouri. April.
- LODISH, H., A. BERK, S. L. ZIPURSKY, P. MATSUDAIRA, D. BALTIMORE and J. DARNELL. 2000. *Molecular Cell Biology*, 4th ed. W. H. Freeman, New York, New York. 1150 pp.
- LUU, D.-T. and C. MAURELL. 2005. Aquaporins in a challenging environment: molecular gears for adjusting plant water status. *Plant, Cell and Environment* 28:85–96.
- MA, L., J. LI, L. QU, J. HAGER, Z. CHEN, H. ZHAO and X. W. DENG. 2001. Light control of *Arabidopsis* development entails coordinated regulation of genome expression and cellular pathways. *The Plant Cell* 13:2589–2607.
- MACEY, R. I. 1984. Transport of water and urea in red blood cells. *American Journal of Physiology* 246(Cell Physiology 15):C195–C203.
- MAGGIO, A. and R. J. JOLY. 1995. Effects of mercuric chloride on the hydraulic conductivity of tomato root systems. *Plant Physiology* 109:331–335.
- MAUREL, C., R. T. KADO, J. GUERN and M. J. CHRISPEELS. 1995. Phosphorylation regulates the water channel activity of the seed-specific aquaporin α -TIP. *The EMBO Journal* 14:3028–3035.
- MAUREL, C. 1997. Aquaporins and water permeability of plant membranes. *Annual review of Plant physiology and Plant Molecular Biology* 48:399–429.
- MICKEL, J. T. 1979. *How to Know the Ferns and Fern Allies*. WCB McGraw-Hill, St. Louis, MO. 229 pp.
- MOHLENBROCK, R. H. 1959. Plant communities in Jackson County, Illinois. *Bulletin of the Torrey Botanical Club* 86(2):109–119.
- 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.
- PHILIP, J. R. 1958. Osmosis and diffusion in tissue: Half-times and internal gradients. *Plant Physiology* 45:275–278.
- PICKETT, F. L. 1931. Notes on xerophytic ferns. *American Fern Journal* 21(2):49–57.

- PRESTON, G. M., T. P. CARROLL, W. B. GUGGINO and P. AGRE. 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* 256:385–387.
- PRESTON, G. M., J. S. JUNG, W. B. GUGGINO and P. AGRE. 1993. The mercury-sensitive residue at cysteine 189 in the CHIP28 water channel. *The Journal of Biological Chemistry* 268:17–20.
- QUI, Q. S., Z. Z. WANG, N. ZHANG, Q. G. CAI and R. X. JIANG. 2000. Aquaporins in the plasma membrane of leaf callus protoplasts of *Actinidia deliciosa* Var. *deliciosa* cv. Hayward. *Australian Journal of Plant Physiology* 27:71–75.
- 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* 62:69–118.
- RAGHAVAN, V. 1989. Developmental biology of fern gametophytes. Cambridge University Press, Cambridge, MA.
- RAVEN, P. H., R. F. EVERT and S. E. EICHHORN. 2003. The biology of plants. 6th Edition. WH Freeman and Company.
- SAMBROOK, J., E. F. FRITCH and T. MANIATIS. 1989. Molecular Cloning: A Laboratory Manual. Volume 3. 2nd edition. Cold Spring Harbor Laboratory Press.
- STEIL, W. N. 1933. New cases of apogamy in certain homosporous leptosporangiate ferns. *Botanical Gazette* 95:164–167.
- STEIL, W. N. 1939. Apogamy, apospory and parthenogenesis in the pteridophytes. *The Botanical Review* 8:433–453.
- STEUDLE, E. 1992. The biophysics of plant water: compartmentation, coupling with metabolic processes, and water flow in plant roots. In *Water and Life: A Comparative Analysis of Water Relationships at the Organismic, Cellular, and Molecular Levels*. Springer-Verlag, Berlin. pp. 173–204.
- STEUDLE, E. and C. PETERSON. 1998. How Does Water Get Through Roots? *Journal of Experimental Botany* 49(322):775–788.
- STOSCHEK, C. M. 1990. Quantitation of Proteins. *Methods in Enzymology: Guide to Protein Purification* 182:50–60.
- SMITH, R. H. 1992. Plant Tissue Culture: Techniques and Experiments. Academic Press, Inc, San Diego, CA. 231 pp.
- SUGA, S., S. KOMATSU and M. MAESHIMA. 2002. Aquaporin isoforms responsive to salt and water stresses and phytohormones in radish seedlings. *Plant Cell Physiology* 43(10):1229–1237.
- TAIZ, L. and E. ZEIGER. 2006. Plant Physiology. 4th Edition. Sinauer Associates, Inc., Sunderland, Massachusetts.
- TYERMAN, S. D., H. J. BOHNERT, E. STEUDLE and J. A. SMITH. 1999. Plant aquaporins: Their molecular biology, biophysics and significance for plant water relations. *Journal of Experimental Biology* 50:1–30.
- VANDELEUR, R., C. NIEMIETZ, J. TILBROOK and S. D. TYERMAN. 2005. Role of aquaporins in responses to root irrigation. *Plant and Soil* 274:141–161.
- VOICU, M. C., J. E. K. COOKE and J. J. ZWIAZEK. 2009. Aquaporin gene expression and apoplastic water flow in bur oak (*Quercus macrocarpa*) leaves in relation to the light response of hydraulic conductance. *Journal of Experimental Botany* 60(14):4063–4065.
- ZHANG, R., A. N. VAN HOEK, J. BIWERSI and A. S. VERKMAN. 1993. A point mutation at cysteine 189 blocks the water permeability of rat kidney water channel CHIP28k. *Biochemistry* 32:2938–2941.

The Effects of Exogenous Cytokinin on the Morphology and Gender Expression of *Osmunda regalis* Gametophytes

GARY K. GREER*, MARGARET A. DIETRICH, JOSEPH A DEVOL, and APRIL REBERT

Grand Valley State University, Biology Department, 1 Campus Drive, Allendale, Michigan 49401

ABSTRACT.—The goal of this study was to determine the function of cytokinin in the morphological development and gender expression of gametophytes of *Osmunda regalis*, a member of Osmundales, sister-group to all other extant leptosporangiate fern families. Gametophytes of *Osmunda regalis* were grown in multispore populations on C-fern nutrient enriched agar containing 0, 1 nM, 1 μ M, and 1 mM kinetin. Higher concentrations of exogenous kinetin reduced gametophyte size (area), disrupted correlations observed in the control between rates of apical notch formation and thallus widening, increased the proportions of asexuals and males, decreased the proportion of females in the population, and correspondingly increased male reproductive effort (antheridia per unit thallus area) and decreased female reproductive effort (archegonia per unit thallus area) compared to the control. Low concentrations of exogenous kinetin increased the proportion of females compared to the control. In the control, thalli with a comparatively deep apical notch tended to be wider (relative to their length) and possess a more circular silhouette relative to thalli with a comparatively shallow apical notch; however, these morphological parameters were independent of gametophyte size and gender. Thus, variance in the rate and planes of cell division and patterns of cell expansion and differentiation, most likely genetic in basis, were observed in the control, and the observed effects of exogenous kinetin were more than a simple “push” towards a phenotype already present in the control.

KEY WORDS.—cytokinin, development, kinetin, leptosporangiate, fern, gametophyte, morphology, *Osmunda regalis*, gender expression

Current models of phytohormonal control of seed plant shoot apical meristems (SAM) emphasize the role of cytokinin (Stahl and Simon, 2010; Kurakawa *et al.*, 2007; Kyojuka, 2007; Sablowski, 2007; Hwang and Sakakibara 2006; Kepinski, 2006; Shani *et al.*, 2006; Hudson, 2005; Rashotte *et al.*, 2005; Higuchi *et al.*, 2004). Cytokinin maintains indeterminism and stimulates cell division in SAM and apical dominance and other source sink relationships. Cytokinins are also involved in a wide range of developmental processes including vascular development and senescence (Mok and Mok, 2001), phyllotaxy (Guilini *et al.*, 2004), cotyledon expansion (Stoynova-Bakalova *et al.*, 2003; Huff and Ross, 1975), and chloroplast maturation (Stetler and Laetsch, 1965), and they are involved in a wide range of responses to abiotic and biotic stimuli, including light responses, drought resistance, ion uptake, pathogen defense and symbiont interaction (Werner and Schmülling, 2009). In contrast, cytokinins have the opposite developmental role in the root apical

*Author for correspondence

meristem, where they control root meristem size via regulation of auxin distribution (which stimulates cell proliferation and maintains totipotency in the root apical meristem (RAM); Stahl and Simon, 2010; Růžička *et al.*, 2009), stimulate elongation and differentiation in the transition zone (Moubayidin *et al.*, 2009; Ioio *et al.*, 2008; Kyojuka, 2007), and stimulate root nodulation (Frugier *et al.*, 2008). Plant reproduction is also governed by SAM behavior; however, mechanisms governing gender expression and reproductive effort (unit investment into reproduction per unit vegetative investment) vary widely among and within plant groups (Tanurdzic and Banks, 2004). In taxa where phytohormones rather than sex-determining genes control gender expression, cytokinins are associated with reproduction (e.g., flowering) and femaleness (Tanurdzic and Banks, 2004; Khryanin, 2002; Lejeune *et al.*, 1988).

In mosses, cytokinins also play a key role in SAM behavior in the gametophyte, governing the transition from a filamentous protonema to a 3-dimensional leafy thallus (Cove *et al.*, 2006). Whereas picomolar concentrations of kinetin initiate the formation of caulonema initials that produce filamentous growth, micromolar concentrations stimulate the assembly of 3-dimensional buds in *Funaria hygrometrica* Hedwig (Schumaker and Dietrich, 1997; Bopp and Jacob, 1986). This change in morphology was initiated by a dramatic swelling of the initial and a subsequent change from periclinal to oblique and anticlinal divisions. Our search of the literature failed to find studies investigating the role of cytokinins in moss reproduction.

Among the majority of leptosporangiate families, morphological development of the gametophyte proceeds from spore germination through filamentous and spathulate forms culminating in a cordate form that exhibits taxon-specific propensities for subsequent elongation and branching (Nayar and Kaur, 1971). The transition from a spathulate to a cordate form reflects an increase in the rate of anticlinal divisions (i.e., perpendicular to the apical surface), as opposed to periclinal and oblique divisions, in a single plane within the single-celled or pluricellular apical meristem and prolonged meristematic activity by its derivatives (von Aderkas and Cutter, 1983). Formation of the apical notch characteristic of a cordate morphology is reproductively significant in the taxa in which it occurs as it always precedes female gender expression (i.e., production of archegonia). In species with an antheridiogen system, sensitivity to antheridiogen diminishes with the onset of an apical notch. Thus, to the extent that cytokinin influences the rate and orientation of cell division of the apical meristem, it governs morphological form and gender expression in leptosporangiate fern gametophytes.

Cytokinins have been identified in leptosporangiate fern sporophytes (Stirk and van Staden, 2003 and citations therein); however, our search of the literature did not find any reports regarding cytokinin functions in these plants. Similarly, we found only two reports regarding the role of cytokinins in leptosporangiate gametophyte development (Menendez *et al.*, 2009; Spiro *et al.*, 2004). Pico and nanomolar concentrations of kinetin (BAP, 6-Benzylaminopurine) induced formation of the notch meristem *Ceratopteris richardii* Brongn. grown in the dark, resulting in a decrease in thallus length:

width ratio (Spiro *et al.*, 2004), partially overcoming etiolation. Exogenous cytokinin did not accelerate the rate of reproductive maturation in this study; however, the possibility of a delay in reproductive maturity or effects on gender expression and reproductive effort (gametangia per unit thallus area) were not explored. In contrast, micromolar concentrations of exogenous BAP delayed formation of an apical notch and production of gametangia in light-grown gametophytes of *Blechnum spicant* (L.) Smith (Menendez *et al.*, 2009). In the same study, endogenous levels of six cytokinins were found to be higher in female than in male gametophytes in this species. These observations establish the presence of cytokinins in sporophytes and gametophytes of leptosporangiate ferns, the influence of light on cytokinin response by gametophytes, and that, like seed plants, cytokinins are associated with the maintenance of an apical meristem and female gender expression of gametophytes.

The study we report here investigated the effects of exogenous kinetin on morphological development, gender expression, and reproductive effort in *Osmunda regalis* L., a member of Osmundales, sister-group to all other extant leptosporangiate ferns (Smith *et al.*, 2006; Pryer *et al.*, 2004). Our goal was to generate information regarding cytokinin that may be useful in reconstructing the evolution of developmental mechanisms in leptosporangiate fern gametophytes.

MATERIALS AND METHODS

Spores of the fern *O. regalis* were collected from a minimum of three sporophytes growing in Pigeon Creek Park, Ottawa County, Michigan, and stored in aggregate for 10 days at 3°C. Preliminary experiments using spore sterilization and antibiologics (Nystatin® and streptomycin) resulted in reduced germination and atypical morphology, namely branched rather than a non-branched, globular form. Thus, the green spores of this species were repeatedly centrifugally rinsed, but, not surface sterilized in our study. No bacterial, fungal, or algal contaminations were observed throughout the study.

Concentrations (1 nM, 1 µM, and 1 mM) of kinetin were prepared with C-fern nutrient media (Carolina Biological Supply) and 1.5% agar with the control group containing only nutrient media. Each treatment was replicated five times. Spores were sown at an approximate density of four per cm² and the plates arranged in a fully randomized design under full spectrum lights producing light levels of approximately 29.5 µmol⁻¹, m⁻², sec⁻¹ at dish level using a 16 hour light: 8 hour dark regime at 20°C.

Data collection.—Six weeks after sowing, gametophytes were harvested haphazardly from the lowest density neighborhoods within each plate, totaling 30–35 gametophytes per treatment. Although efforts were made to create an even density of gametophytes within each plate, variation occurred. Gametophytes in high-density neighborhoods are subject to competitive interactions resulting in reduced growth rate (Greer, 1993; Huang *et al.*, 2004) and more vulnerable to damage during harvest and were therefore avoided. The harvested gametophytes were fixed with Clarion® mounting

medium as semi-permanent slides. Dissecting microscopes were used under uniform magnification to digitally photograph each gametophyte. Morphological traits were measured in pixels using the trace measurements feature in SigmaScan Pro 5.0 (Fox and Urich, 1993) following the methodology of Greer and Curry (2004) and included thallus area (size), thallus length, thallus width and notch depth. Thallus length extends from the caudal "tail" to the apical notch. Notch depth was measured as an extension of the thallus length line terminated by a line that connects the anterior apex of each thallus lobe. Thallus width was measured as the longest possible line perpendicular to the thallus length line. Silhouettes are shown at the same scale; scale shown with silhouette taken from 1 nM kinetin treatment. Three morphological metrics, thallus length / width ratio (LW), notch depth / thallus length ratio (NDL), and shape factor ($SF = \text{circularity} = 4\pi \times (\text{object area} / \text{perimeter}^2)$; 0 = line, 1.0 = a perfect circle) were used to assess developmental status, namely development of an apical notch. As a fern gametophyte develops, its NDL and SF are expected to increase and its LW decrease as a notch-bearing apical meristem develops and, subsequently, widening growth outpaces lengthening growth. Although included here for comparative purposes, Greer *et al.* (2009) rejected shape factor as a reliable metric of developmental status. Each gametophyte was also scored for the number of antheridia and archegonia present.

Data analyses.—One-way parametric or non-parametric (Kruskal-Wallis) ANOVA were used to test for treatment effects on thallus size and shape. Bonferroni multiple comparisons were used following significant parametric ANOVA and Tamhane's T2 multiple comparisons were used following significant Kruskal-Wallis ANOVA. Spearman's ranked correlations were used to compare relationships among thallus size and the three morphological metrics within each kinetin treatment group.

Chi-square tests of independence including all four kinetin concentrations were used to test for treatment effects on gender ratios. When a multi-treatment chi-square was found significant, pair-wise chi-square tests of independence were performed between all six treatments pairings. Parametric and Kruskal-Wallis ANOVA were used to test for treatment effects on numbers of antheridia and archegonia per gametophyte after adjusting for thallus size and each of the three morphological metrics; i.e., size and morphologically-based measures of reproductive effort. Bonferroni and Tamhane's multiple comparisons were used accordingly.

Assumptions of normality and homogeneity were tested using Kolmogorov-Smirnov's and Levene's tests respectively. P-values were considered significant when ≤ 0.05 and marginally significant when between 0.05 and 0.10. When warranted, P-values were adjusted using Bonferroni-Holm's correction for multiple comparison error rates.

RESULTS

Treatment effects on morphology.—All gametophytes were cordate at harvest. Of the morphological traits assessed, only gametophyte area differed

among kinetin treatments (Table 1). Gametophytes exposed to the highest kinetin concentration (1 mM) were approximately 50% smaller than gametophytes all other treatment groups (Fig. 1a, e–h). Visually consistent but statistically non-significant declines in LW and SF, and to a lesser extent NDL, were observed with increasing kinetin concentration (Fig. 1b–h).

Thallus area did not correlate with the three morphological metrics within any treatment group. Among the three morphological metrics, correlations involving NDL were the strongest and most frequently significant, whereas correlations involving SF were the weakest and least frequently significant (Table 2). Thallus NDL correlated negatively with LW in all treatments (maximum $R^2 = -0.404$ in the 1nM Kinetin treatment) except within the highest (1 mM) kinetin treatment. Similarly, NDL correlated negatively with SF in the control and lowest (1 nM) kinetin treatment (Table 2). LW correlated positively with SF (maximum $R^2 = 0.258$) only in the 1 μ M kinetin treatment. Thus, control thalli possessing deeply formed apical notches tended to have wider thallus widths relative to their lengths and more linear silhouettes than thalli with shallowly formed apical notches (Fig. 2), and these relationships weakened (i.e., became non-significant) at higher (1 μ M and 1 mM) kinetin concentrations (Fig. 1).

Of the three metrics used to assess the morphology of *O. regalis* gametophytes, NDL correlated more strongly with LW and SF, than either of the latter with one another. A similar observation was made by Greer *et al.* (2009) in assessing gametophyte morphology of *O. regalis* and *Athyrium filix-femina* (L.) Mertens, thus, NDL appears to be the most reliable of these metrics for assessing the developmental status of leptosporangiate species with gametophytes that form an apical-notch. However, each metric provides a slightly different insight and we recommend using all three metrics when assessing gametophyte morphology and reproductive effort.

TABLE 1. ANOVA of morphological traits of *Osmunda regalis* gametophytes exposed to 0, 1 nM, 1 μ M, and 1 mM concentrations of kinetin.

Parametric ANOVA	Groups	Df	MS	F	P
Thallus Area	Between	3	5.31×10^{11}	40.04	<0.001
	Within	128	1.32×10^{10}		
	Total	131			
Thallus Length / Width (LW)	Between	3	0.006	1.88	0.135
	Within	128	0.003		
	Total	131			
Notch depth / Thallus Length (NDL)	Between	3	0.001	0.039	0.990
	Within	128	0.012		
	Total	131			
Thallus Shape Factor (SF)	Between	3	0.006	1.32	0.271
	Within	128	0.004		
	Total	131			

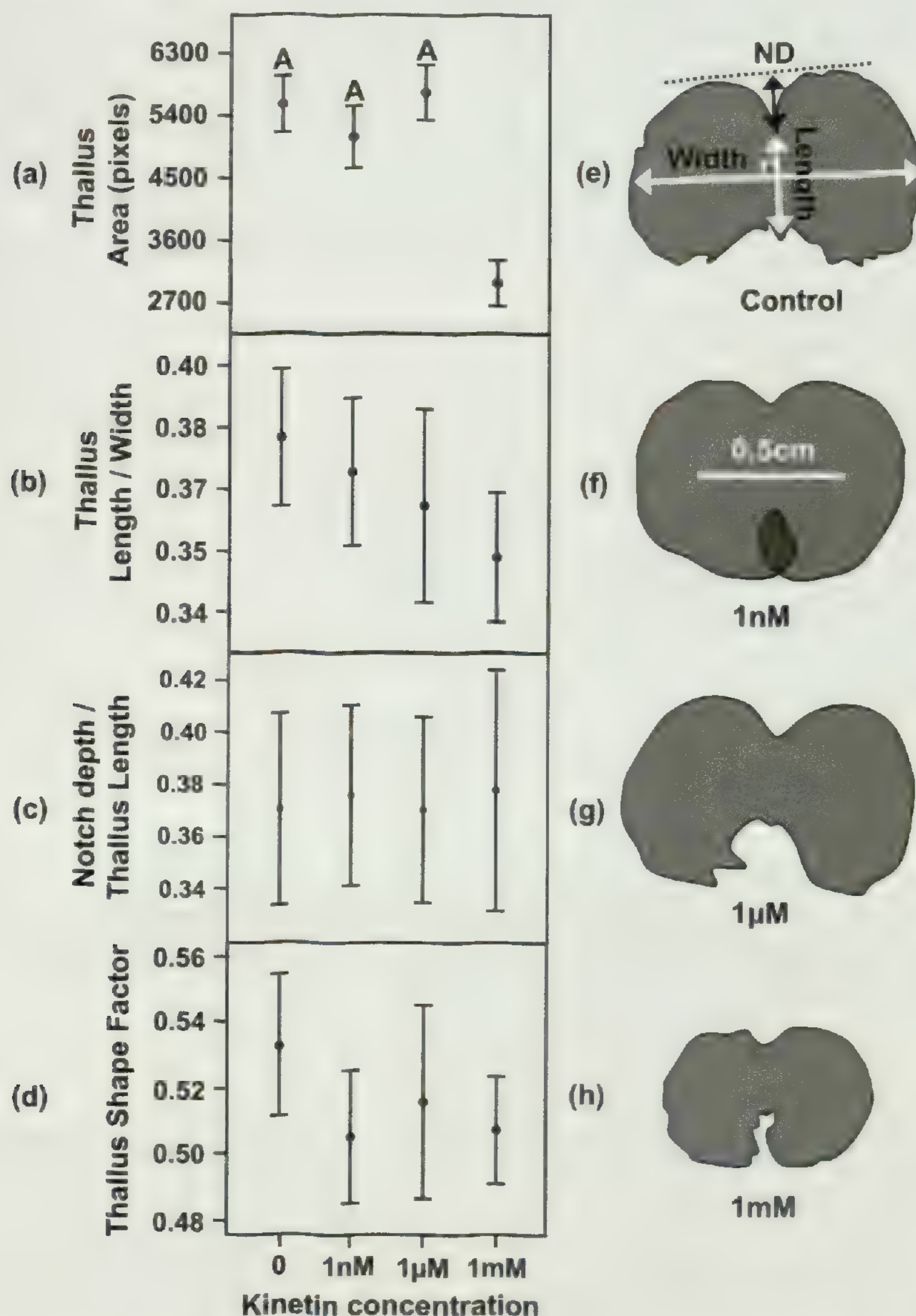


FIG. 1. Morphological measurements (a–d) and representative silhouettes (e–i) of *O. regalis* gametophytes measured in pixels in response to 0 (control), 1 nM, 1 μ M, and 1 mM kinetin treatments. Treatments with the same letter are not significantly different ($P > 0.05$) from one another based on Bonferroni multiple comparison tests (a–d). Linear morphological measurements are shown with the silhouette taken from the control and are described in the methods.

Treatment effects on reproductive traits.—No differences in thallus area, ND, LW or SF were observed among gender categories within the control or 1 mM kinetin treatments (Table 3).

Gender ratios differed only between the highest (1 mM) kinetin treatment group and all lower treatment groups with one exception (Fig. 3). The proportion of asexuals and males in the 1 mM kinetin treatment group increased significantly relative to the control by 48% and 876% (0.48 and 8.76-fold) increase, respectively (Fig. 3). Expected values for males in the lower kinetin treatment groups fell below five therefore results from chi-square tests for this gender should be viewed with caution. Correspondingly, the

TABLE 2. Spearman's ranked correlation coefficients (R^2) between morphological traits among *Osmunda regalis* gametophytes exposed to 0 (control), 1 nM, 1 μ M and 1 mM kinetin treatments. Asterisks indicate P -value ≤ 0.05 . N = number of gametophytes per treatment group.

	Thallus Length / Width (LW)	Notch Depth / Thallus Length (NDL)	Thallus Shape Factor (SF)
Control (N = 42)			
Area	-0.022	-0.021	-0.0003
LW		-0.320*	0.058
NDL			-0.149*
1 nM Kinetin (N = 26)			
Area	-0.011	0.027	-0.032
LW		-0.404*	0.258*
NDL			-0.309*
1 μ M Kinetin (N = 32)			
Area	-0.230*	-0.003	-0.016
LW		-0.325*	0.001
NDL			-0.076
1 mM Kinetin (N = 32)			
Area	-0.0007	-0.041	0.005
LW		0.047	0.082
NDL			-0.062

proportions of females in the 1 mM kinetin treatment group decreased significantly (58%) relative to the control (Fig. 3). The only other kinetin treatment with detectable effects on gender expression was the lowest (1 nM) kinetin treatment, where the proportion of females was marginally ($P < 1.0$) greater than in the control or 1 mM kinetin treatment (Fig. 3). Differences in the proportion of hermaphrodites were non-significant among all treatment groups.

Significant differences in the number of antheridia per antheridium-bearing gametophyte were detected only when corrected for thallus area (Table 4, Fig. 4a–d). Gametophytes in the 1 mM treatment groups possessed more antheridia after correcting for size than in all lower kinetin treatment groups (Fig. 4a). Similarly, gametophytes in the second highest (1 μ M) kinetin treatment group possessed more antheridia than those in the control (Fig. 4a).

In contrast, no significant differences in the number of archegonia per archegonium-bearing gametophyte were observed when corrected for thallus area, but were observed when corrected for each of the three morphological metrics (Table 5, Fig. 4e–h). Gametophytes in the 1mM treatment group possessed fewer archegonia, when corrected for NDL, LW or SF, than those in the control and lower kinetin treatments (Table 5, Fig. 4e–h). Interpretation of gametophyte reproductive effort is straightforward when gametangium production is adjusted for thallus area; however, adjustments using morphological metrics that are themselves fractions (e.g., number of archegonia / (notch depth / thallus length)) require careful interpretation as there are three means by which such a measure of reproductive effort can change in the same

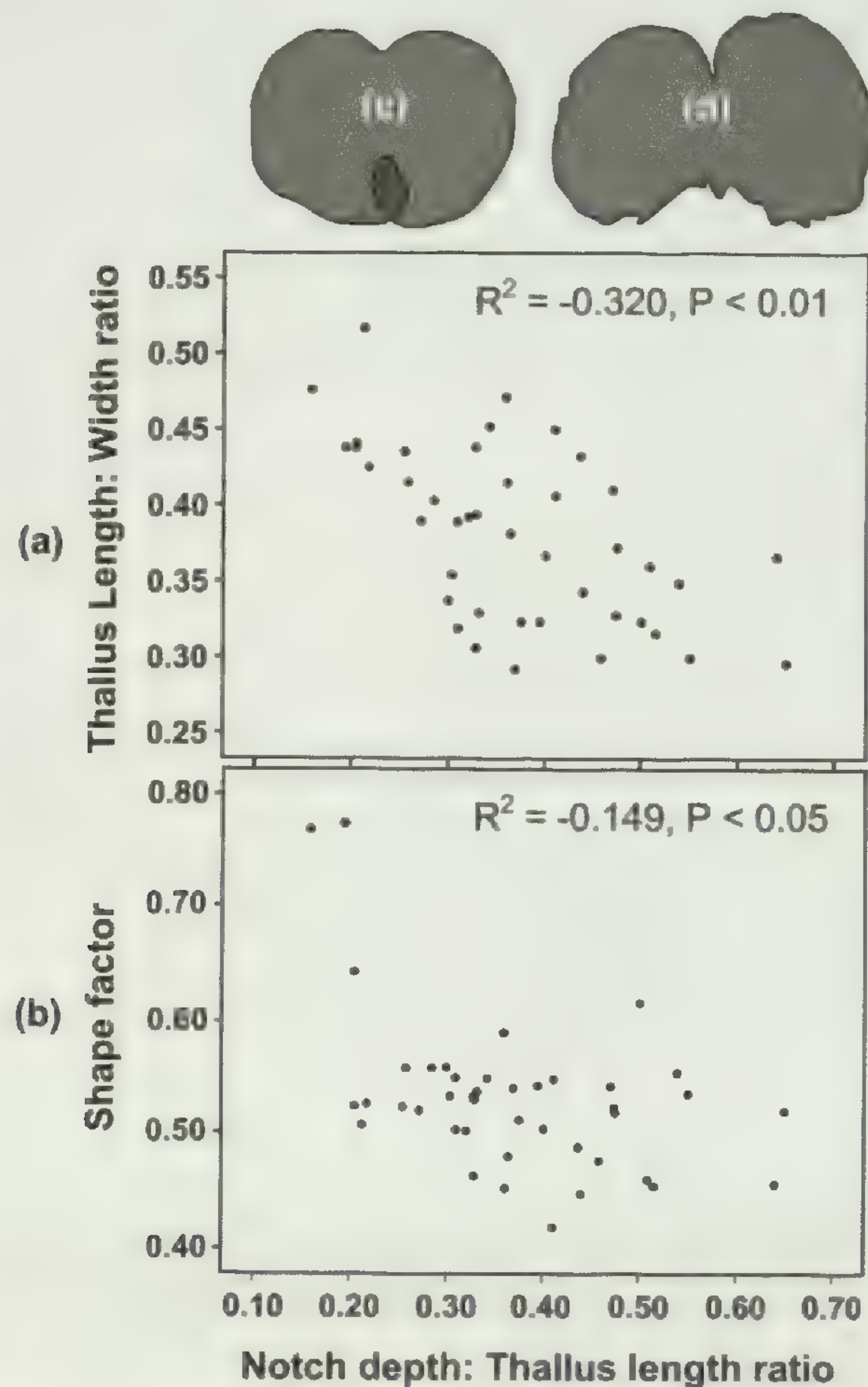


FIG. 2. Significant correlations among morphological metrics within the control. Scatterplots of notch depth: thallus length ratio versus (a) thallus length: width ratio and (b) shape factor. R^2 and P -values taken from Table 2. Silhouettes of representative thalli possessing comparatively low NDL and high LW and SF (c) and comparatively high NDL and low LW and SF (d); shown at same scale.

direction. Three comparisons between the morphological and reproductive data support the conclusion that the decline in the 1 mM kinetin treatment in archegonium production when corrected for morphological status reflects a biologically meaningful decrease in reproductive effort: (1) thallus LW declined (visually) in stepwise manner (Fig. 1b), whereas archegonia / LW remained essentially constant among the lower kinetin treatments and declined 51% in the 1 mM kinetin treatment relative to the control (Fig. 4b); (2) NDL showed no visual change with increasing kinetin concentration (Fig. 1c) yet archegonia / NDL declined 46% in the 1 mM treatment relative to the control (Fig. 4c); (3) SF decline began (visually) with the 1 nM kinetin treatment (Fig. 1d), whereas archegonia / SF declined 53%, relative to the control, only at 1mM kinetin treatment (Fig. 4d).

DISCUSSION

At the higher concentrations used in this study (1 μ M and 1 mM), exogenous kinetin reduced the size (area) of *O. regalis* gametophytes, disrupted the

TABLE 3. Kruskal-Wallis ANOVA of thallus size and morphology among gender categories in the control and 1 mM kinetin treatments. Only one male occurred within the control; however, exclusion of males did not change the outcome of the analysis and were therefore retained here. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

Trait	Gender	Control				1 mM Kinetin			
		N	Mean rank	χ^2	P	N	Mean rank	χ^2	P
Area	Asexual	8	19.25	3.506	0.320	9	14.44	0.742	0.863
	Male*	1	21.00			7	16.29		
	Female	22	20.18			7	18.14		
	Hermaphrodite	11	24.00			9	17.44		
NDL	Asexual	8	22.00	2.037	0.565	9	13.67	0.714	0.870
	Male*	1	15.00			7	16.43		
	Female	22	21.23			7	19.86		
	Hermaphrodite	11	22.27			9	16.78		
LW	Asexual	8	17.88	1.005	0.800	9	14.67	0.793	0.793
	Male*	1	24.00			7	17.00		
	Female	22	21.77			7	19.29		
	Hermaphrodite	11	23.36			9	15.78		
SF	Asexual	8	21.63	0.349	0.951	9	18.22	1.726	0.631
	Male*	1	11.00			7	17.00		
	Female	22	23.50			7	16.29		
	Hermaphrodite	11	18.34			9	14.56		

*Exclusion of males did not change the significance of these analyses and were therefore retained.

positive correlation between apical notch formation (NDL) and the rate of thallus widening (LW and SF) observed in the control, increased the proportions of asexuals and males and decreased the proportion of females in the population, and correspondingly increased male reproductive effort and decreased female reproductive effort. In the control, thalli with a comparatively deep apical notch tended to be wider (relative to length) and possess a more circular silhouette relative to thalli with a comparatively shallow apical notch; however, morphological status was independent of gametophyte size. These observations reveal variance, potentially genetic in basis, in the prevailing rate and planes of cell division and patterns of cell expansion and differentiation. Variations in gametophyte size and morphology in the control were not associated with differences in gender, therefore the effects of exogenous kinetin treatment on notch development, thallus widening, gender expression, and reproductive effort were more than a simple “push” towards a phenotype already present in the control. In contrast, Huang *et al.* (2004) observed the following size hierarchy among one year-old gametophytes of *Osmundastrum cinnamomeum* (L.) C. Presl: female – hermaphrodite – male – asexual, with females three times larger than hermaphrodites. *Osmundastrum* is sister to all other genera within extant Osmundales (Metzgar *et al.*, 2008). Assuming *O. regalis* exhibits similar gender-based size hierarchies at the same age and densities as *Osmundastrum*, latent differences in size or, correspondingly,

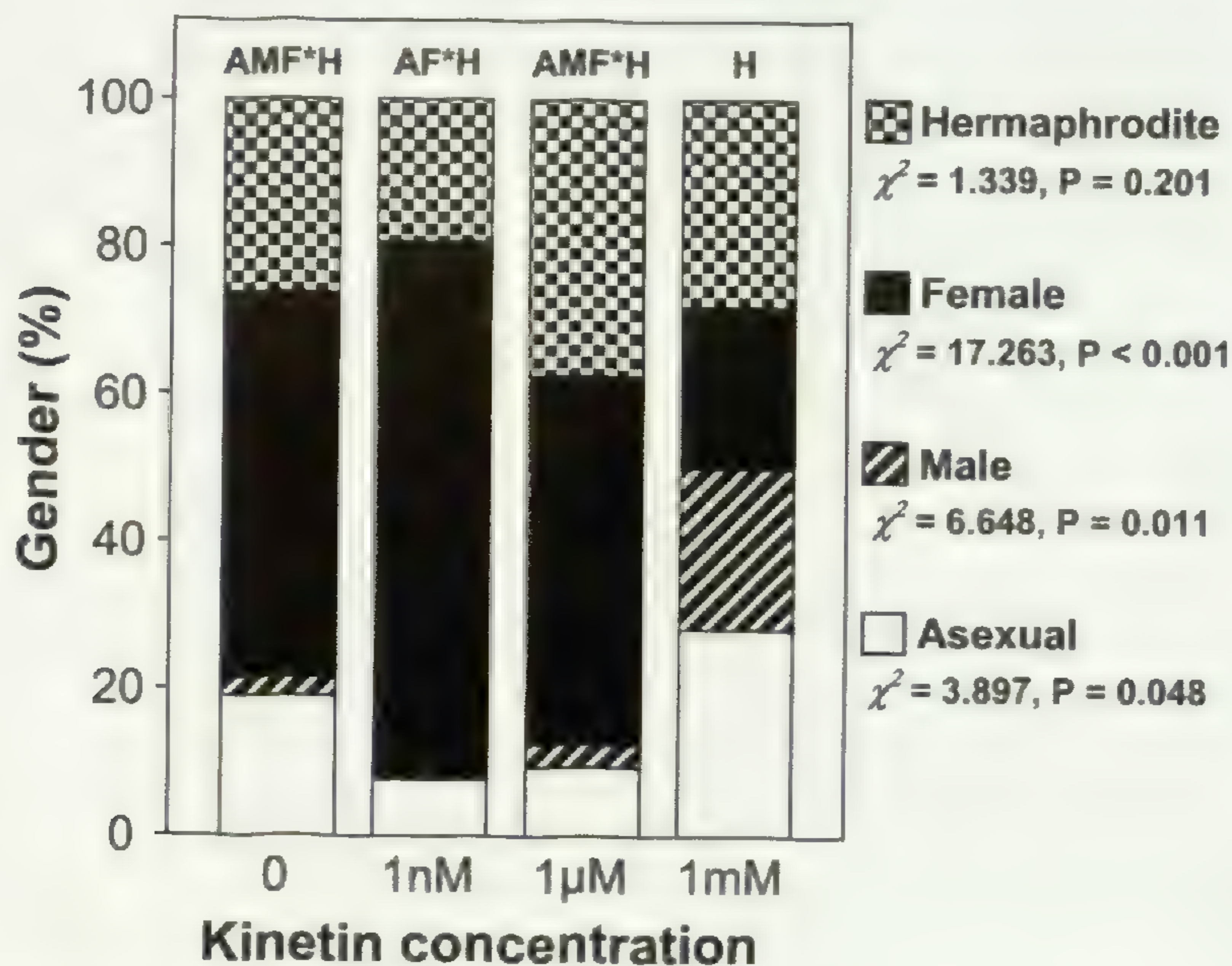


FIG. 3. Gender ratios in multisporous populations of *O. regalis* exposed to 0 (control), 1 nM, 1 μ M, and 1 mM kinetin treatments. Results for chi-square tests of independence for each gender across all kinetin treatments are listed below each legend. Treatments that possess the same letter are not significantly different from one another ($P > 0.05$) based on pair-wise chi-square tests of independence following Bonferroni-Holm's correction for multiple comparison error rates; however, the proportion of female gametophytes was marginally greater ($P < 0.10$) in the 1 nM kinetin treatment than in the control and 1 μ M kinetin treatment, as indicated by the asterisk.

growth rate in six week-old gametophytes have on gender expression increase with gametophyte age.

Production of archegonia in *O. regalis*, as with all known cordate-forming leptosporangiate ferns, is preceded by the formation of a pluricellular apical meristem and corresponding apical notch. Reduction of female reproductive effort by exogenous kinetin was largely independent of thallus area and closely associated with measures of cordate morphology (i.e., NDL, LW and SF), reflecting the activity of the apical meristem.

In contrast, the stimulating effect of high levels of exogenous kinetin on the frequency of males and on male reproductive effort (i.e., the rate of antheridium production) was associated with its reducing effect on thallus area. All gametophytes in this experiment were observed only once, six-weeks after spore sowing, and all were cordate, therefore the timing of antheridium development relative to the development of an apical notch is unknown. The sequence of gender expression in Osmundales is poorly known. A male to hermaphrodite sequence was reported for *Osmundastrum cinnamomea* (Huang *et al.*, 2004) which, as noted above is sister to all other extant genera within Osmundales, and *O. regalis* (Klekowski, 1973), and *Todea barbara* (L.) T. Moore (von Aderkas and Cutter, 1983); however, the relationship between apical notch formation and antheridium production was not reported in these studies. In a time-series study of multisporous populations of congeners *O. lancea* Thunb. and *O. japonica* Houtt., Hiyama *et al.* (1992) observed antheridia only after, or corresponding to, the formation of a cordate

TABLE 4. ANOVA of morphologically adjusted rates of antheridium production among male and hermaphrodite *O. regalis* gametophytes exposed to 0 (control), 1 nM, 1 μ M and 1 mM concentrations of kinetin. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

Parametric ANOVA	Groups	df	MS	F	P
Antheridia / NDL	Between	3	4.917	0.306	0.821
	Within	42			
	Total	45			
Kruskal-Wallace ANOVA	Kinetin Concentration	N	Mean Rank	χ^2	P
Antheridia / Area	0	12	14.75	15.56	0.002
	1 nM	5	17.20		
	1 μ M	13	22.15		
	1 mM	16	33.13		
Antheridia / LW	0	12	18.17	2.856	0.414
	1 nM	5	23.80		
	1 μ M	13	24.38		
	1 mM	16	26.69		
Antheridia / SF	0	12	20.00	1.380	0.710
	1 nM	5	22.80		
	1 μ M	13	26.15		
	1 mM	16	24.19		

morphology. Similarly antheridia were observed only after the formation of an apical notch in a time-series of *O. cinnamomeum* isolates, (Hollingsworth *et al.*, in press). Thus, antheridium production in *Osmundastrum* and at least two species of *Osmunda* appears to be dependent upon the formation of an apical notch. If cordate dependence of antheridium production exists in *O. regalis* as well, it would explain why the effect of exogenous kinetin on antheridium production was independent of NDL, LW or SF. The rate of antheridium production in *O. regalis* also appears to be largely independent of NDL, LW and SF among cordate gametophytes, but is dependent on area as evidenced in the control. This may reflect the fact that antheridia were produced in the basal region, the region most independent of the influence of the apical meristem. As was observed in the control, variations in size and morphology were not associated with differences in gender in the highest (1 μ M) kinetin treatment. Thus, the effects of kinetin on female and male reproductive efforts appear to be independent of latent, potentially genetic, differences in the rates of growth and development. The absence of males in the lowest kinetin treatment (1 nM) group and corresponding increase in the proportion of females relative to the control and 1 μ M kinetin treatment supports the hypothesis that cytokinin effects on gender expression are concentration dependent.

The morphological and reproductive changes in *O. regalis* induced by high levels of exogenous kinetin are most readily explained by one or more of the following effects on the apical meristem: (1) an increase in the ratio of anticlinal versus periclinal-oblique divisions, (2) reduced expansion of derivatives, and (3) a delay in the differentiation of the derivatives.

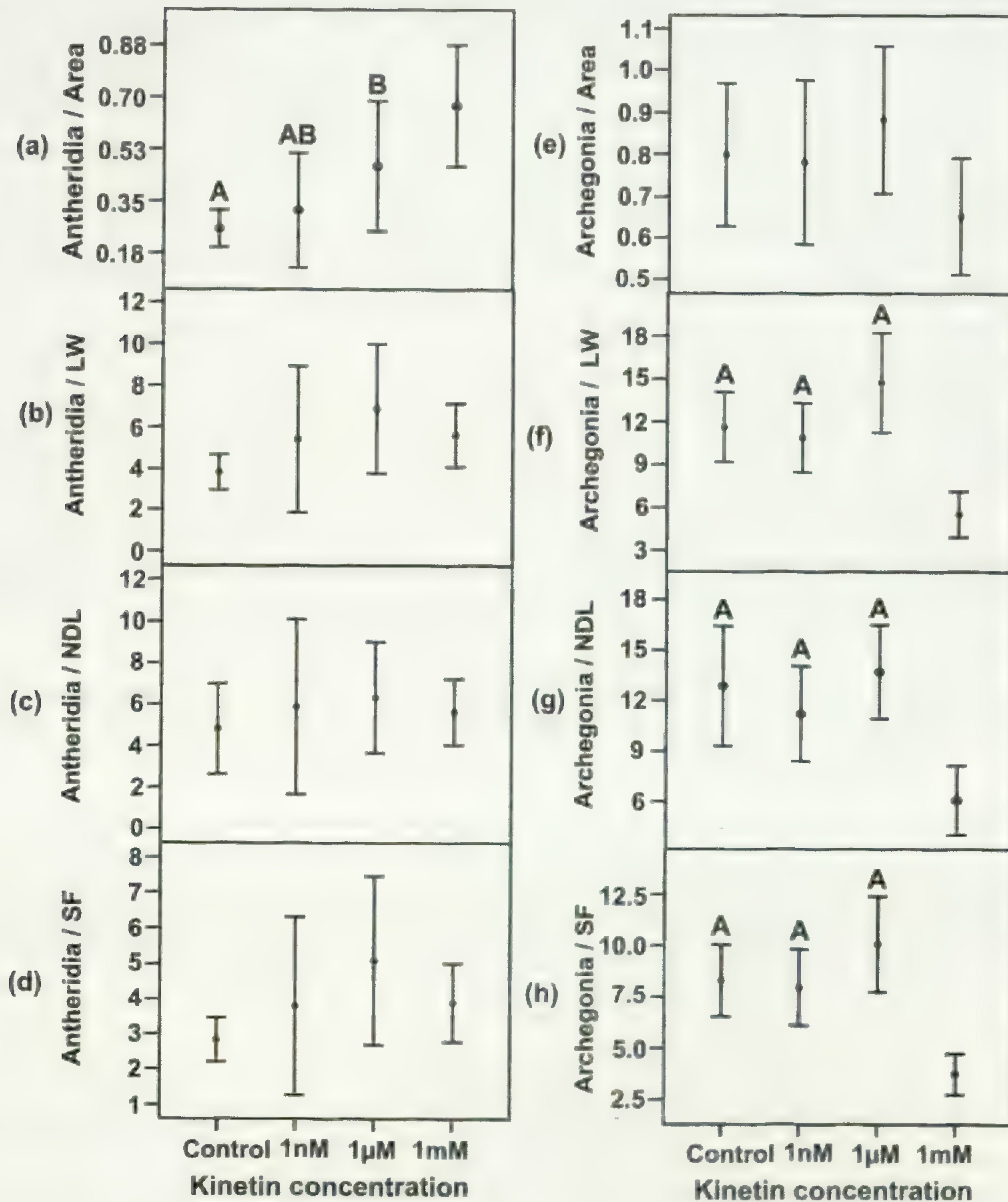


FIG. 4. Production of gametangia per *O. regalis* gametophyte after correcting for size (area) and morphology in response to 0 (control), 1 nM, 1 μ M, and 1 mM kinetin treatments. Archegonia per pixel area was multiplied 100 fold for visual purposes. Treatments that possess the same letter are not significantly different ($P > 0.05$) from one another based on Bonferroni and Tamhane's T2 multiple-comparisons tests.

Greer *et al.* (2009) also increased the cytokinin: gibberellin ratio in *O. regalis*; however, they did so by lowering endogenous gibberellin levels. Nevertheless, they also observed an increase in the proportion of asexual and male gametophytes, suggesting that the cytokinin: gibberellin ratio may be more important than absolute phytohormone levels in determining the specifics of cell division and differentiation. If a high cytokinin: gibberellin ratio is necessary for production of archegonia, then these observations emphasize that exogenous cytokinins do not precisely mimic the effects of endogenous cytokinins as revealed by the control in the present study.

In contrast to the present study using kinetin, exogenous cytokinin (BAP) accelerated the development of an apical notch and significantly decreased

TABLE 5. ANOVA of morphologically adjusted rates of archegonium production among female and hermaphrodite *O. regalis* gametophytes exposed to 0 (control), 1 nM, 1 μ M, and 1 mM concentrations of kinetin. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

Parametric ANOVA	Groups	df	MS	F	P
Archegonia / Thallus Area	Between	3	0.185	0.891	0.449
	Within	97			
	Total	100			
Kruskal-Wallis ANOVA	Kinetin Concentration	N	Mean Rank	χ^2	P
Archegonia / LW	0	33	52.15	14.52	0.002
	1 nM	24	52.33		
	1 μ M	28	62.04		
	1 mM	16	27.31		
Archegonia / NDL	0	33	69.57	26.24	<0.001
	1 nM	24	77.08		
	1 μ M	28	82.56		
	1 mM	16	37.81		
Archegonia / SF	0	33	53.64	15.71	0.001
	1 nM	24	53.13		
	1 μ M	28	60.71		
	1 mM	16	25.38		

LW in dark-grown gametophytes of *Ceratopteris richardii* (Spiro *et al.*, 2004). Similarly, exogenous BAP reduced LW and delayed the production of both antheridia and archegonia in *Blechnum spicant* and endogenous levels of six cytokinins were higher in females than in males (Menendez *et al.*, 2009). The different results between these studies of core-leptosporangiate species and the present study of a member of the Osmundales may underscore phylogenetically relevant differences in phytohormonal controls of development.

ACKNOWLEDGMENTS

We wish to thank Sheila Blackman and two anonymous reviewers for useful comments on earlier versions of this manuscript.

LITERATURE CITED

- BOPP, M. and JACOB, H. J. 1986. Cytokinin effect on branching and bud formation in *Funaria*. *Planta* 169:462–464.
- COVE, D., M. BEZANILLA, P. HARRIES and R. QUATRANO. 2006. Mosses as model systems for the study of metabolism and development. *Annu. Rev. Plant Bio.* 57:497–520.
- FOX, E. C. and G. URICH. 1993. *Sigma-scan user's manual*. Jandel Scientific, San Rafael, California.
- FRUGIER, F., S. KOSUTA, J. D. MURRAY, M. CRESPI and K. SZCZYGLOWSKI. 2008. Cytokinin: secret agent of symbiosis. *Trends Plant Sci.* 13:115–120.
- GREER, G. K. and D. CURRY. 2004. Pheromonal interactions among cordate gametophytes of the lady fern, *Athyrium filix-femina*. *Am. Fern J.* 94:1–8.
- GREER, G. K., M. DIETRICH, S. STEWART, J. DEVOL and A. REBERT. 2009. Morphological functions of gibberellins in leptosporangiate fern gametophytes: insights into the evolution of form and gender expression. *Bot. J. Linn. Soc.* 159:599–615.

- GREER, G. K. 1993. The influence of soil topography and spore-rain density on gender expression in gametophyte populations of the homosporous fern *Aspidotis densa* (Brack. in Wilkes) Lellinger. *Am. Fern J.* 84:54–59.
- HOLLINGSWORTH, S., E. ANDRES and G. GREER. In press. Pheromonal Interactions Among Gametophytes of *Osmundastrum cinnamomeum* and the Origins of Antheridiogen Systems in Leptosporangiate Ferns. *Int. J. Plant Sci.*
- GUILINI, A., J. WANG and D. JACKSON. 2004. Control of phyllotaxy by the cytokinin-control response regulator homolog *ABPHYL1*. *Nature* 430:1031–1034.
- HIGUCHI, M., M. S. PISHKE, M. P. MÄHÖNEN, K. MIYAKAWI, Y. HASHIMOTO, M. SEKI, M. KOBAYASHI, K. SHINOZAKI, T. KATO, S. TABATA, Y. HELARRIUTA, M. SUSSMAN and T. KAKIMOTO. 2004. *In planta* functions of the *Arabidopsis* cytokinin receptor family. *Proc. Nat. Acad. Sci. USA* 101:8821–8826.
- HIYAMA, T., R. IMAICHI and M. KATO. 1992. Comparative development of gametophytes of *Osmunda lancea* and *O. japonica* (Osmundaceae): Adaptation of Rheophillous gametophyte. *The Botanical Magazine, Tokyo* 105:215–225.
- HUANG, Y., H. CHOU and W. CHIOU. 2004. Density affects gametophyte growth and sexual expression in *Osmunda cinnamomea* (Osmundaceae, Pteridophyta). *Ann. Bot.* 94:229–232.
- HUDSON, A. 2005. Plant meristems: mobile mediators of cell fate. *Curr. Biol.* 15:803–805.
- HUFF, A. K. and C. W. ROSS. 1975. Promotion of radish cotyledon enlargement and reducing sugar content by zeatin and red light. *Plant Physiol.* 56:429–433.
- HWANG, I. and H. SAKAKIBARA. 2006. Kinetin biosynthesis and perception. *Physiol. Plantarum* 126:528–538.
- IOIO, R. D., F. S. LINHARES and S. SABATINI. 2008. Emerging role of cytokinin as a regulator of cell fate. *Curr. Opin. Plant Biol.* 11:23–27.
- KEPINSKI, S. 2006. Integrating hormone signaling and patterning mechanisms in plant development. *Curr. Opin. Plant Biol.* 9:28–34.
- KHYRANIN, V. N. 2002. Role of phytohormones in sex determination in plants. *Russ. J. Plant Physiol.* 49:608–613.
- KLEKOWSKI, E. J. JR. 1973. Genetic load in *Osmunda regalis* populations. *Am. J. Bot.* 60:146–154.
- KURAKAWA, T., N. UEDA, M. MAEKAWA, K. KOBAYASHI, M. KOSHIBA, Y. NAGATO, H. SAKAKIBARA and J. KYOSUKA. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445:652–655.
- KYOZUKA, J. 2007. Control of shoot and root meristem function by cytokinin. *Curr. Opin. Plant Biol.* 10:442–446.
- LEJEEUNE P., J. KINET and G. BERNIER. 1988. Cytokinin fluxes during floral induction in the long day plant *Sinapis alba* L. *Plant Physiol.* 86:1095–1098.
- MENENDEZ, V., M. A. REVILLA, M. A. FAL and H. FERNANDEZ. 2009. The effect of cytokinins on growth and sexual organ development in the gametophyte of *Blechnum spicant* L. *Plant Cell Tiss. Org.* 96:245–250.
- METZGAR, J. S., J. E. SKOG, E. A. ZIMMER and K. M. PRYER. 2008. The Paraphyly of *Osmunda* is Confirmed by Phylogenetic Analyses of Seven Plastid Loci. *Syst. Bot.* 33:31–36.
- MOK, D. and M. C. MOK. 2001. Kinetin metabolism and action. *Annu. Rev. Plant Physiol.* 52:89–118.
- MOUBAYIDIN, L., R. DI MAMBRO and S. SABATINI. 2009. Cytokinin-auxin crosstalk. *Trends Plant Sci.* 14:557–562.
- NAYAR, B. K. and S. KAUR. 1971. Gametophytes of homosporous ferns. *Bot. Rev.* 37:295–396.
- PRYER, K. M., E. SCHUETTPLEZ, P. G. WOLF, H. SCHNEIDER, A. R. SMITH and R. CRANFILL. 2004. Phylogeny and evolution of ferns (monilphytes) with a focus on the early leptosporangiate divergences. *Am. J. Bot.* 91:1582–1598.
- RASHOTTE, A. M., H. SOOK, B. B. MAXWELL and J. J. KIEBER. 2005. The interaction of kinetin with other signals. *Physiol. Plantarum* 123:184–194.
- RŮŽIČKA, K., M. ŠIMÁŠKOVÁ, J. DUCLERCQ, J. PETRÁŠEK, E. ZAŽIMALOVÁ, S. SIMON, J. FRIML, M. C. E. VAN MONTAGU and E. BENKOVA. 2009. *Proc. Nat. Acad. Sci.* 106:4284–4289.
- SABLOWSKI, R. 2007. The dynamic stem cell niches. *Curr. Opin. Plant Biol.* 10:639–644.
- SCHRAUDOLF, H. 1964. Relative activity of the gibberellins in the antheridium induction of *Anemia phillitidis*. *Nature* 201:98–99.

- SCHUMAKER, K. S. and M. DIETRICH. 1997. Programmed changes in form during moss development. *The Plant Cell* 9:1009–1107.
- SHANI, E., O. YANAI and N. ORI. 2006. The role of hormones in shoot apical function. *Curr. Opin. Plant Biol.* 9:484–489.
- SMITH, A. R., K. M. PRYER, E. SCHEUTTPELZ, P. KORRALL, H. SCHNEIDER and P. WOLF. 2006. A classification for extant ferns. *Taxon* 55:705–731.
- SPIRO, M. D., B. TORABI and C. N. CORNELL. 2004. Kinetins induce photomorphogenic development in dark-grown gametophytes of *Ceratopteris richardii*. *Plant Cell Physiol.* 45(9):1252–1260.
- STAHL, Y. and R. SIMON. 2010. Plant primary meristems: shared functions and regulatory mechanisms. *Curr. Opin. Plant Biol.* 13:53–58.
- STETLER, D. A. and W. M. LAETSCH. 1965. Kinetin-induced chloroplast maturation in cultures of tobacco tissue. *Science* 149:1387–1388.
- STIRK, W. A. and J. VAN STADEN. 2003. Occurrence of cytokinin-like compounds in to aquatic ferns and their exudates. *Environ. Exp. Bot.* 49:77–85.
- STOYONOVA-BAKALOVA, E., E. KARANOV, P. PETROV and M. A. HALL. 2003. Cell division and cell expansion in cotyledons of *Arabidopsis* seedlings. *New Phytol.* 162:471–479.
- TANURDZIC, M. and J. A. BANKS. 2004. Sex-determining mechanisms in plants. *The Plant Cell* 16:61–71.
- VON ADERKAS, P. and E. G. CUTTER. 1983. The role of the meristem in gametophyte development of the osmundaceous fern *Todea barara* (L.) Moore. *Bot. Gaz.* 144:519–524.
- WERNER, T. and T. SCHMÜLLING. 2009. Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12:1–12.

Optimization of Protocol for Isolation of Genomic DNA from Leaves of *Selaginella* Species Suitable for RAPD Analysis and Study of their Genetic Variation

SAYANTANI DAS, MAUMITA BANDYOPADHYAY, and SUBIR BERA*

Centre of Advanced Studies, Department of Botany, University of Calcutta, 35, B.C.Road, Kolkata- 700019, India

ABSTRACT.—A simple and efficient protocol for isolating genomic DNA from leaves of *Selaginella* spp. (*S. delicatula*, *S. repanda*, *S. bryopteris*, *S. plana*, *S. monospora*) was developed, involving a modified CTAB protocol of Rogers and Benedich (1994). Increasing the incubation time with the precipitation buffer (1X CTAB) from 1–3 hours to 12–14 hours helped achieve higher quantity genomic DNA from the specimens, when compared with DNA extracted by protocols reported by Dellaporta *et al.* (1983), Murray and Thompson (1980) and Doyle and Doyle (1987). The DNA yield ranged from 846–1836 µg/ml from fresh and herbaria-preserved leaf samples. The DNA samples were found suitable for genetic diversity analysis with Random Amplified Polymorphic DNA (RAPD) markers. Nine random primers (OPA A17, OPB 4, OPB13, OPC 2, OPC 11, OPD 5, OPG 2, OPG 19 and OPK 10) were studied, of which two primers (OPD 5 and OPG 2) yielded reproducible amplification profile of polymorphic fragments.

KEY WORDS.—DNA extraction, RAPD, *Selaginella*, modified protocol

Selaginella (spike moss) is an enigma in the plant kingdom. At present only one genus is recognized in the Selaginellaceae, i.e., *Selaginella* (Family Selaginellaceae, Order Selaginellales, Class Lycopsidea). The genus *Selaginella* is cosmopolitan in distribution and contains approximately 700 species that include temperate, tropical, frost-tolerant arctic, and drought-tolerant desert species. Such extremes are very rarely found in the same genus, and hence the family Selaginellaceae has been treated differently and sub-divided into myriad taxa by researchers (Spring, 1850; Braun, 1857; Baker, 1883; Hieronymus, 1901; Walton and Alston, 1938; Jermy, 1986).

Selaginella shows morphological variation within species and as such it is difficult to distinguish species depending on traditional morphology only. Thus, researchers have concentrated on molecular phylogenetic analysis to gain information about its evolutionary relationships. A recent molecular phylogenetic analysis of the genus has revealed that rates of molecular evolution among species are remarkably high, including when compared to the angiosperm families (Korall and Kenrick, 2004). Although many subtle morphological and developmental differences exist between species, few of these differences are phylogenetically useful markers for classifying the species in a way that is consistent with molecular data (Korall and Kenrick

* Corresponding author: berasubir@yahoo.co.in

2002, 2004). In this perspective, studies involving isolation and characterization of DNA are very useful, since they open up the possibility for detection of an evolutionary pattern that implies both morphological and genetic changes. Molecular marker based phylogenetic studies (e.g., RAPD, ISSR, SNP, etc.) have been utilized in complementing and supplementing morpho-taxonomy in many cases. The success of these procedures relies on inexpensive, rapid and simple DNA extraction methods (Weishing *et al.*, 1995), as they require large amounts of high quality genomic DNA.

The main aim of the present study is to evaluate different protocols of DNA isolation and to standardize a protocol for obtaining better DNA yield and amplification quality for RAPD analysis from milligram amounts of living and herbarium *Selaginella* leaf specimens. The use of dried herbarium specimens is essential due to the unavailability of suitable quantity of required plant material from the living plant materials. The detriment is that satisfactory quantity and quality of DNA from herbarium specimens cannot be obtained due to the rapid degradation of plant material during preservation. DNA isolation from dried specimens usually requires some modifications to frequently used protocols (Rogers, 1994) to ensure quality DNA extraction from even very small amounts of dry herbarium tissues are available. Here we report a DNA isolation protocol from milligram amounts of both living and herbarium plant materials that has been standardized and proved to be suitable for RAPD analysis.

MATERIALS AND METHODS

Sample collection.—*Selaginella* samples including *S. delicatula* (Desv. ex Poir) Alston, *S. repanda* (Desv. ex Poir) Spring, *S. bryopteris* (L.) Baker, *S. plana* (Desv. ex Poir) Hieron., *S. monospora* Spring were collected from the Darjeeling district (West Bengal) and Nainital (Uttarakhand) regions of India. Herbarium specimens were obtained from the Calcutta University Herbarium (CUH).

Genomic DNA isolation.—Genomic DNA was extracted from fresh and dried leaf samples using several reported protocols, including those of Dellaporta *et al.* (1983) (Protocol 1); Doyle and Doyle (1987) (Protocol 2); Murray and Thompson (1980) (Protocol 3); Rogers and Benedich (1994) (Protocol 4). A modified Rogers and Benedich (1994) protocol was devised to increase DNA yield. The essential modification is an increase in the incubation time which involves incubation of supernatant containing DNA after a second chloroform-isoamyl alcohol extraction with 1X CTAB overnight, instead of 1–3 hours as reported in the original protocol, and an additional step of RNase treatment was added, when required.

The modified protocol of Rogers and Benedich, 1994 (Protocol 5) that was standardized is as follows: fresh leaves (0.5 g) frozen at -20°C for 7–15 days or herbarium or dried leaves of *Selaginella* (0.2 g) were freeze dried in liquid nitrogen and crushed in a mortar and pestle. The crushed powder was transferred to a 50 ml tube and mixed with 2X CTAB extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP) with

β -mercaptoethanol, warmed at 55°C before use. The mixture was incubated while shaking at 66°C for 45 minutes. Then an equal volume of chloroform: isoamyl alcohol (24:1) was mixed and gently shaken for 10 minutes at room temperature. The mixture was centrifuged for 20 minutes at 10,000 rpm and the supernatant was recovered to which 1/10 volume of 10% CTAB warmed at 55°C was added. Equal volume of chloroform:isoamyl alcohol (24:1) was added again and mixed gently. The mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was pipetted into a new tube and 2 volumes of 1X CTAB were added to it; after mixing, it was incubated overnight at room temperature. The mixture was centrifuged for 20 minutes at 10,000 rpm. The pellet thus obtained was dissolved in high salt TE to which 2 volumes of ice-cold ethanol were added and the mixture was incubated overnight at -20°C, and later centrifuged at 10,000 rpm for 10 minutes. The pellet was washed with cold 80% ethanol before centrifugation for 5 minutes at 10,000 rpm. The pellet containing nucleic acids thus obtained was dried and redissolved in 30 μ l TE and stored.

In the case of DNA extraction from fresh *Selaginella* specimens, an additional step of RNase treatment was required. The RNase treatment was not an essential step for the herbarium specimens as RNA interference is absent in the case of preserved specimens.

The extracted genomic DNA was tested for purity index (A_{260}/A_{280} absorbance ratio) on UV-VIS Spectrophotometer and for size, purity and integrity on 1% agarose gel at 80V for 40 minutes.

Polymerase Chain Reaction.—PCR reactions for RAPD analysis were performed in a 25 μ l volume containing 100 ng genomic template DNA, 2.5 μ l of reaction buffer, 100 mM dNTP mix, 2.5 ng primer (random primer, Operon Technologies), and 3 U/ μ l *Taq* polymerase. Amplification was performed in a Gradient Thermal Cycler (Eppendorf). The reaction mixtures were amplified in an initial step of 94°C for 3 min and then subjected to 35 cycles of the following program: 94°C for 1 min, 37°C for 1 min, 72°C for 1 min. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide and photographed on a UV transilluminator. Amplification products generated by a few decamer primers from OP series (Operon Technologies) are presented in the current study.

RESULTS

When a modified version of the original CTAB protocol (Rogers and Benedich, 1994) was used for the extraction of DNA from the dried herbarium and living leaves of different species of *Selaginella*, DNA yield and quality was significantly increased. The extraction protocol of Dellaporta *et al.* (1983) and Doyle and Doyle (1987) did not yield quantifiable amounts of genomic DNA, while the extraction protocol of Murray and Thompson (1980) yielded comparatively less quantifiable DNA, which was not suitable for RAPD analysis. The spectrophotometric results for the five different species of

TABLE 1. Yield of genomic DNA measured by quantity and purity index of *Selaginella* obtained from five different species using different protocols.

Plant sample	Modified Rogers and Benedich protocol (1994)		Original Rogers and Benedich protocol (1994)		Murray and Thompson protocol (1980)	
	Quantity ($\mu\text{g/ml}$)	Purity index (A_{260}/A_{280} ratio)	Quantity ($\mu\text{g/ml}$)	Purity index (A_{260}/A_{280} ratio)	Quantity ($\mu\text{g/ml}$)	Purity index (A_{260}/A_{280} ratio)
<i>Selaginella delicatula</i>	1164	1.84	486	1.92	294	1.36
<i>Selaginella bryopteris</i>	924	1.69	390	1.71	246	1.41
<i>Selaginella plana</i>	1836	2.01	696	1.96	366	1.60
<i>Selaginella monospora</i>	846	1.71	318	2.65	276	1.53
<i>Selaginella repanda</i>	912	1.87	450	2.08	234	1.34

Selaginella obtained using the original protocols of Rogers and Benedich (1994), Murray and Thompson (1980) and the modified protocol of Rogers and Benedich (1994) are given in Table 1.

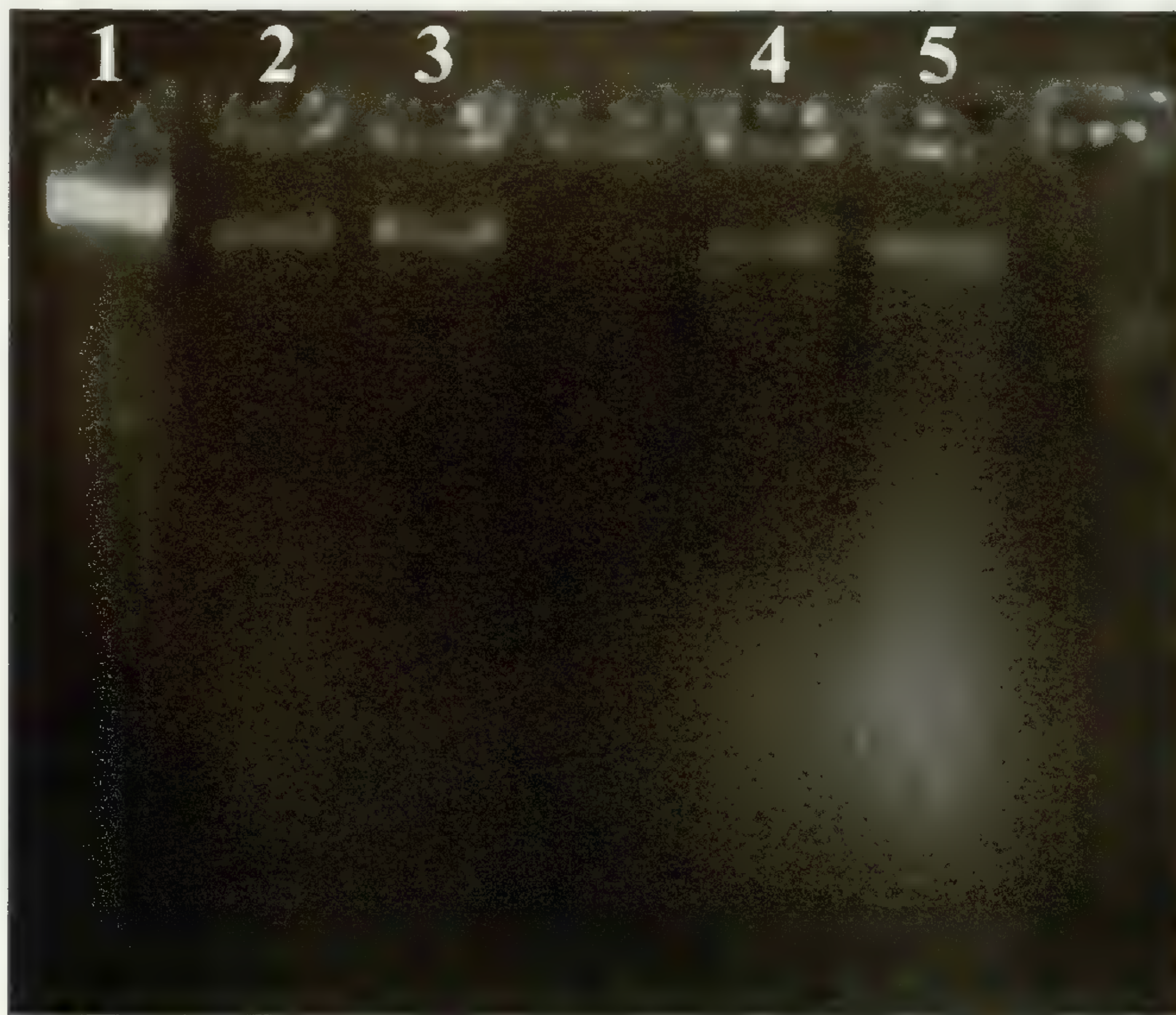
For RAPD analysis, initial PCR amplification of genomic DNA from the five species of *Selaginella* was done. Among the nine random primers tried, two primers from OP series (OPD 5 and OPG 2) were selected for RAPD analysis which could amplify the template DNA from the five leaf samples of *Selaginella* using PCR. From the study we obtained a number of RAPD bands from two different primers for the samples of *Selaginella* studied (Table 2).

DISCUSSION

A cost effective DNA extraction procedure greatly facilitates genetic diversity analysis. The present study demonstrates that the DNA extraction procedure significantly affects yield and quality in *Selaginella*, as well as the

TABLE 2. Primers used in RAPD analysis.

Primer	Sequence	Total number of amplified bands	Total number of polymorphic bands	Percentage of polymorphic bands (%)
OPA A17	5'- GAG CCC GACT - 3'	Absent		
OPB 4	5'- GGA CTG GAGT - 3'	Absent		
OPC 11	5'- AAA GCT GCG C - 3'	Absent		
OPD 5	5'- TGA GCG GAC A - 3'	26	21	84.7
OPG 2	5'- GGC ACT GAG G- 3'	19	9	47.3
OPG 19	5'- GTC AGG GCA A- 3'	Absent		
OPK 10	5'- GTG CAA CGT G - 3'	Absent		
OPC 2	5'- GTG AGG CGT C - 3'	Absent		
OPB 13	5'- TTC CCC CGC T - 3'	Absent		



- Lane 1 - *Selaginella plana*
- Lane 2 - *Selaginella monospora*
- Lane 3 - *Selaginella delicatula*
- Lane 4 - *Selaginella repanda*
- Lane 5 - *Selaginella bryopteris*

FIG. 1. Electrophoretic analysis of total DNA from leaves of *Selaginella plana*, *Selaginella monospora*, *Selaginella delicatula*, *Selaginella repanda*, and *Selaginella bryopteris* extracted by the modified protocol of Roger and Benedich (1994).

efficiency of RAPD amplification. Upon gel electrophoresis a clear continuous band of DNA was obtained showing that the quality of DNA had improved and was consistently suitable for PCR amplification for RAPD analysis (Fig. 1). After PCR optimization for RAPD analysis, the generated amplification products were found to be of good quality and could be used to discriminate the genetic polymorphisms present in different species of *Selaginella* used in the study. Yield of genomic DNA was increased by certain modifications of the protocol. The modification involved increasing the time of incubation in 1X CTAB buffer. CTAB being a cationic detergent, it can form a CTAB–nucleic acid precipitate at room temperature, when the salt concentration is lower than 0.5 M. We observed that increasing the incubation time of the chloroform-isoamyl alcohol extract in precipitation buffer (1X CTAB solution) may cause more nucleic acids to be selectively precipitated, thus increasing the net yield.

We also observed that, in general, the Rogers and Benedich (1994) protocol yielded higher quantities of DNA compared to the protocol of Murray and

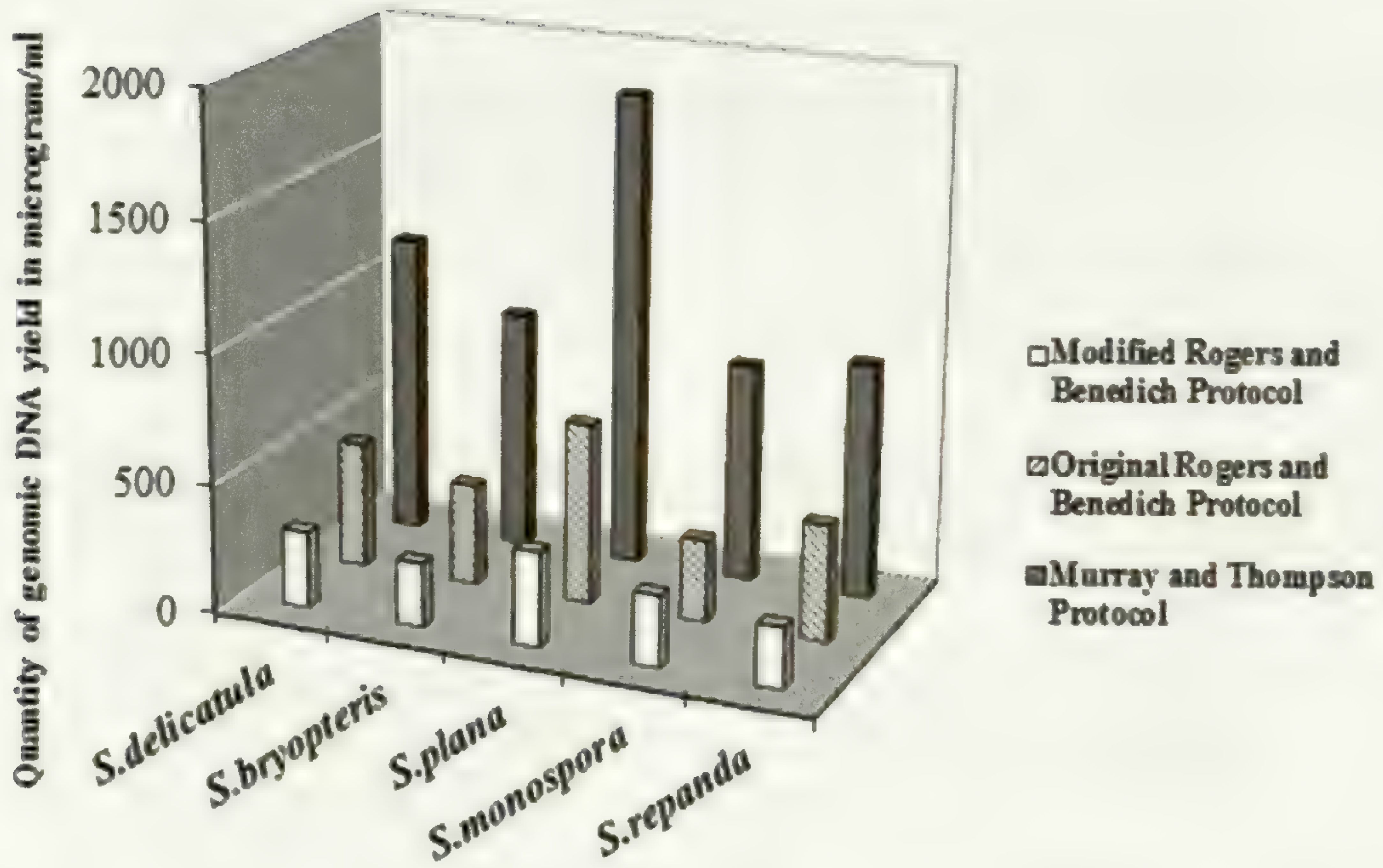


FIG. 2. Graphical representation showing the quantity of DNA obtained from different protocols for the five different species of *Selaginella*.

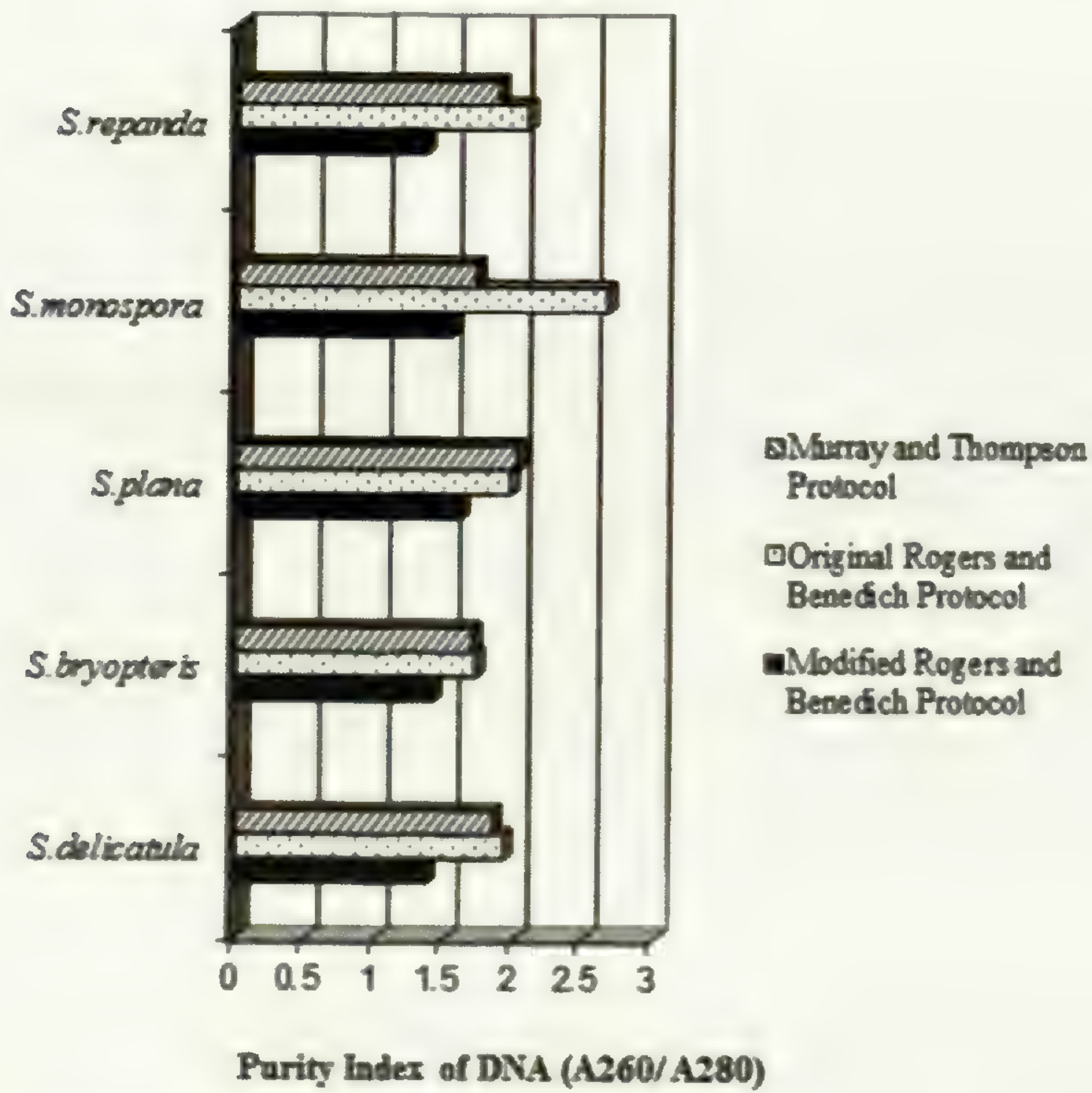


FIG. 3. Graphical representation showing the purity index of DNA obtained from different protocols for the five different species of *Selaginella*.

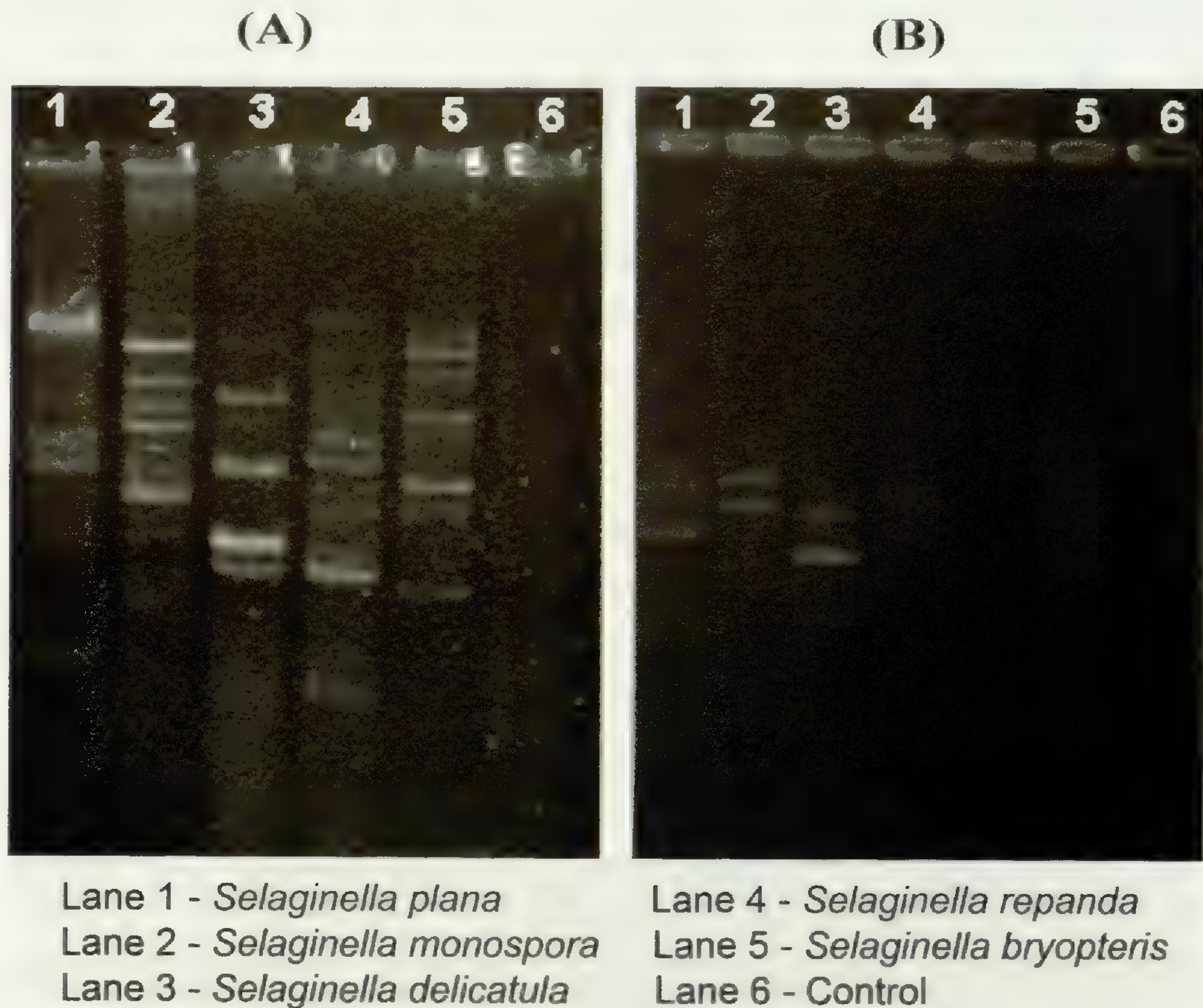


FIG. 4. PCR amplification profile of five different species of *Selaginella* using the primer OPD 5 using genomic DNA extracted from two different protocols, (A) modified Roger and Benedich (1994), and (B) original Roger and Benedich (1994).

Thompson (1980). The modified Rogers and Benedich (1994) protocol, on the other hand, yielded better quantity (2–3 times more) and quality genomic DNA than the original protocol of Rogers and Benedich (1994) (Fig. 2 and Fig. 3). The isolation protocols of Dellaporta *et al.* (1983) and Doyle and Doyle (1987) did not yield quantifiable amounts of genomic DNA.

The present study revealed that though the modified protocol of Rogers and Benedich (1994) required longer time (nearly three days) than the other methods followed, better quality DNA was extracted using this protocol (Fig. 1). Since the quantity and purity of extracted genomic DNA plays a crucial role for analysis of molecular diversity and optimization of different parameters for PCR (Weeden *et al.*, 1992; Staub *et al.*, 1996), the modified DNA extraction protocol of Rogers and Benedich proved to be most useful, as both the quality and quantity of genomic DNA significantly increased, yielding better results in RAPD-PCR analyses compared to the original DNA extraction protocol of Rogers and Benedich (Fig. 4 a and b).

Further analyses using more RAPD primers are necessary to obtain molecular markers for distinguishing the different subgenera of *Selaginella*, which would give valuable information regarding genetic diversity of *Selaginella* species.

ACKNOWLEDGMENTS

The authors wish to thank the Programme Coordinator, CAS and the Head, Department of Botany, University of Calcutta, for facilities provided. The authors would also like to acknowledge UGC-UPE for financial assistance. Thanks are also due to Prof. Radhanath Mukhopadhyay, Department of Botany, University of Burdwan for his assistance in identifying the plant species.

LITERATURE CITED

- BAKER, J. G. 1883. A synopsis of the genus *Selaginella*. *J. Bot.* 21:1–5, 42–46, 80–84, 97–100, 141–145, 210–213, 240–244.
- BRAUN, A. 1857. Appendix plantarum novarum et minus cognitarum. pp. 12–22, in *Horto regio botanico Berolinensi coluntur*.
- DELLAPORTA, S. L., J. WOOD and J. B. HICKS. 1983. A plant DNA mini-preparation: version II. *Plant. Mol. Biol. Rep.* 1:19–21.
- 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.
- HIERONYMUS, G. 1901. Selaginellaceae. Vol. 1(4), Pp. 621–716, In A. Engler and K. Prantl, eds., *Die Natürlichen Pflanzenfamilien*. W. Engelmann, Leipzig, Germany.
- JERMY, A. C. 1986. Subgeneric names in *Selaginella*. *Fern Gaz.* 124:371–383.
- KORALL, P. and P. KENRICK. 2002. Phylogenetic relationships in Selaginellaceae based on *rbcl* sequences. *Am. J. Bot.* 89:506–17.
- KORALL, P. and P. KENRICK. 2004. The phylogenetic history of Selaginellaceae based on DNA sequences from the plastid and nucleus: extreme substitution rates and rate heterogeneity. *Mol. Phylogenet. Evol.* 31:852–64.
- MURRAY, M. and W. F. THOMPSON. 1980. Rapid isolation of molecular weight plant DNA. *Nucleic Acid.* 8:4321–5.
- ROGERS, S. O. 1994. Phylogenetic and taxonomic information from herbarium and mummified DNA. Vol. 48, In R. P. Adams, J. Miller, E. Golenberg and J. E. Adams, eds. *Conservation of plant genes II: Utilization of ancient and modern DNA*. Miss Bot Gard, Monogr.
- ROGERS, S. O. and A. J. BENEDICH. 1994. Extraction of total cellular DNA from plants, algae and fungi. Pp. 1–8, In S. B. Gelvin and R. A. Schilerpoort, eds. *Plant Molecular Biology Manual*. Kluwer Academic, Dordrecht, The Netherlands.
- STAUB, J. J., J. BACHER and K. POETER. 1996. Sources of potential errors in the application of random amplified polymorphic DNAs in cucumber. *Hort. Sci.* 31:262–266.
- SPRING, A. F. 1850. Monographie de la famille des Lycopodiacees, Second partie. *Mémoires de l'académie Royale des Sciences, des Lettres et des Beaux-arts de Belgique.* 24:1–358.
- WALTON, J. and A. H. G. ALSTON. 1938. Lycopodiinae. Pp. 500–506, In F. Verdoorn, ed., *Manual of Pteridology*. Martinus Nijhoff, The Hague, The Netherlands.
- WEEDEN, N. F., M. TIMMERMAN, M. HERMMAT, B. E. KNEEN and M. A. LODHI. 1992. Applications of RAPD Technology to plant Breeding. Symposium Proceeding, Minneapolis. Pp. 12–17.
- WEISHING, K., H. NYBOM, K. WOLFF and W. MEYER. 1995. DNA isolation and purification. Pp. 44–59, In: *DNA fingerprinting in plants and fungi*. CRC Press, Boca Raton, Florida.

Molecular Evidence on the Origin of *Osmunda* *×mildei* (Osmundaceae)

C. TSUTSUMI*, Y. HIRAYAMA, and M. KATO

Department of Botany, National Museum of Nature and Science, Amakubo,
Tsukuba 305-0005, Japan

Y. YATABE-KAKUGAWA

Botanical Gardens, Graduate School of Science, the University of Tokyo, Hakusan,
Tokyo 112-0001, Japan

S.-Z. ZHANG

Shenzhen FairyLake Botanical Garden, Luohu District, Shenzhen, Guangdong, China 518004

ABSTRACT.—The southern Chinese *Osmunda ×mildei* has been suggested to be an intersubgeneric hybrid, i.e., *O. japonica* (subgenus *Osmunda*) \times *O. angustifolia* (subgenus *Plenasium*) or *O. japonica* \times *O. vachellii* (subgenus *Plenasium*). These interpretations were based on morphological, cytological, and/or chloroplast DNA data, yet the parents of the hybrid remained unclear. Molecular phylogenetic relationships inferred here from chloroplast *rbcL* sequences and three nuclear DNA markers show that *O. ×mildei* is most likely a hybrid between the paternal *O. japonica* and the maternal *O. vachellii*.

KEY WORDS.—EST, intersubgeneric hybrid, *Osmunda japonica*, *Osmunda ×mildei*, *Osmunda vachellii*, *rbcL*

The genus *Osmunda* of the leptosporangiate fern family Osmundaceae has natural hybrids (Kato, 2009; Tsutsumi *et al.*, 2011). One such is *Osmunda ×mildei* C.Chr. (= *O. bipinnata* Hook., a later homonym of *O. bipinnata* L.), which nearly became extinct in its known range in Hong Kong. However, this hybrid was recently found in Shenzhen, Guangdong, and Mt. Qiyun, Jiangxi (Zhang *et al.*, 2008), and less than 10 individuals are known. It was also found in Zhangjiajie, Hunan (Y.-H. Yan, pers. comm.). It can propagate via spores in experimental conditions (J.-F. Yang, unpubl. data), but it is uncertain if the individuals were derived from spore propagations or from independent formations of the hybrid. *Osmunda ×mildei* is characterized by subcoriaceous, bipinnate-bipinnatifid leaves with round, entire pinnules, and fertile pinnae inserted below the middle of the leaf. For the origin of *O. ×mildei*, two possibilities were proposed (Fig. 1). Based on karyological and morphological analyses, He *et al.* (2006) suggested that *O. ×mildei* is a hybrid of *O. japonica* Thunb. (subgenus *Osmunda*) and *O. angustifolia* Ching (subgenus *Plenasium*). Zhang *et al.* (2008) observed the absence of chromosome pairings at meiosis and resulting abortive spores in *O. ×mildei*, and suggested that it is a sterile F1 hybrid, but argued that the parents were *O.*

* Corresponding author: (e-mail: tsutsumi@kahaku.go.jp)

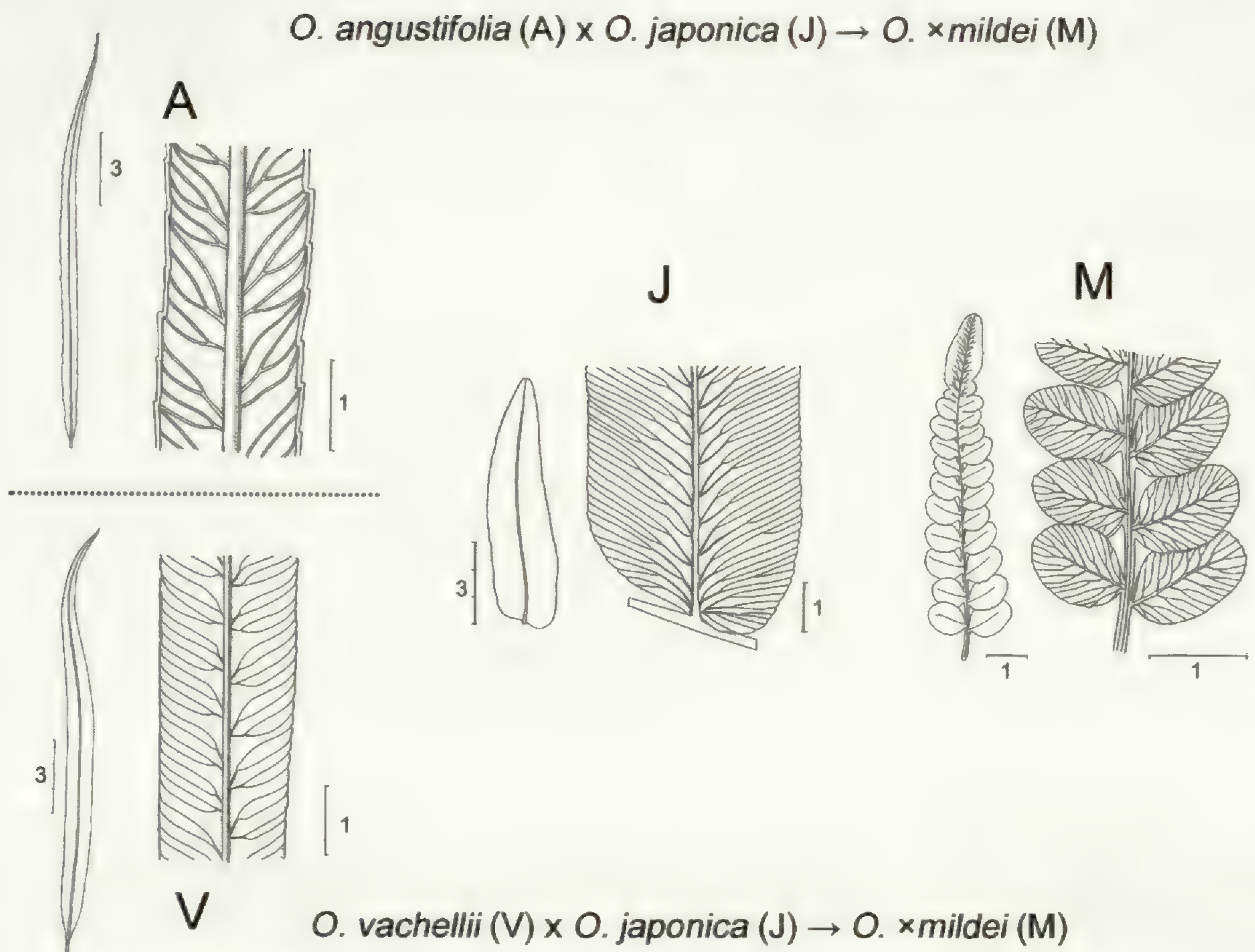


FIG. 1. Candidate parentage of *Osmunda* × *mildei*. A: *O. angustifolia*: pinna (on left side) and part (on right side). J: *O. japonica*: pinnule and part. M: *O.* × *mildei*: pinna and part. V: *O. vachellii*: pinna and part. Scale unit is cm. Two proposed crossings are illustrated at the top and bottom.

japonica and *O. vachellii* Hook. (subgenus *Plenasium*), because *O. vachellii* co-occurs with *O.* × *mildei*, but *O. angustifolia* does not occur in some of the localities of *O.* × *mildei* (Fig. 2). Gou *et al.* (2008) also proposed that *O. vachellii* is the maternal progenitor of *O.* × *mildei*, based on inferences from chloroplast DNA sequence data. Under either parentage hypothesis *O.* × *mildei* is likely an intersubgeneric hybrid between the subgenera *Osmunda* and *Plenasium*.

There are three more known hybrids reported in Osmundaceae (Kato, 2009). Eastern North American *O.* × *ruggii* Tryon is *O. regalis* L. (subgenus *Osmunda*) × *O. claytoniana* L. (subgenus *Claytosmunda*) (Tryon, 1940; Wagner *et al.*, 1978; Whetstone and Atkinson, 1993; Li and Haufler, 1994). Japanese *O.* × *nipponica* Makino is *O. japonica* (subgenus *Osmunda*) × *O. claytoniana* (subgenus *Claytosmunda*) (Ito, 1964), but Sugimoto (1979) suggested that it is an intergeneric hybrid, i.e., *O. japonica* × *Osmundastrum cinnamomeum* (L.) C.Presl. Neither is given molecular evidence. Finally, Japanese *O.* × *intermedia* (Honda) Sugim. is *O. japonica* × *O. lancea* Thunb. (both in subgenus *Osmunda*; Tagawa, 1959; Iwatsuki, 1995; Tatuno and Yoshida, 1966; Yatabe *et al.*, 2009).

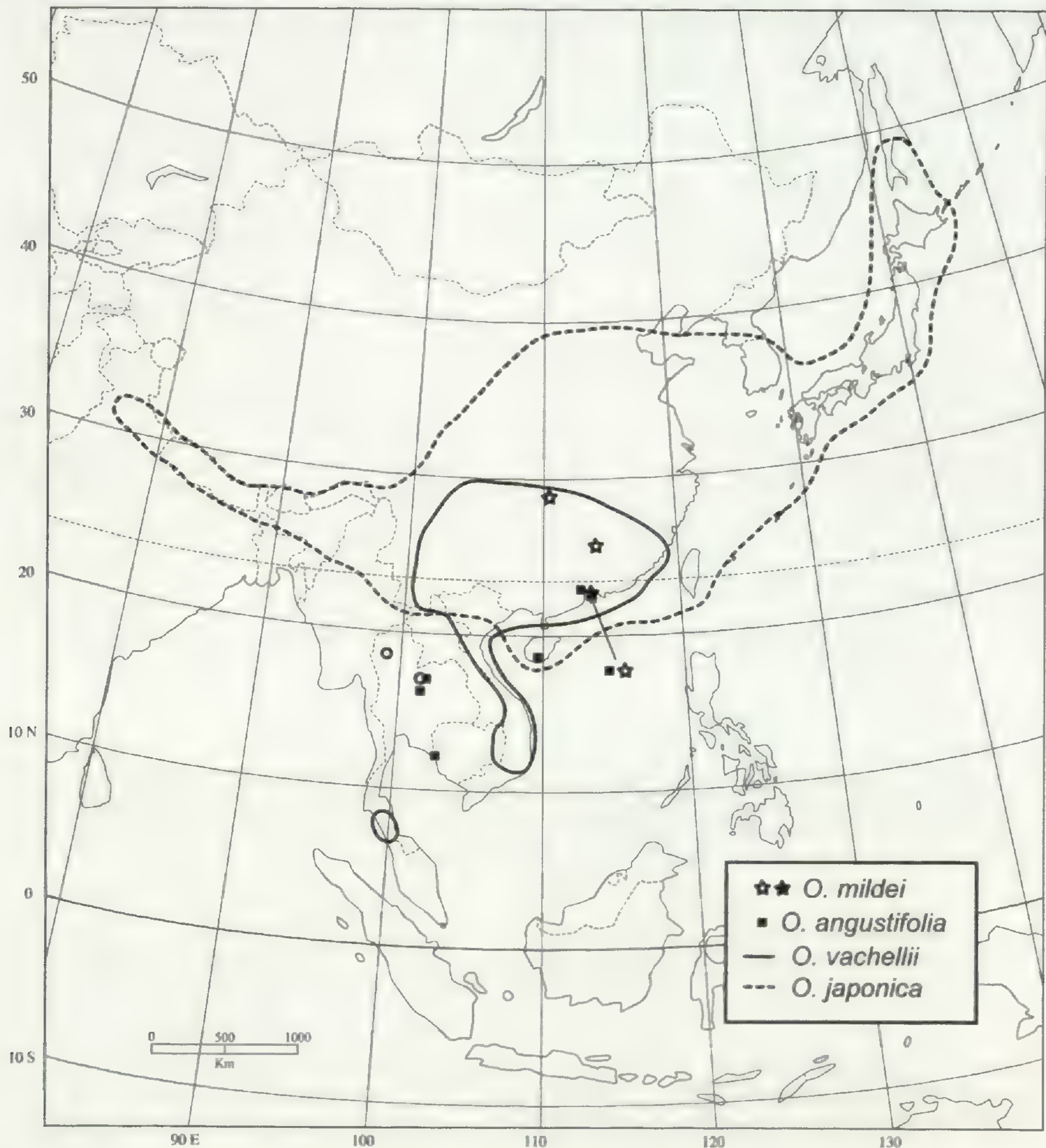


FIG. 2. Map showing distribution of *Osmunda angustifolia* (solid squares), *O. japonica* (broken line), *O. ×mildei* (asterisks) and *O. vachellii* (solid line). Solid asterisk shows the place of *O. ×mildei* sampled in this study.

The evidence offered to support the parentage of *O. ×mildei* in previous studies is inconclusive, because it lacked nuclear sequence data. Combining nuclear and chloroplast DNA data are useful to identify a maternal and a paternal parent. This study examines the aforementioned alternative origins of the hybrid, based on chloroplast *rbcL* sequences and three nuclear DNA sequences.

MATERIALS AND METHODS

Materials.—Samples of three species of subgenus *Plenasium* (*O. vachellii*, *O. banksiifolia* (C.Presl) Kuhn, *O. angustifolia*), the putative intersubgeneric

hybrid *O. ×mildei*, one species of subgenus *Claytosmunda* (*O. claytoniana*), *Osmundastrum cinnamomeum*, and *Todea barbara* (L.) T.Moore, along with three species of subgenus *Osmunda* (*O. japonica*, *O. lancea*, *O. regalis*), were collected in the field or botanical gardens. The sources of materials used are shown in Table 1.

Sequencing of chloroplast and nuclear DNA.—Leaf fragments were used for molecular analysis. DNA was extracted from fresh or silica-gel-dried material using a QIAGEN DNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instruction. Three nuclear DNA markers (EST_L058, EST_L110, EST_L258) selected from the expressed sequence tag library developed by Yatabe *et al.* (2009) and the chloroplast locus *rbcL* were analyzed. Primers for amplification and sequencing, and detailed information on the three nuclear markers are shown in Table 2 and Table 3, respectively. PCR was performed using a Perkin-Elmer 9700 DNA thermal cycler (Applied Biosystems, Foster, CA) with *Ex Taq* DNA polymerase (TaKaRa Bio, Tokyo, Japan) and Ampdirect Plus (Shimadzu, Kyoto, Japan) in 35 denaturation, annealing, and elongation cycles (30 sec at 94°C; 30 sec at 50°C for the *rbcL* and 58°C in the three nuclear markers; and 90 sec at 72°C) with a final elongation step (7 min at 72°C). The PCR products were purified with ExoSAP-IT (USB corporation, Cleveland, OH) following the manufacturer's instructions. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The raw sequence data were assembled using Seqman II (Dnastar, Madison, WI). For the sample of *O. ×mildei*, PCR of the nuclear DNA markers was performed with PrimeSTAR Max DNA polymerase (TaKaRa Bio) in 35 cycles (10 sec at 98°C, 5 sec at 55°C and 5 sec at 72°C). The PCR products were cloned using a pGEM-T Vector System I (Promega, Madison, WI) and at least 10 clones were sequenced. Minor variants from single clones, presumably sequencing errors, were observed. Therefore a consensus sequence was used for each allele type; the differences between each consensus sequence and the original clones are shown in Table 4. The assembled sequences were aligned by Clustal X program (Thompson *et al.*, 1997) and then aligned manually.

Molecular phylogeny.—Phylogenetic analyses were performed by maximum parsimony (MP) and Bayesian analysis. Registered sequences of Osmundaceae in Genbank were added into the analyses (see Table 1). Maximum parsimony (MP) inference was conducted with PAUP* 4.0b10 (Swofford, 2002). The bases that could not be identified were treated as unknown (N), and gaps were treated as missing data. All characters were equally weighed and heuristic searches were conducted with 1000 random addition replicates involving TBR branch swapping. Bootstrap values were calculated from 1000 pseudoreplicates, each with 100 random additions. For the Bayesian analyses, MrModeltest 2.0 (Nylander, 2004) was used to determine the nucleotide substitution model. Bayesian searches were conducted by MCMC with two independent sets of four chains, each run for ten million generations, sampling every 100 generations by MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The nucleotide model selected was: *rbcL*, GTR + I + G; EST_L058, HKY + I; EST_L110, GTR + I; EST_L258, GTR + I. The program

TABLE 1. Materials used in this study. * shows Genbank accession numbers of each locus.

Genus, subgenus, species	Sample ID	Source and voucher	<i>rbcL</i> *	EST_L058*	EST_L110*	EST_L258*
<i>O. × mildei</i> C.Chr.		Cult. in Shenzhen Fairy Lake Botanical Garden (originated from China, Guangdong, Shenzhen)	AB672746	See Table 4	See Table 4	See Table 4
<i>Osmunda</i> subg. <i>Claytoniana</i>						
<i>O. claytoniana</i> L.	CL1	Cult. in Tsukuba Botanical Garden (originated from Japan); <i>C. Tsutsumi</i> s.n. (TNS)	AB672747	AB672752	AB672790	AB672828
	CL2	China, Yunnan, Gongshan, Chichi; <i>L.-Y. Kuo 9485</i> (TAIF)	AB639186	AB672753	AB672791	AB672829
	CLg1	Cult. in Missouri Botanical Garden; <i>G. Yatskievych 99-0302</i>	AB024950	-	-	-
	CLg2	Cult. in Botanical Gardens, Nikko, University of Tokyo; <i>S. Nemoto 99-307</i>	AB024951	-	-	-
<i>Osmunda</i> subg. <i>Osmunda</i>						
<i>O. japonica</i> Thunb.	J1	Cult. in Tsukuba Botanical Garden (originated from Japan); <i>C. Tsutsumi</i> s.n. (TNS)	AB494711	AB672754	AB672792	AB672830
	J2	Japan, Miyazaki Pref., Kobayashi; <i>G. Kokubogata GK9416</i> (TNS)	AB494714	AB672755	AB672793	AB672831
	J3	Japan, Kagoshima Pref., Amami Island, Mt. Yuwan; <i>M. Kato et al. s.n.</i> (TNS:764134)	AB494712	AB672756	AB672794	AB672832
	J4	China, Fujian, Mt. Wuyi; <i>A. Ebihara s.n.</i> (TNS:762111)	AB494712	AB672757	AB672795	AB672833
	J5	China, Yunnan, Gongshan, Chichi; <i>L.-Y. Kuo 9468</i> (TAIF)	AB639162	AB672758	AB672796	AB672834
	J6	Bhutan, Sha-ngawang; <i>S. Matsumoto W.G.M.124</i> (TNS)	AB494711	AB672759	AB672797	AB672835
	J7	India, Meghalaya State, Shillong Peak ridge, by site of ruined "Peak Lodge"; <i>C. R. Fraser-Jenkins s.n.</i> (TNS:740377)	AB639163	AB672760	AB672798	AB672836
	J8	India, Central Himalaya, from Fern Garden, Department of Botany, Government Postgraduate College, Pithoragarh, 262502. (Originated from Champawat); <i>C. R. Fraser-Jenkins s.n.</i> (TNS:777785)	AB639164	AB672761	AB672799	AB672837
<i>O. lancea</i> Thunb.	Jg1	Japan, Shizuoka Pref.; <i>Y. Yatabe 99-0303</i>	AB024947	-	-	-
	L1	Cult. in Tsukuba Botanical Garden (transplanted from Shizuoka Pref.); <i>C. Tsutsumi s.n.</i> (TNS)	AB494711	AB672762	AB672800	AB672838
	L2	Japan, Kochi Pref.; <i>A. Ebihara et al., KC2007-1408</i> (TNS:766627)	AB494713	AB672763	AB672801	AB672839
	L3	Japan, Miyazaki Pref.; <i>T. Minamitani s.n.</i> (TNS)	AB494711	AB672764	AB672802	AB672840
	Lg1	Cult. in Botanical Gardens, Koishikawa, University of Tokyo; <i>K. Hirai 99-0305</i>	AB024952	-	-	-

TABLE 1. Continued.

Genus, subgenus, species	Sample ID	Source and voucher	<i>rbcL</i> *	EST_L058*	EST_L110*	EST_L258*
<i>O. regalis</i> L.	R1	India, Kerala, Kozhikode Dist., Vellarimala; A. K. Pradeep 73207 (TNS:771612)	AB639165	AB672765	AB672803	AB672841
	R2	India, Karnataka, Shimoga Dist., Kemmanagundi near Shimoga; J. Murata & H. Murata s.n. (TI)	AB639166	AB672766	AB672804	AB672842
	R3	India, Kerala, Kozhikode Dist., Vellarimala; A. K. Pradeep 86941 (TNS)	AB639167	AB672767	AB672805	AB672843
	R4	India, Pachmarhi (cult. in National Botanical Research Institute Botanic Garden); M. Kato s.n. (TNS)	AB639168	AB672768	AB672806	AB672844
	R5	Cult. in the home garden of H. P. Nootboom in Netherlands	AB639169	AB672769	AB672807	AB672845
	R6	Netherlands, 12 km S of Venlo, Limburg prov.; P. Hovenkamp s.n. (L)	AB639170	AB672770	AB672808	AB672846
	R7	Germany, Rhineland-Palatinate, Palatinate Forest Biosphere Reserve, 15 km S of Kaiserslautern; M. Zink s.n.	AB639171	AB672771	AB672809	AB672847
	R8	Macedonia, Strumica, Kolesino, Orman; A. Tuji et al. s.n. (TNS:737424)	AB639172	AB672772	AB672810	AB672848
	R9	Macedonia, Strumica, Kolesino, Orman; A. Tuji et al. s.n. (TNS:737429)	AB639173	AB672773	AB672811	AB672849
	R10	Cameroun, Mbalmayo, Ebogo, Nyong River; M. Kato et al. CMR-141 (TNS)	AB639174	AB672774	AB672812	AB672850
	R11	Madagascar, Perinet, Andasibe; T. Nakamura s.n. (TNS)	AB639175	AB672775	AB672813	AB672851
	R12	Madagascar, Perinet, Vohmana; T. Nakamura s.n. (TNS)	AB639176	AB672776	AB672814	AB672852
	R13	USA, New York, N of New York City; R. Moran 8179	AB639177	AB672777	AB672815	AB672853
	R14	USA, Indiana, Laporte Co., Ambler Flatwoods Nat. Pres.; G. Yatskievych 07-91(TNS:771606)	AB639178	AB672778	AB672816	AB672854
	R15	USA, Missouri, Reynolds Co., Maury Pond; G. Yatskievych 07-16 (TNS:771608)	AB639179	AB672779	AB672817	AB672855
	R16	USA; L.-Y. Kuo s.n. (TNS)	AB639180	AB672780	AB672818	-
	R17	Mexico; R. C. Monica s.n.	AB639181	AB672781	AB672819	AB672856
	R18	Argentina, Misiones, Monte Carlos, Colonia Guatambu, Bafiado junto al arroyo Caragatay; E. I. Meza Torres, et al. No. 1243 (TNS)	AB639182	AB672782	AB672820	AB672857

TABLE 1. Continued.

Genus, subgenus, species	Sample ID	Source and voucher	<i>rbcL</i> *	EST_L058*	EST_L110*	EST_L258*
	Rg1	Madagascar	AB076258	-	-	-
	Rg2	United Kingdom	AB076259	-	-	-
	Rg3	Cult. in Missouri Botanical Garden; <i>G. Yatskievych 99-304</i>	AB024948	-	-	-
<i>Osmunda</i> subg. <i>Plenasium</i>						
<i>O. angustifolia</i> Ching		Cult. in Shenzhen Fairy Lake Botanical Garden	AB672748	AB672783	AB672821	AB672858
<i>O. banksiifolia</i> (C.Presl) Kuhn	B1	Cult. in Tsukuba Botanical Garden (originated from Okinawa Pref., Japan)	AB024956	AB672784	AB672822	AB672859
	Bg1	Japan, Miyazaki Pref.; <i>K. Hirai 90-559</i>	AB024956	-	-	-
	Bg2	Japan, Tokyo, Ogasawara; <i>K. Hirai 92-267</i>	AB024955	-	-	-
<i>O. javanica</i> Blume	V1	Cult. in UKM, Malaysia; <i>N. Murakami 98-M12</i>	AB024953	-	-	-
<i>O. vachellii</i> Hook.	Vg1	Cult. in Shenzhen Fairy Lake Botanical Garden	AB672749	AB672785	AB672823	AB672860
		Cult. in UKM, Malaysia; <i>N. Murakami 98-M13</i>	AB024954	-	-	-
<i>Leptopteris</i>						
<i>L. hymenophyllum</i> (A.Rich.) C.Presl		New Zealand; <i>M. Ito & T. Asakawa 97Yg09</i>	AB024957	-	-	-
<i>L. wilksiana</i> (Brack.) Christ		New Caledonia; <i>M. Ito & T. Asakawa 97Zm06</i>	AB024958	-	-	-
<i>Osmundastrum</i>						
<i>O. cinnamomeum</i> (L.) C.Presl	Cl1	Cult. in Tsukuba Botanical Garden; <i>C. Tsutsumi s.n.</i> (TNS)	AB672750	AB672786	AB672824	AB672861
	Clg1	Cult. in Missouri Botanical Garden; <i>G. Yatskievych 98-301</i>	AB024949	-	-	-
	Clg2	Cult. in Botanical Gardens, Nikko, University of Tokyo; <i>Hasebe 27624</i> (TI)	D14882	-	-	-
<i>Todea</i>						
<i>Todea barbara</i> (L.) T.Moore	T1	Cult. in Cibodas Botanical Garden; <i>C. Tsutsumi s.n.</i> (TNS)	AB672751	AB672787	AB672825	AB672862
	Tg1	Cult. in Cibodas Botanical Garden (originated from New Zealand); <i>Y. Yatabe 99-0306xs</i>	AB024959	-	-	-

TABLE 2. Primers used for the analyses of three nuclear markers.

Marker	Primer	Primer sequence	Reference
EST_L058	EST_L058F	ATAAGGTTTCGCCCTCGAAT	Yatabe <i>et al.</i> 2009
	EST_L058R	TCTTGCAGTTGCGAGTTCAC	Yatabe <i>et al.</i> 2009
EST_L110	EST_L110F (P108)	CATTGCACATCGGAGATGAT	Yatabe <i>et al.</i> 2009
	EST_L110R (P107)	CACAGCTGAAACACCCTGAA	Yatabe <i>et al.</i> 2009
EST_L258	EST_L258F	TCATGGCGACTGTGAAGAAG	Yatabe <i>et al.</i> 2009
	EST_L258R	CGCCCTTTGGATTTACGATA	Yatabe <i>et al.</i> 2009

Tracer (Rambaut and Drummond, 2009) was used to check the runs had reached stationarity and effective sample size of all the parameters was high (>100). The first 2.5 million generations before sufficient stationary generations were discarded as burn-in periods and the rest of trees were used to calculate posterior probabilities. *Osmundastrum cinnamomeum* and *Todea barbara* were used as outgroups (Yatabe *et al.*, 1999; Metzgar *et al.*, 2008).

RESULTS

Bayesian and maximum parsimony analyses of each dataset produced congruent topologies (Bayesian consensus trees shown in Figs. 3–6). Maximum parsimony analyses resulted in three shortest trees of a length of 146 steps (CI = 0.77, HI = 0.23, RI = 0.93) for chloroplast *rbcL* (1227 bp) with 101 parsimony-informative characters, 78 shortest trees of a length of 47 steps (CI = 0.89, HI = 0.11, RI = 0.88) for nuclear EST_L058 (198 bp) with 18 parsimony-informative characters, ten shortest MP trees of a length of 125 steps (CI = 0.91, HI = 0.09, RI = 0.91) for nuclear EST_L110 (572 bp) with 57 parsimony-informative characters, and two shortest trees of a length of 122 steps (CI = 0.89, HI = 0.12, RI = 0.94) for nuclear EST_L258 (361 bp) with 59 parsimony-informative characters.

TABLE 3. Total lengths of three nuclear markers, coding and non-coding regions in *Osmunda japonica* (J3 in Table 1), and identified genes with one of the highest E-value (< 0.001) obtained by Blast search (blastn) using sequences of EST libraries (Yatabe *et al.* 2009).

Marker	Accession no.	Total length (bp)	Coding region (bp)	Non-coding region (bp)	Putative gene (species)	GenBank hit accession no. (E-value)
EST_L058	FS993661	198	101	97	glycolate oxidase (gox) (<i>Zantedeschia aethiopica</i>)	AY173074 (2 × 10 ⁻¹²⁴)
EST_L110	FS993713	548	148	400	glycerol-3-phosphate (<i>Zea mays</i>)	EU964956 (9 × 10 ⁻⁷⁷)
EST_L258	FS993861	317	79	238	ribosomal protein L17 (<i>Castanea sativa</i>)	AF334838 (9 × 10 ⁻¹⁰²)

TABLE 4. Allele types, numbers of clones used, and variations between a consensus sequence and the original clones for *Osmunda* × *mildei* samples in three nuclear markers examined.

	Allele	Genbank accession No.	Number of clones:				
			Included in consensus seq.	Identical to consensus seq.	Differing by one subst'n	Differing by two subst'ns	Differing by three subst'ns
EST_L058	A	AB672788	11	7	4	-	-
	B	AB672789	4	4	-	-	-
EST_L110	A	AB672826	10	4	3	1	2
	B	AB672827	4	2	1	-	1
EST_L258	A	AB672863	6	3	3		
	B	AB672864	7	4	1	2	

The *rbcL* sequence of *O.* × *mildei* is identical to that of *O. vachellii* and also was similar to those of *O. banksiifolia* and *O. javanica* Blume (Fig. 3). The *Osmunda* × *mildei* sequence was more distantly related to *O. angustifolia* (but with low support), and very far from *O. japonica* and other species of subgenus *Osmunda*.

In the three nuclear markers, the *O.* × *mildei* sample has two distinct allele types (Figs. 4–6). One type (Type A) had the same sequence as some plants of *O. japonica*, while the other (Type B) had the same sequence as *O. vachellii* (in EST_L58 and L110 in Figs. 4 and 5) or a sequence very similar to it (in EST_L258 in Fig. 6). In each of the three nuclear-marker trees, *O.* × *mildei* was more closely related to *O. vachellii* than to *O. angustifolia*.

DISCUSSION

Our trees constructed from the chloroplast gene and nuclear DNA sequences agree with previous trees in the monophyly of the three subgenera of *Osmunda* (Yatabe *et al.*, 1999; Gou *et al.*, 2008; Metzgar *et al.*, 2008). The *rbcL* phylogenetic relationships of the subgenera are the same as Yatabe *et al.*'s (1999) from the same gene and Metzgar *et al.*'s (2008) from seven chloroplast loci including *rbcL*, and different from Gou *et al.*'s (2008) *rbcL* relationships. The relationships deduced from the three nuclear EST markers are not consistent with each other, but the relationship of the EST_L110 agrees with the *rbcL* relationship in the subgenera *Osmunda* and *Plenasium* being sister to each other.

All the phylogenetic trees inferred from the three nuclear EST markers show that *Osmunda* × *mildei* has two distinct allele types, and one is identical to those of *O. japonica*, and the other formed a monophyletic clade with those of *Plenasium* species (Figs. 4–6). It is suggested that *O.* × *mildei* is an intersubgeneric hybrid between the subgenera *Osmunda* and *Plenasium*. The Type B alleles of *O.* × *mildei* have the same EST_L058, EST_L110 sequences and the closest EST_L258 sequences to those of *O. vachellii*, suggesting that *O.* × *mildei* is most likely derived by hybridization of *O. japonica* and *O. vachellii*.

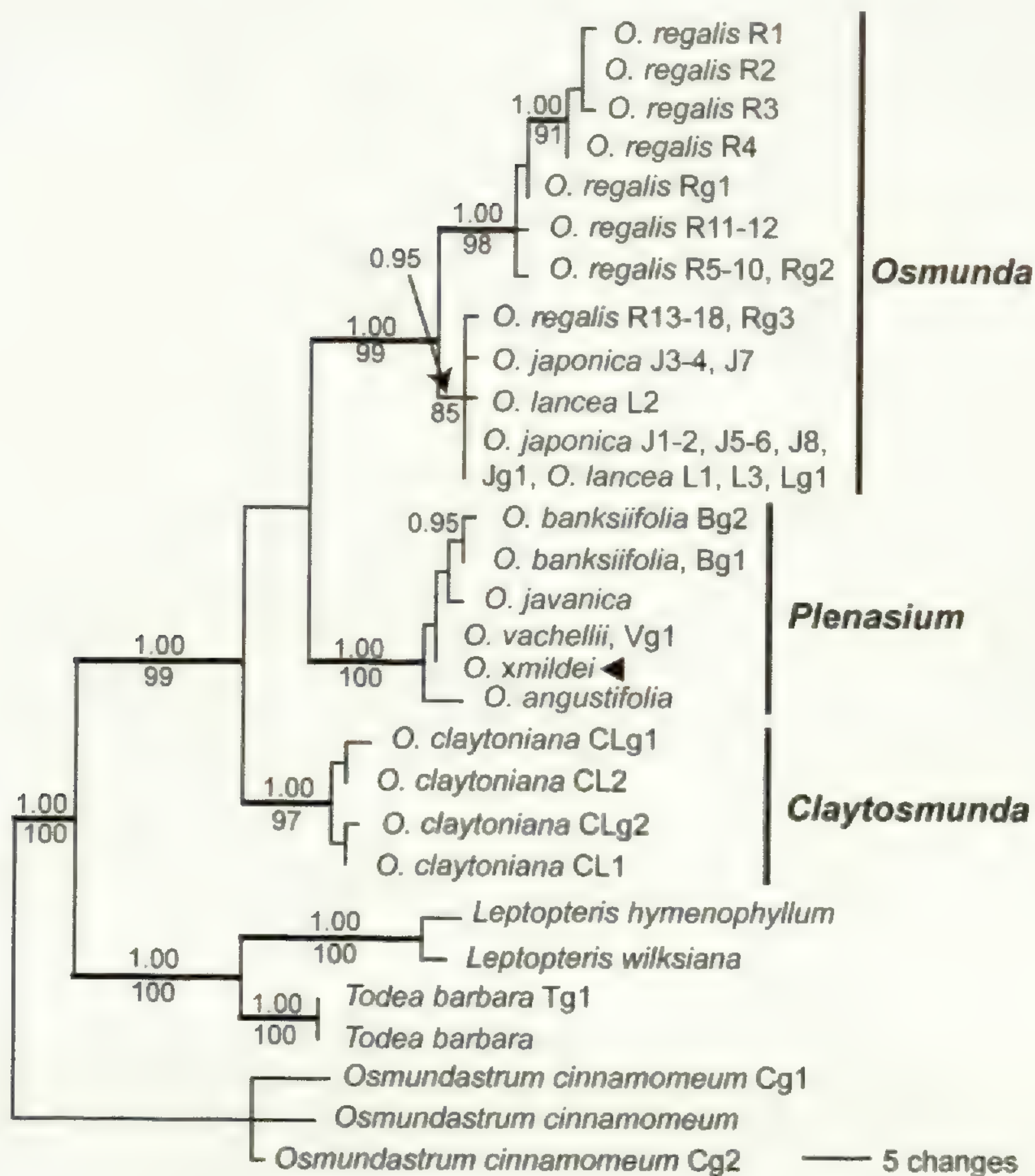


FIG. 3. Bayesian consensus tree based on chloroplast *rbcL* (1227 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities $p > 0.95$ and bootstrap values >90). Arrowhead indicates *O. xmildei*. Abbreviations of materials follow Table 1.

(Figs. 4–6). The chloroplast *rbcL* sequence of *O. xmildei* is identical to that of *O. vachellii* (Fig. 3), suggesting that it is the maternal progenitor of *O. xmildei*; hence *O. japonica* is paternal. This suggested parentage agrees with Zhang *et al.* (2008) and Gou *et al.* (2008), who suggested *O. vachellii* as the maternal parent, based on chloroplast DNA data and distributional data. *Osmunda banksiifolia* is also very closely related to *O. xmildei*, however, comparative morphology does not support that *O. banksiifolia* is a parent, because it has prominently dentate pinnae, whereas *O. vachellii* and *O. xmildei* (and also *O. japonica*) are both distinct with entire or somewhat serrate pinnae or pinna-segments.

This study analyzed a sample of *O. xmildei* from Shenzhen, Guangdong (Fig. 2). It has very low spore viability and a very low offspring reproduction rate even in carefully controlled culture conditions (Zhang *et al.*, 2008; J.-F. Yang *et al.*, unpubl. data). Considering the low reproductive ability and a few isolated localities in southern China, it is possible that *O. xmildei* is of multiple origins, although no molecular evidence is available. *Osmunda x ruggii* of eastern North America (Connecticut and Virginia, USA) is an

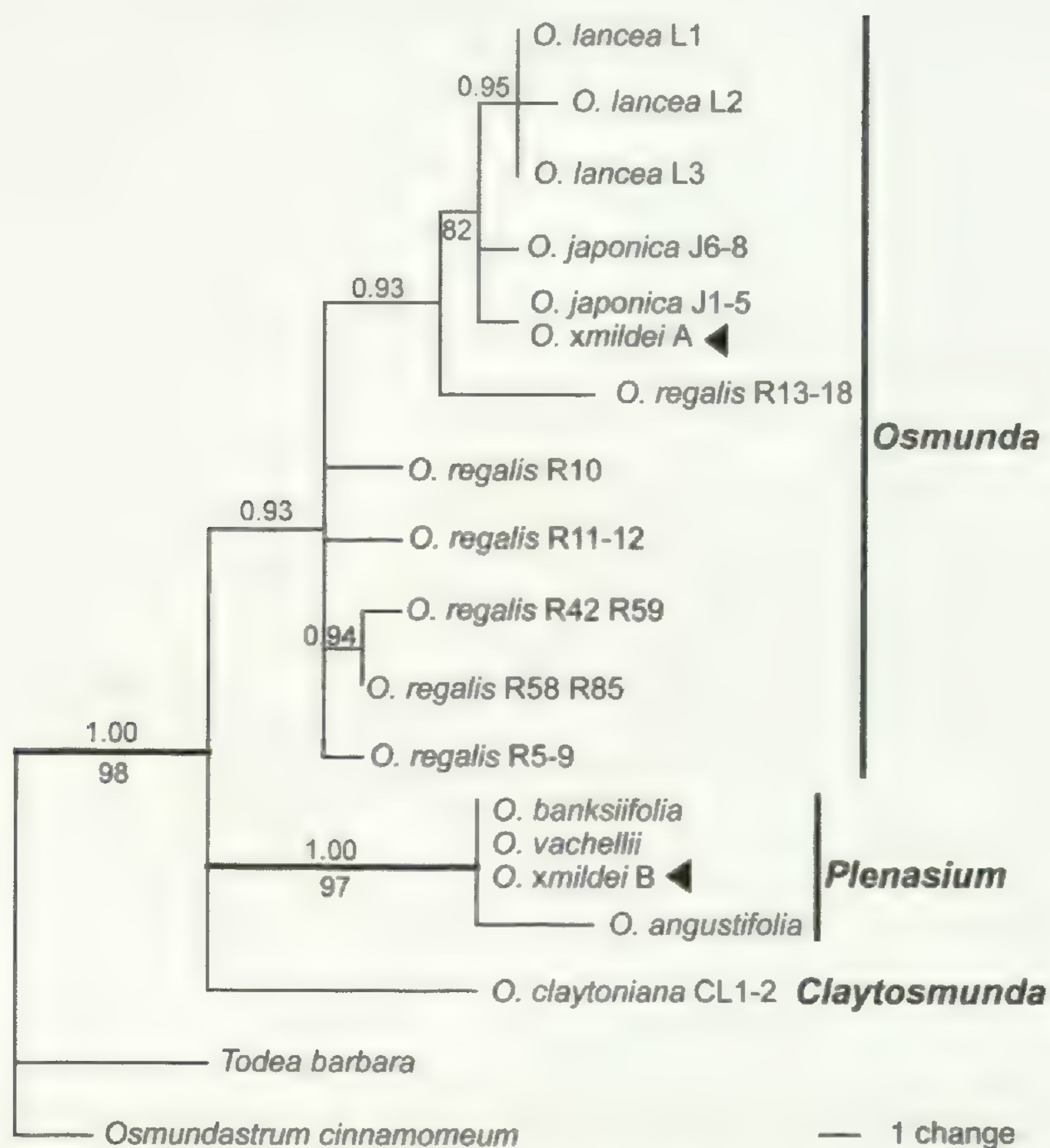


FIG. 4. Bayesian consensus tree based on nuclear EST_L058 (198 bp). Figures above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities >0.95 and bootstrap values >90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O. ×mildei*. Numbers of clones of each allele type are in Table 4.

intersubgeneric sterile hybrid derived from *O. regalis* (subgenus *Osmunda*) and *O. claytoniana* (subgenus *Claytosmunda*; Tryon, 1940; Wagner *et al.*, 1978; Whetstone and Atkinson, 1993; Li and Haufler, 1994). Like *O. ×mildei*, *O. ×ruggii* grows together with the parents in a few localities (Wagner *et al.*, 1978). Wagner *et al.* (1978) described that transplants produced fertile pinnae, but produced aborted spores and only univalent chromosomes at meiosis, suggesting its high sterility. *Osmunda ×intermedia*, which is widely distributed across Japan, is suggested to be an intrasubgeneric hybrid of multiple origins from *O. japonica* and *O. lancea*, and it is self-reproducible (Shimura, 1972; Yatabe *et al.*, 2009). The differing levels of fertility and sterility in the three hybrids may reflect close or remote phylogenetic affinities (Yatabe *et al.*, 1999; Metzgar *et al.*, 2008).

Osmunda japonica is distributed in eastern Asia extending west to the Himalayas and south to northern Vietnam (Kato, 2007). It is a likely parent for *O. ×mildei*, *O. ×nipponica* and *O. ×intermedia*, all three of which occur within the distributional range of *O. japonica*. *Osmunda vachellii*, the other parent for *O. ×mildei*, also co-occurs with *O. ×mildei* (Fig. 2). From this

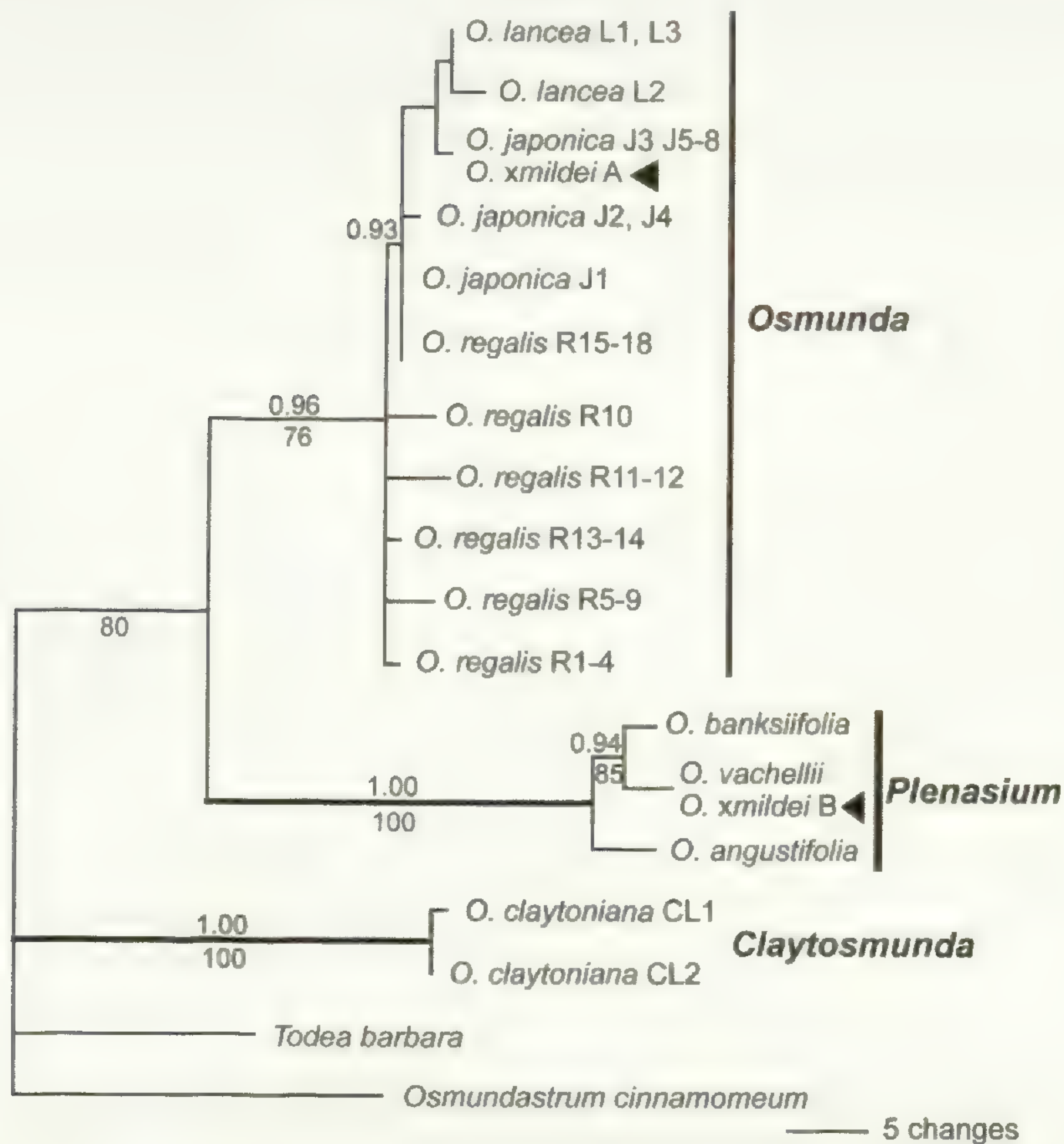


FIG. 5. Bayesian consensus tree based on nuclear EST_L110 (572 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities >0.95 and bootstrap values >90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O. ×mildei*. Numbers of clones of each allele type are in Table 4.

distributional pattern, along with the distributions of *O. ×ruggii* and its parents, we suggest that overlap of the parental species allowed the interspecific hybridization relatively recently. On the contrary, a fertile tetraploid species of hybrid origin between *O. japonica* and *O. regalis* (subgenus *Osmunda*), occurs in northern Central Laos distant from the distribution ranges of both parents, and in northern Myanmar, distant from Central India where *O. regalis* occurs (Kato, 2007; Tsutsumi *et al.*, 2011). Tsutsumi *et al.* (2011) suggested that the hybrid species arose when the distribution ranges of the parents overlapped, a pattern different from the current pattern.

ACKNOWLEDGMENTS

We thank S. Akiyama, M. K. Bhattacharya, W.-L. Chiou, A. Ebihara, C. R. Fraser-Jenkins, P. Hovenkamp, N. Katayama, S. Kobayashi, S. Koi, G. Kokubugata, L.-Y. Kuo, T. Minamitani, R. C. Monica, R. Moran, H. Murata, J. Murata, T. Nakamura, H. P. Nootboom, T. Oka, A. K. Pradeep, S. Suddee, A. Tuji, T. Wongprasert, G. Yatskievych, X.-C. Zhang, and M. Zink for their help during the trips or providing materials. We also thank H. Liu, Shenzhen Fairy Lake Botanical Garden for

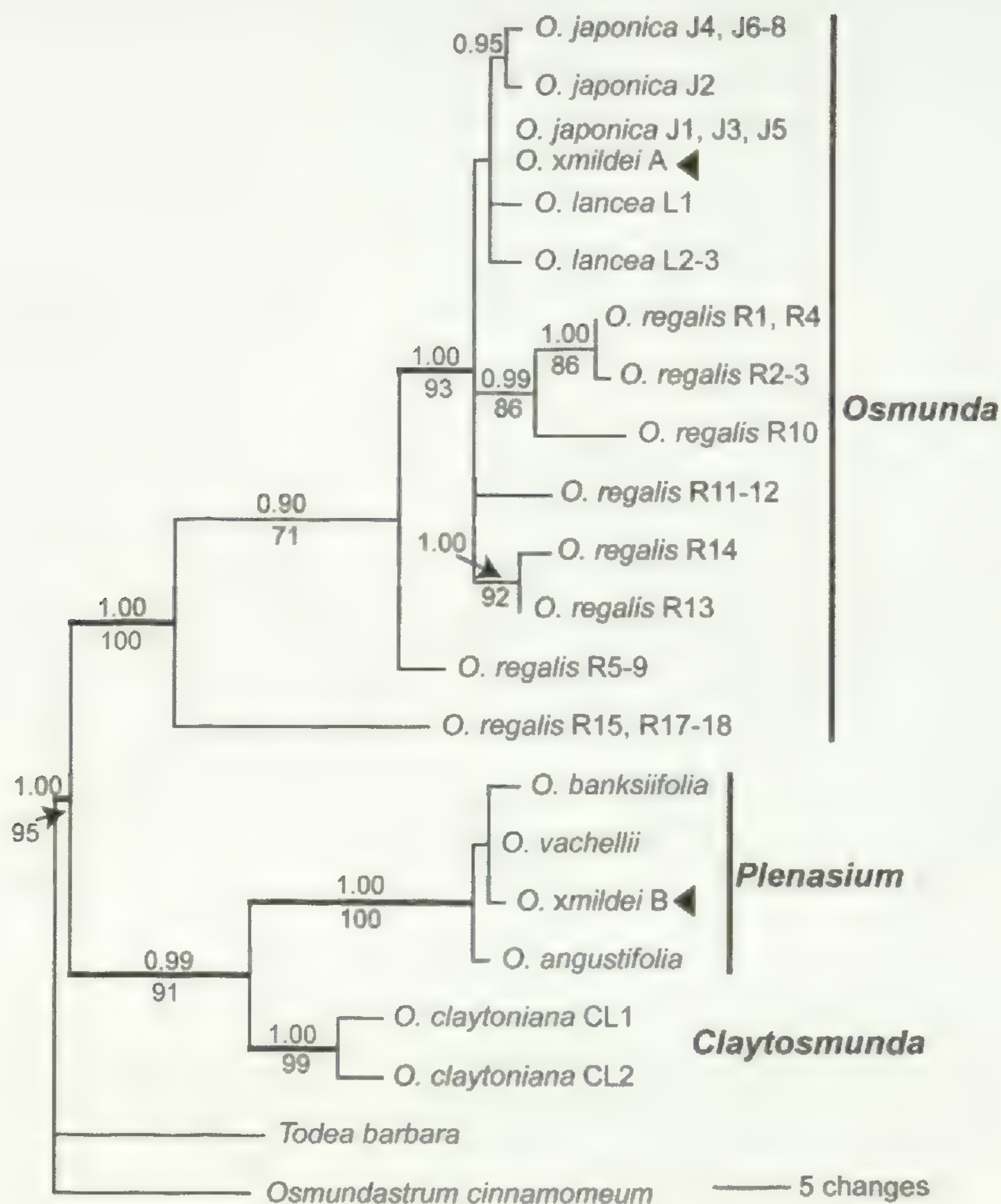


FIG. 6. Bayesian consensus tree based on nuclear EST L258 (361 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities $p > 0.95$ and bootstrap values > 90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O. xmildei*. Numbers of clones of each allele type are in Table 4.

sending a copy of a reference cited here and M. Nakajima for the illustrations. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

LITERATURE CITED

- GOU, C.-Y., S.-Z. ZHANG and S.-L. GENG. 2008. Phylogenetic position and genetic relationship of *Osmunda mildei* (Osmundaceae); evidence from *rbcL* gene and *trnL-trnF* region. *Acta Bot. Boreal.-Occident. Sin.* 28:2178–2183.
- HE, Z.-C., Y. LI, B. YAN, M. ZHENG and S.-Z. ZHANG. 2006. Karyotype analysis of five species in *Osmunda* (Osmundaceae). *Acta Phytotax. Sin.* 44:617–626.
- HUELSENBECK, J. P. and F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
- ITO, H. 1964. Type specimens of ferns in Makino Herbarium. *J. Jap. Bot.* 39:247–249.
- IWATSUKI, K. 1995. Osmundaceae. In K. Iwatsuki et al (eds.), *Flora of Japan*. Vol. 1. Pteridophyta and Gymnospermae, pp. 31–33. Kodansha, Tokyo, Japan.

- KATO, M. 2007. Distribution of Osmundaceae. *Bull. Natl. Mus. Nat. Sci., ser. B (Bot.)* 33:81–90.
- KATO, M. 2009. Hybrids in the fern genus *Osmunda* (Osmundaceae). *Bull. Natl. Mus. Nat. Sci., ser. B (Bot.)* 35:63–69.
- LI, J.-W. and C. H. HAUFLE. 1994. Phylogeny, biogeography, and population biology of *Osmunda* species: insights from isozymes. *Amer. Fern J.* 84:105–114.
- METZGAR, J. S., J. E. SKOG, E. A. ZIMMER and K. M. PRYER. 2008. The paraphyly of *Osmunda* is confirmed by phylogenetic analyses of seven plastid loci. *Syst. Bot.* 33:31–36.
- RAMBAUT, A. and A. J. DRUMMOND. 2009. Tracer v1.5. <http://beast.bio.ed.ac.uk/Tracer>
- RONQUIST, F. and J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
- SHIMURA, Y. 1972. Study of reproduction of *Osmunda* × *intermedia* Sugimoto. *J. Geobot.* 20:38–42.
- SUGIMOTO, J. 1979. Keys to Herbaceous Plants of Japan. III. Pteridophyta. Revised and enlarged ed. Inoue Book Company, Tokyo.
- SWOFFORD, D. L. 2002. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Sinauer, Sunderland, USA.
- TAGAWA, M. 1959. Coloured Illustrations of the Japanese Pteridophyta. Hoikusha, Osaka.
- TATUNO, S. and H. YOSHIDA. 1966. Kalyologische Untersuchungen über Osmundaceae I. Chromosomen der Gattung *Osmunda* aus Japan. *Bot. Mag., Tokyo* 79:244–252.
- THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN and D. G. HIGGINS. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 25:4876–4882.
- TRYON, R. M. JR. 1940. An *Osmunda* hybrid. *Amer. Fern J.* 30:65–68.
- TSUTSUMI, C., S. MATSUMOTO, Y. YATABE, Y. HIRAYAMA and M. KATO. 2011. A new allotetraploid species of *Osmunda* (Osmundaceae). *Syst. Bot.* 36(4):836–844.
- WAGNER, W. H. JR., F. S. WAGNER, C. N. MILLER, JR. and D. H. WAGNER. 1978. New observations on the royal fern hybrid *Osmunda* × *ruggii*. *Rhodora* 80:92–106.
- WHETSTONE, R. D., T. A. ATKINSON. 1993. Osmundaceae Berchtold & J. Presl. *In* Flora of North America Editorial Committee (ed.). 1993. Flora of North America. Vol. 2. Pteridophytes and gymnosperms, pp. 107–109. Oxford University Press, New York.
- YATABE, Y., H. NISHIDA and N. MURAKAMI. 1999. Phylogeny of Osmundaceae inferred from *rbcL* nucleotide sequences and comparison to the fossil evidence. *J. Plant Res.* 112:397–404.
- YATABE, Y., C. TSUTSUMI, Y. HIRAYAMA, K. MORI, N. MURAKAMI and M. KATO. 2009. Genetic population structure of *Osmunda japonica*, rheophilous *O. lancea* and their hybrids. *J. Plant Res.* 122:585–595.
- ZHANG, S.-Z., Z.-C. HE, C.-R. FAN and B. YAN. 2008. A cytogenetic study of five species in the genus *Osmunda*. *J. Syst. Evol.* 46:490–498.

The Tree Fern Highland Lace is a Cultivar of *Sphaeropteris cooperi*

DANIEL G. YANSURA

330 Carmel Ave., Pacifica, CA, 94044-2407, USA

BARBARA J. HOSHIZAKI

557 N. Westmoreland Ave., Los Angeles, CA, 90004-2210, USA

ABSTRACT.—The tree fern Highland Lace had an unusual introduction into cultivation almost thirty years ago in Eastern Australia and was initially identified as *Sphaeropteris tomentosissima* (Copel.) R.M.Tryon. Since then, it has been introduced to Europe and the US, and it remains a popular tree fern found in both public and private collections. We re-examined this fern, comparing it to a herbarium type specimen, and conclude that it is not *S. tomentosissima*, but is most likely a variant form of *Sphaeropteris cooperi* (F.V. Mueller) R.M.Tryon. Sequence analysis of chloroplast DNA [*rbcL*, *atpA* and *trnL* (UAA) intron] confirmed this species identification.

KEY WORDS.—Highland Lace, *Sphaeropteris tomentosissima*, *Sphaeropteris cooperi*, *Sphaeropteris excelsa*, tree ferns, chloroplast DNA sequence analysis, *rbcL*, *atpA*, *trnL* (UAA) intron

A distinctive tree fern with narrow pinnules and relatively small fronds appeared in Australian cultivation in the 1980s. It was a robust grower and its reduced pinnules imparted a lacy look to the leaves. Compared to most tree ferns, it was smaller, but it also seemed to bear more leaves in its crown. It originated as an unknown contaminant in a sporing pot at a wholesale nursery on the north coast of New South Wales, Australia. The late Rod Hill, an Australian tree fern enthusiast, made an attempt to identify the species and his closest match was *Sphaeropteris tomentosissima* (Copel.) R.M. Tryon, which grows in the highlands of west central New Guinea.

In the 1980s this plant spread among tree fern collectors and commercial growers in Australia and by the 1990s it was being grown in Europe and the United States. It is called either Highland Lace, New Guinea Treefern or *Sphaeropteris tomentosissima*. Its lacier appearance compared to other cultivated tree ferns has led to its high popularity, and it was awarded a first place and a trophy at the Los Angeles International Fern Society's annual Exotic Plant Show in 1997 and 2003 (Lois and Kurt Rossten, Huntington Beach, California).

Despite the enthusiasm for this new addition to the limited list of commercially available cultivated tree ferns, the identity of this fern was always a bit suspect, as noted by the question mark next to the species name on Rod Hill's former web site (Treeferns Down Under). We have re-examined this fern, and based on scale morphology and chloroplast DNA sequence analysis, conclude that it is actually a variant form of *Sphaeropteris cooperi* (F.V.Muell.) R.M.Tryon, rather than *S. tomentosissima*.

TABLE 1. Voucher information and GenBank accession numbers for tree ferns examined.

Species	Provenance	ID number	GenBank accession and reference
<i>S. tomentosissima</i>	Papua New Guinea	UC640117	
<i>S. tomentosissima</i>	Papua New Guinea	Conant 4581 (LSC)	Korall et al., 2006 <i>atpA</i> - AM176460 Korall et al., 2007 <i>rbcL</i> - AM177352 <i>trnL</i> intron - AM410304
<i>S. cooperi</i>	Highland Lace "Cultivated" Australia	Yansura 1 (UC)	<i>rbcL</i> - JN106035 <i>atpA</i> - JN106039 <i>trnL</i> intron - JN106036
<i>S. cooperi</i>	"Cultivated" Australia	Yansura 2 (UC)	<i>rbcL</i> - HM347350 <i>atpA</i> - JF690125 <i>trnL</i> intron - JF742607
<i>S. cooperi</i>	Flecker Botanical Gardens, Cairns Australia	Yansura 3 (UC)	<i>rbcL</i> - JN106038 <i>atpA</i> - JN106040 <i>trnL</i> intron - JN1060367

MATERIALS AND METHODS

The type specimen of *Sphaeropteris tomentosissima* (*Cyathea tomentosissima* Copel.) was examined and stipe scales were photographed at high resolution at the University and Jepson Herbaria at the University of California Berkeley (Brass 9116; UC 640117).

Leaf material for isolating chloroplast DNA was obtained from three sources: a cultivated plant of Highland Lace and a cultivated *Sphaeropteris cooperi*, both from the US (Hoshizaki's and Yansura's gardens); and four *S. cooperi* plants in the Flecker Botanical Gardens in Cairns, Australia. The latter four plants were carefully checked to be sure they had stipe scales consistent with *S. cooperi* as the garden had one plant labeled *Sphaeropteris excelsa* (Endlicher) R.M. Tryon, which could be confused with *S. cooperi* except for the scale differences. DNA was extracted from leaf material using the DNeasy Plant Mini Kit from QIAGEN (Valencia, California, USA), and the purified DNA was then used as a template to amplify three plastid loci (*rbcL*, *atpA*, *trnL* intron) using the polymerase chain reaction (PCR). The reaction was carried out with the appropriate set of primers and Cloned Pfu DNA polymerase from New England Biolabs (Ipswich, MA, USA) according to manufacturer's protocols. The PCR products were purified using the MinElute Reaction Cleanup kit from QIAGEN and then subjected to DNA sequencing on an ABI3730xl DNA Analyzer. All sequences (the four plants from the Flecker Botanical Garden had one common sequence) were deposited in GenBank (Table 1).

The beginning of the *rbcL* gene and the *atpB-rbcL* spacer were amplified with the primers *atpBR* or *atpBR1* and *RBCL158R*, the middle of the *rbcL* gene with primers *brun1* and *brun2*, and the 3' end as well as the *rbcL-accD* spacer with primers *RBCL1187F* and *ACCD887R*. The *atpA* gene was amplified by

TABLE 2. Primers used in amplification and sequencing. F = forward; R = reverse; S = sequencing.

Loci primer	Usage	Sequence (5'-3')	Reference
<i>rbcL</i> atpBR	F	TGAGCTTTGGCAATATTATTG	This study
<i>rbcL</i> atpBR1	F	TAATCTCTTGACCCGCTGGGTTAC	This study
<i>rbcL</i> RBCL158R	R S	AAGATTCCGCAGCTACTGCAGCTCC	Pryer, 2004
<i>rbcL</i> brun1	F S	CATTACCTTCACGAGCAAGGTCACGG	This study
<i>rbcL</i> RBCL1187F	F S	GGAACYTTGGGACATCCTTGG	Korall,2007
<i>rbcL</i> ACCD887R	R	TTATCACABCGMGCCCATTAATCC	Korall,2007
<i>rbcL</i> rbcf1	S	CCAAAATTGGGCTTATCTGCT	This study
<i>rbcL</i> rbcf2	S	CTAGCTTGGCCTTCTATTGCCG	This study
<i>atpA</i> ESATPF415F	F S	CARGTTCGACAGCAAGTYTCTCG	Schuettpelz,2006
<i>atpA</i> ESTRNR46F	R S	GTATAGGTTTCRARTCCTATTGGACG	Schuettpelz,2006
<i>atpA</i> atpAf	S	GACAGACTGGTAAAACAGCAGTAG	This study
<i>atpA</i> atpAr	S	TTGCCGGTCGAATGCCAGCATTAA	This study
<i>trnL</i> trn1	R S	ATTTGAACTGGTGACACGAGGATT	This study
<i>trnL</i> trn2	F S	CGAAATCGGTAGACGCTACGGACT	This study
<i>trnL</i> trn5	S	CTACCCTGTTCTGTTGGGGAT	This study
<i>trnL</i> trn6	S	TCCACGGGGGCTATTCCAACG	This study
<i>trnL</i> trn9	S	TCGAGTCTCTGTACCTATC	This study

PCR using the primers ESATPF415F and ESTRNR46F, and the *trnL* intron and flanking sequences were amplified with the primers trn1 and trn2. All primers used for PCR amplification and sequencing are listed in Table 2.

RESULTS

Our first indication that this tree fern might be misidentified was based on the stipe scales, which did not match the published description for *Sphaeropteris tomentosissima* (Holttum, 1963). Comparison with the type specimen reaffirmed that the two plants were very different in scales, leaf, and other details (Fig. 1 and 2A–C). Surprisingly, however, the stipe scales on Highland Lace closely match those of the commonly cultivated Australian tree

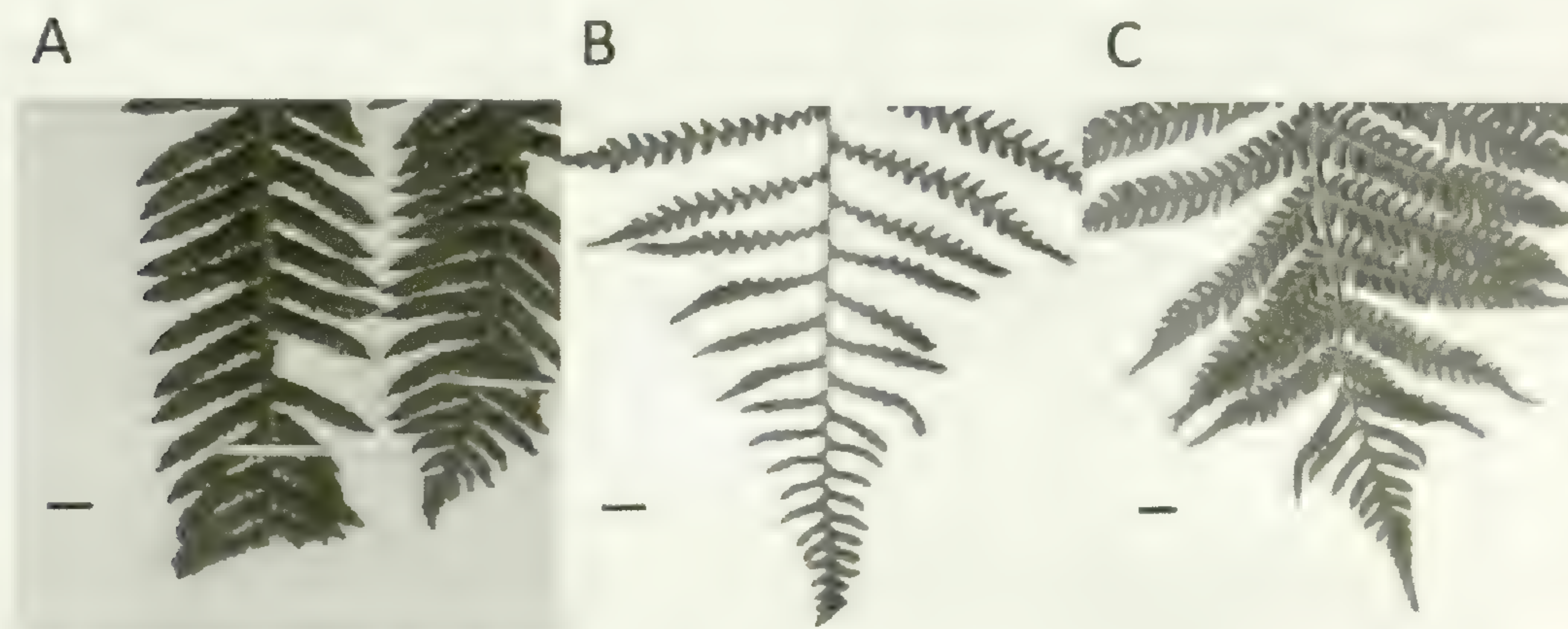


FIG. 1. A comparison of the pinnae. A: *Sphaeropteris tomentosissima*; B: Highland Lace; C: *Sphaeropteris cooperi*. The bars represent 1 cm.

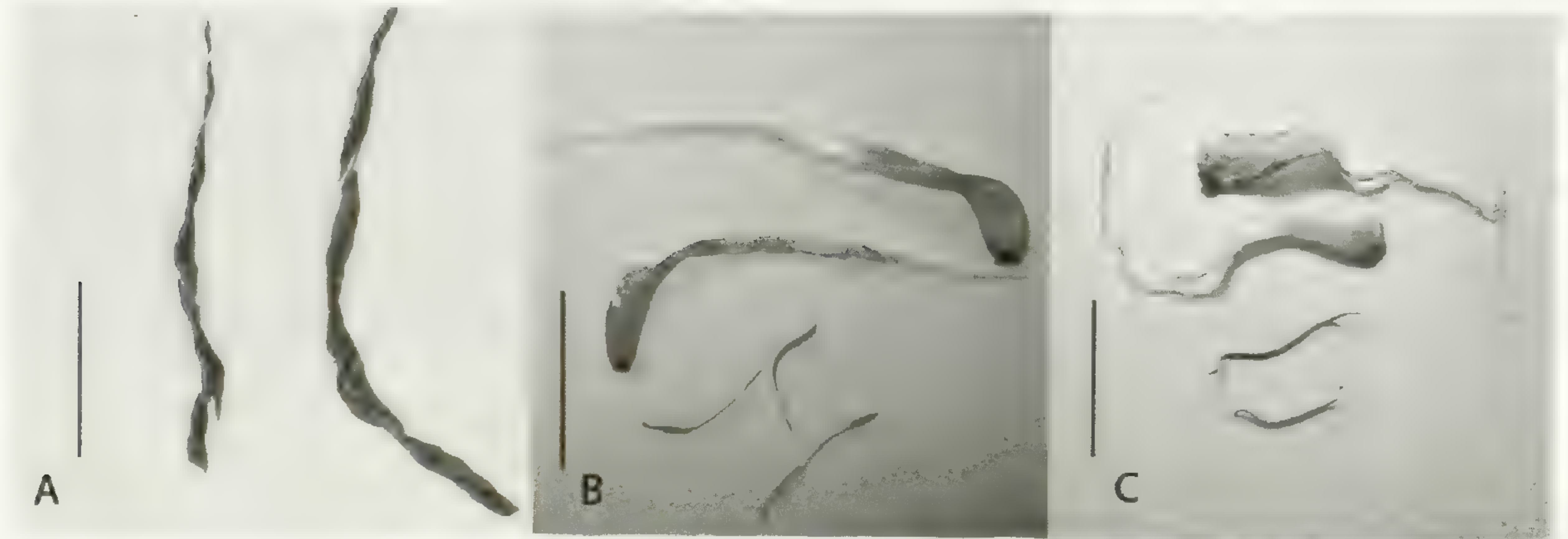


FIG. 2. A comparison of the stipe scales of *Sphaeropteris tomentosissima*, Highland Lace, and *Sphaeropteris cooperi*. A: Scales of *S. tomentosissima* are brown and twisted. B and C: Highland Lace and *S. cooperi* respectively both have large pale scales with dark setae on the edge as well as smaller narrow dark-red scales. The bars represent 1 cm.

fern *Sphaeropteris cooperi* despite their distinct differences in pinnae and leaf shape (Fig. 1).

The scales of Highland Lace were compared to *Sphaeropteris cooperi* and *Sphaeropteris tomentosissima*. The *S. tomentosissima* stipe scales are brown, twisted, and have edges bearing setae of the same color as the scales. In contrast, the broader stipe scales of Highland Lace and *S. cooperi* are whitish to light tan, with their margins usually bearing a very narrow row of dark reddish brown marginal cells and setae of the same color. Additionally, Highland Lace and *S. cooperi* have small narrow dark reddish brown scales on the stipe, which are absent on *S. tomentosissima* (Fig. 2A–C). Also particularly noticeable on *S. tomentosissima* are the very dense mats of small woolly scales on the abaxial side of all rachises (Fig. 3A–C), which are not present on *S. cooperi* or Highland Lace. The comparison of scales alone is highly suggestive that Highland Lace is much more closely related to *S. cooperi* than to *S. tomentosissima*.

In order to ascertain if Highland Lace is possibly *Sphaeropteris cooperi* and to further rule out *Sphaeropteris tomentosissima*, we compared *rbcL* DNA

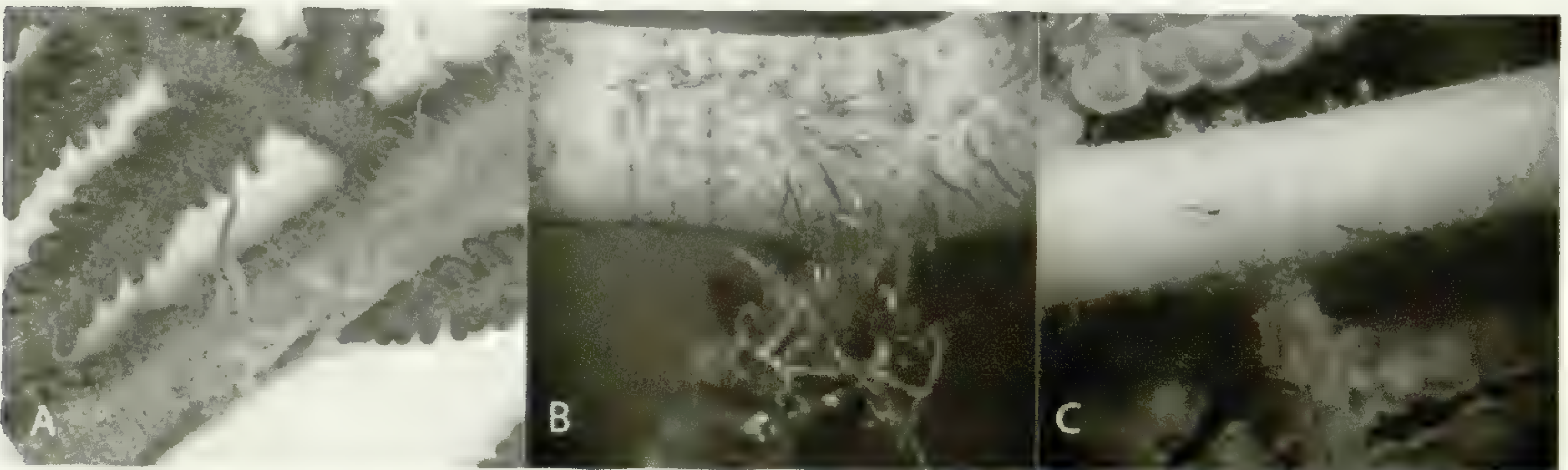


FIG. 3. A comparison of the costae of *Sphaeropteris tomentosissima*, Highland Lace and *Sphaeropteris cooperi*. A: *S. tomentosissima* has a dense mat of small woolly scales on the abaxial side. B and C: Highland Lace and *S. cooperi* respectively do not have the dense mat of woolly scales.

TABLE 3. Dissimilarity matrix indicating the number of base-pair changes observed for the three loci, *rbcL*, *atpA* and *trnL* intron. Numbers in parenthesis indicate the total base pairs compared.

		Highland Lace	Cultivated <i>S. cooperi</i>	Flecker <i>S. cooperi</i>	<i>S. tomentosissima</i>
<i>S. tomentosissima</i>	<i>rbcL</i>	4 (1309)	4 (1309)	4 (1309)	-
	<i>atpA</i>	1 (1514)	1 (1514)	1 (1514)	-
	<i>trnL</i>	2 (554)	2 (554)	2 (554)	-
Flecker <i>S. cooperi</i>	<i>rbcL</i>	0 (1428)	0 (1428)	-	
	<i>atpA</i>	0 (1521)	0 (1521)	-	
	<i>trnL</i>	0 (554)	0 (554)	-	
Cultivated <i>S. cooperi</i>	<i>rbcL</i>	0 (1428)	-		
	<i>atpA</i>	0 (1521)	-		
	<i>trnL</i>	0 (554)	-		
Highland Lace	<i>rbcL</i>	-			
	<i>atpA</i>	-			
	<i>trnL</i>	-			

sequence data for both species (Newmaster *et al.*, 2006; Korall *et al.*, 2007). The Highland Lace sequence differed from the partial gene sequence of *S. tomentosissima* in GenBank by four changes over the 1309 base pair (bp) length, further evidence that they were different species (Table 3). This sequence was then searched on GenBank and surprisingly the top BLAST match for Highland Lace was a sequence from *Sphaeropteris excelsa* rather than the expected *S. cooperi*. The 1309 bp sequence of *S. excelsa* (AM410213) was identical to that portion in Highland Lace, while the *S. cooperi* sequence (SCU05944) differed by four changes over 1320 bp.

This *rbcL* sequence comparison seemed to indicate that Highland Lace was closer to *S. excelsa* than to either *S. cooperi* or *S. tomentosissima*. However, the scales on Highland Lace did not match this conclusion. Highland Lace and *S. cooperi* have both broad pale scales as well as small narrow dark red scales on its stipe, while *S. excelsa* has only broad pale scales. Additionally, Highland Lace (and *S. cooperi*) has small narrow dark red scales on its costa and costule while *S. excelsa* has a mat of whitish scales and hairs (Hoshizaki and Yansura, 2005). Since the scales on Highland Lace matched those of *S. cooperi* rather than *S. excelsa*, we decided to obtain additional *S. cooperi rbcL* sequence data from a cultivated plant and from four plants from the Flecker Botanical Gardens (Table 1). All five sequences were identical over the complete *rbcL* gene of 1428 bp, and these were exact matches for the Highland Lace gene.

In order to further confirm the identity of Highland Lace, the chloroplast *atpA* gene sequence (Schuettpetz *et al.*, 2006) was obtained from this plant, from the cultivated *S. cooperi* plant and from the four tree ferns in the Flecker Botanical Garden. All six sequences matched perfectly over the complete gene sequence of 1521 bp, while the GenBank partial sequence for *Sphaeropteris tomentosissima* differed by one change over 1514 bp (Table 3), resulting in one

amino acid change (T213N). There were no reference *atpA* sequences in GenBank for *S. cooperi* or *S. excelsa*.

As a final step, we obtained DNA sequences for the *trnL* (UAA) intron (Taberlet *et al.*, 2007). Highland Lace perfectly matched that of cultivated *S. cooperi* and the four Flecker Botanical Gardens specimens over the intron's 554 bp (Table 3), and these sequences also matched exactly the partially overlapping 534 bp of *S. cooperi* in GenBank (EU554328) and *S. excelsa* over 525 bp (AM410341). The *S. tomentosissima* sequence in GenBank differed by two bp over the complete 554 bp overlap (Table 3).

Sphaeropteris excelsa and *S. cooperi* are closely related (Tryon and Tryon, 1959; Tryon, 1970; Jones and Clemesha, 1981) and share at least partial common *rbcL* and *trnL* (UAA) intron DNA sequences. These *S. excelsa* sequences were subsequently reconfirmed using leaf material from a cultivated plant (Hoshizaki and Yansura, 2005). The phylogenetic relationship between these species is unknown, but less conserved non-coding (Shaw *et al.*, 2005; Kress and Erickson, 2007) or nuclear sequences (Sang, 2002) could resolve this question.

DISCUSSION

The identification of tree ferns is especially difficult when the country of origin is not known (Pryer *et al.*, 2010). While Australia has only eleven native species (Jones and Clemesha, 1980), the possibility of non-native spore arriving from nearby New Guinea or from the collections of tree fern enthusiasts within the country is certainly reasonable. The unique appearance of Highland Lace, in particular the reduced pinnules, almost certainly led Rod Hill to identify it as the non-Australian species *S. tomentosissima*. Upon re-examining Highland Lace, the traditional use of stipe scales for tree fern identification suggested that this identity was incorrect.

The more recently developed approach of using chloroplast or nuclear DNA sequences as barcodes for species identification (Kress *et al.*, 2005; Chase *et al.*, 2005; CBOL Plant Working Group, 2009) has been shown to complement traditional analyses based on morphological characters. While DNA sequence analysis is becoming a more widely used tool for this purpose, the public database is still somewhat limited in terms of species coverage. There are only about 150 *rbcL* sequences from *Sphaeropteris*, *Cyathea* and *Alsophila* in GenBank, while worldwide there are over 600 Cyatheaceae tree fern species (Large and Braggins, 2004). However, an enlarged DNA database will eventually provide a more robust system.

The confirmation that Highland Lace is *S. cooperi* required the use of both morphological characters and DNA sequence analysis. The early study of stipe scales showed that Highland Lace was not *Sphaeropteris tomentosissima*, but it did not demonstrate that it was *S. cooperi*. To do so was more tenuous considering that there are approximately 120 *Sphaeropteris* species worldwide (Large and Braggins, 2004), many with similar scale morphologies.

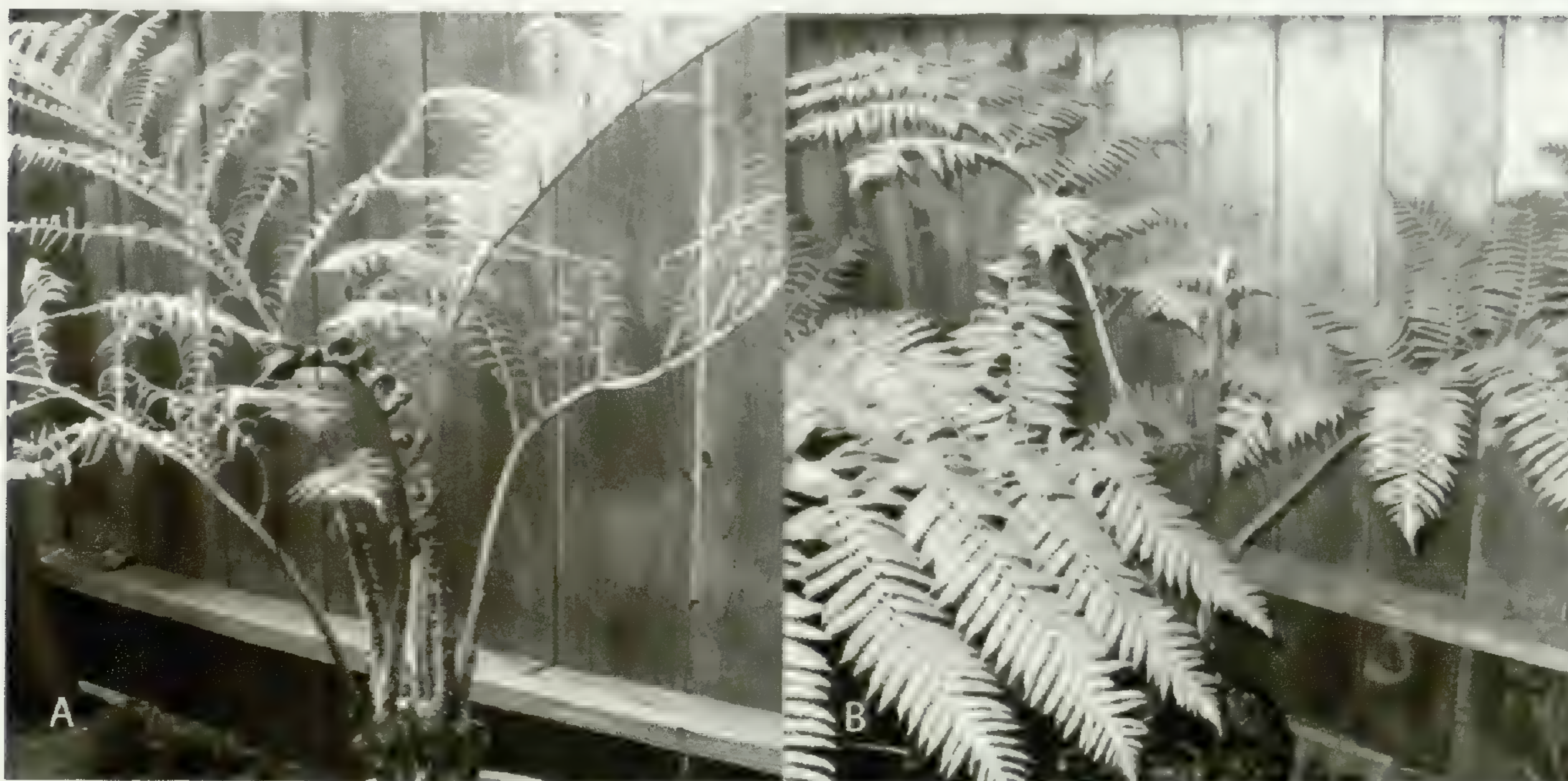


FIG. 4. An overall view of the tree ferns Highland Lace (A) and "Wild-type" *Sphaeropteris cooperi* (B) showing the significant differences in their general appearance.

Our first DNA sequence analysis based on *rbcL* confirmed that Highland Lace was not *S. tomentosissima*, but the effort to determine if it was related to *S. cooperi* resulted in the discovery of a GenBank voucher that was misidentified (see Results for details). As a result, new reference sequences were made for *S. cooperi*, which all proved identical to the Highland Lace sequence. Further DNA sequence analysis based on the chloroplast *atpA* gene and the *trnL* (UAA) intron also confirmed that Highland Lace is *S. cooperi* (Table 3).

As a practical way to identify a tree fern species, DNA barcoding is an important tool, but with the limited data available, it cannot be used exclusively. The initial Highland Lace *rbcL* sequence quickly showed that this tree fern was not *S. tomentosissima*. However, given the sequences that currently exist in GenBank, DNA barcoding could not distinguish whether *S. excelsa* or *S. cooperi* was the correct species. Morphologically specific features, particularly the leaf scales in tree ferns, still play an important role in fern identification. The use of morphological characters that initiated this investigation later led to the discovery of the error in the database and its subsequent correction, and scale characteristics ultimately allowed us to choose *S. cooperi* as the correct species. The interplay of these two methods was important throughout this study.

At first glance, it is difficult to think that Highland Lace and *S. cooperi* are actually the same species because their general appearances are so strikingly different (Fig. 4). *Sphaeropteris cooperi* is native to eastern coastal Australia and is known to be variable in form (producing cultivars including Brentwood, Robusta, Allyn Lace, and Allyn Kiest). Most of these variants, however, are quite modest compared to what is observed in Highland Lace with its conspicuously contracted, recurved margins and the reduced size of the

pinnules. *Sphaeropteris cooperi* shares this ability to produce multiple variants with a limited number of other ferns. Species such as *Athyrium filix-femina* (L.) Roth and *Polystichum setiferum* (Forssk.) Moore ex Woyнар are also known to produce many variants that have contracted or reduced blade surfaces, recurved margins, and smaller dimensions, plus many more deviations from the typical shape (Rickard, 2000; Hoshizaki and Moran, 2001).

A search of the literature suggests that this unusual tree fern may have been reported earlier. A description of *Sphaeropteris cooperi* (in Flora of Australia, 1998) mentions the existence of an unnamed narrow pinnule variant:

“An occasionally cultivated form of *Cyathea (Sphaeropteris) cooperi* from central and northern Queensland has narrow recurved abaxially glaucous pinnule lobes, with the majority of rhizome and stipe scales lacking any brown coloration. The sori in this form are commonly restricted to the basal part of each pinnule, at least on younger plants. The Victorian collection may be an isolated accidental occurrence rather than a sample from a naturalized population.”

This is possibly the same plant as Highland Lace. However, in the Flora of Australia description, the stem and stipe scales of this fern are said to lack any brown coloration while the Highland Lace specimens in the US have dark red-brown margins, bearing setae. The sori on Highland Lace also may extend well beyond the basal part of each pinnule to near the tip in the US specimens. If Rod Hill’s website is correct concerning the origin of this unusual fern in a spore pan (but in New South Wales instead of Queensland), we may consider this form an accidental occurrence. However it cannot be ruled out that this aberrant plant may also exist in the wild.

ACKNOWLEDGMENTS

We are indebted to Chris Goudey and Neil Shirley, both of Australia, for their help in providing information pertaining to this cultivar. We also thank the Flecker Botanical Gardens in Cairns, Australia for providing leaf samples from several *Sphaeropteris cooperi* plants for DNA analysis. Lastly, we would like to acknowledge the generous help we received at the University and Jepson Herbaria at the University of California Berkeley: Alan Smith and Andrew Doran gave us general assistance in locating specimens; Kelly Agnew took and then provided us with the high-resolution photographs used in this article.

LITERATURE CITED

- CBOL PLANT WORKING GROUP. 2009. A DNA barcode for land plants. *Proc. Natl. Acad. Sci.* 106:12794–12797.
- CHASE, M. W., N. SALAMIN, M. WILKINSON, J. M. DUNWELL, R. P. KESANAKURTHI, N. HAIDAR and V. SAVOLAINEN. 2005. Land plants and DNA barcodes: short-term and long-term goals. *Phil. Trans. R. Soc. B* 360:1889–1895.
- FLORA OF AUSTRALIA. 1998. *Ferns Gymnosperms and Allied Groups*. 48:203. Australian Biological Resources Study/CSIRO Publishing.
- HOLTTUM, R. E. 1963 *Cyatheaceae*. *Flora Malesiana, II Pteridophyta* 1:128.
- HOSHIZAKI, B. J. and D. G. YANSURA. 2005. Cooper’s tree fern and Norfolk Island tree fern (*Cyathea cooperi* (F. v. M. ell.) Domin and *Cyathea brownii* Domin). *Fiddlehead forum* 32(3):17–20.

- HOSHIZAKI, B. J. and R. C. MORAN. 2001. *Fern Grower's Manual*. Timber Press, Portland, Oregon, USA.
- JONES, D. L. and S. C. CLEMESHA. 1980. *Australian Ferns and Fern Allies*. Reed Books PTY LTD, NSW, Australia.
- KORALL, P., D. S. CONANT, J. S. METZGAR, H. SCHNEIDER and K. M. PRYER. 2007. A molecular phylogeny of scaly tree ferns (Cyatheaceae). *Am. J. Bot.* 94:873–886.
- KORALL, P., K. M. PRYER, J. S. METZGAR, H. SCHNEIDER and D. S. CONANT. 2006. Tree Ferns: Monophyletic groups and their relationships as revealed by four protein-coding plastid loci. *Mol. Phylogenet. Evol.* 39:830–845.
- KRESS, J. W., K. J. WURDACK, E. A. ZIMMER, L. A. WEIGT and D. H. JANZEN. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA* 102:8369–8374.
- KRESS, J. W. and D. L. ERICKSON. 2007. A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. *PLoS ONE* 2:e508.
- LARGE, M. F. and J. E. BRAGGINS. 2004. *Tree Ferns*. Timber Press, Portland, Oregon, USA.
- NEWMASER, S. G., A. J. FAZEKAS and S. RAGUPATHY. 2006. DNA barcoding in land plants: evaluation of *rbcL* in a multigene tiered approach. *Can. J. Bot.* 84:335–341.
- PRYER, K. M., E. SCHUETTELPELZ, 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. *Am. J. Bot.* 91:1582–1598.
- PRYER, K. M., E. SCHUETTELPELZ, L. HUIET, A. L. GRUSZ, C. J. ROTHFELS, T. AVENT, D. SCHWARTZ and M. D. WINDHAM. 2010. DNA barcoding exposes a case of mistaken identity in the fern horticultural trade. *Mol. Ecol. Resour.* 10:979–985.
- M. RICKARD 2000. *The Plantfinder's Guide to Garden Ferns*. David & Charles Publishers Brunel House, Newton Abbot, Devon, UK.
- SANG, T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. *Crit. Rev. Biochem. Mol. Biol.* 37(3):121–147.
- SCHUETTELPELZ, E., P. KORALL and K. M. PRYER. 2006. Plastid *atpA* data provide improved support for deep relationships among ferns. *Taxon* 55:897–906.
- SHAW, J., E. B. LICKY, J. T. BECK, S. B. FARMER, W. LIU, J. MILLER, K. C. SIRIPUN, C. T. WINDER, E. E. SCHILLING and R. L. SMALL. 2005. The Tortoise and the Hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *Am. J. Bot.* 92:142–166.
- TABERLET, P., E. COISSAC, F. POMPANON, L. GIELLY, C. MIQUEL, A. VALENTINI, T. VERMAT, G. CORTHER, C. BROCHMANN and E. WILLERSLEV. 2007. Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucleic Acids Res.* 35:e14 1–8.
- TRYON, R. and A. TRYON. 1959. Observations on Cultivated Ferns: The Hardy Species of Tree ferns (*Dicksonia* and Cyatheaceae). *Am. Fern J.* 49(4):129–142.
- TRYON, R. 1970. The classification of the Cyatheaceae. *Contr. Gray Herb.* 200:3–53.

Elaphoglossum montanum, a New Species from Southern Brazil

MARIA A. KIELING-RUBIO

Universidade Federal do Rio Grande do Sul, PPG Botânica, Prédio 43433, CEP: 91501-970 Porto Alegre, Brazil. e-mail: angelrubio@ig.com.br

PAULO G. WINDISCH

Universidade Federal do Rio Grande do Sul, PPG Botânica, CP 15020, UFRGS Campus do Vale; 91501-970 Porto Alegre, Brazil. e-mail: pteridos@gmail.com

ABSTRACT.—*Elaphoglossum montanum*, a new fern species of the Atlantic Forest in southern Brazil, is described, illustrated, and compared to the most similar species. It belongs to the *Elaphoglossum* “*Subulate scales clade*” and occurs in the upper montane forest regions in the States of Rio Grande do Sul and Santa Catarina, between 600 and 1400 m.

KEY WORDS.—Atlantic Forest, southern Brazil, pteridophytes, taxonomy, floristic diversity

Elaphoglossum Schott ex J. Sm. contains ca. 600 species and ranks as one of the largest and most complex genera of ferns (Mickel and Atehortúa, 1980). It is pantropical but it is most diverse in the Neotropics, where ca. 80% of the species occur (Moran *et al.*, 2007). In Brazil, the highest species diversity is in the Atlantic Forest biome (Windisch and Kieling-Rubio, 2010), which is considered by Tryon (1972) as one of the three main centers of fern endemism and speciation in Tropical America. In southern Brazil (States of Paraná, Santa Catarina, Rio Grande do Sul), about 40 species of *Elaphoglossum* occur (Windisch and Kieling-Rubio, 2010), most of them occurring in humid forests, especially in montane and submontane areas.

Mickel and Atehortúa (1980) considered the genus *Elaphoglossum* as presenting nine sections, based on morphological characters. Part of these sections was supported by the molecular phylogeny presented by Skog *et al.* (2004) and Rouhan *et al.* (2004). Among the clades recovered by those studies is the “*Subulate scales clade*”. Rouhan *et al.* (2004) indicated more details studies should precede a formal taxonomic definition of this group within the genus *Elaphoglossum*.

During a study on the genus *Elaphoglossum* for Brazil, we found a new species with subulate scales and hydathodes on the laminar margin, belonging to the “*Subulate scales clade*”, *sensu* Skog *et al.* (2004), which we describe as follow.

Elaphoglossum montanum Kieling-Rubio & P.G. Windisch, *sp. nov.* TYPE.—BRAZIL. Santa Catarina: Lauro Müller, Serra do Rio do Rastro (28°23'58.1"S 49°33'0.3"W), 1372 m, 10 Mar 2011, *Kieling-Rubio & Windisch 900* (holotype ICN; isotypes B, RB). **Figs. 1–2.**

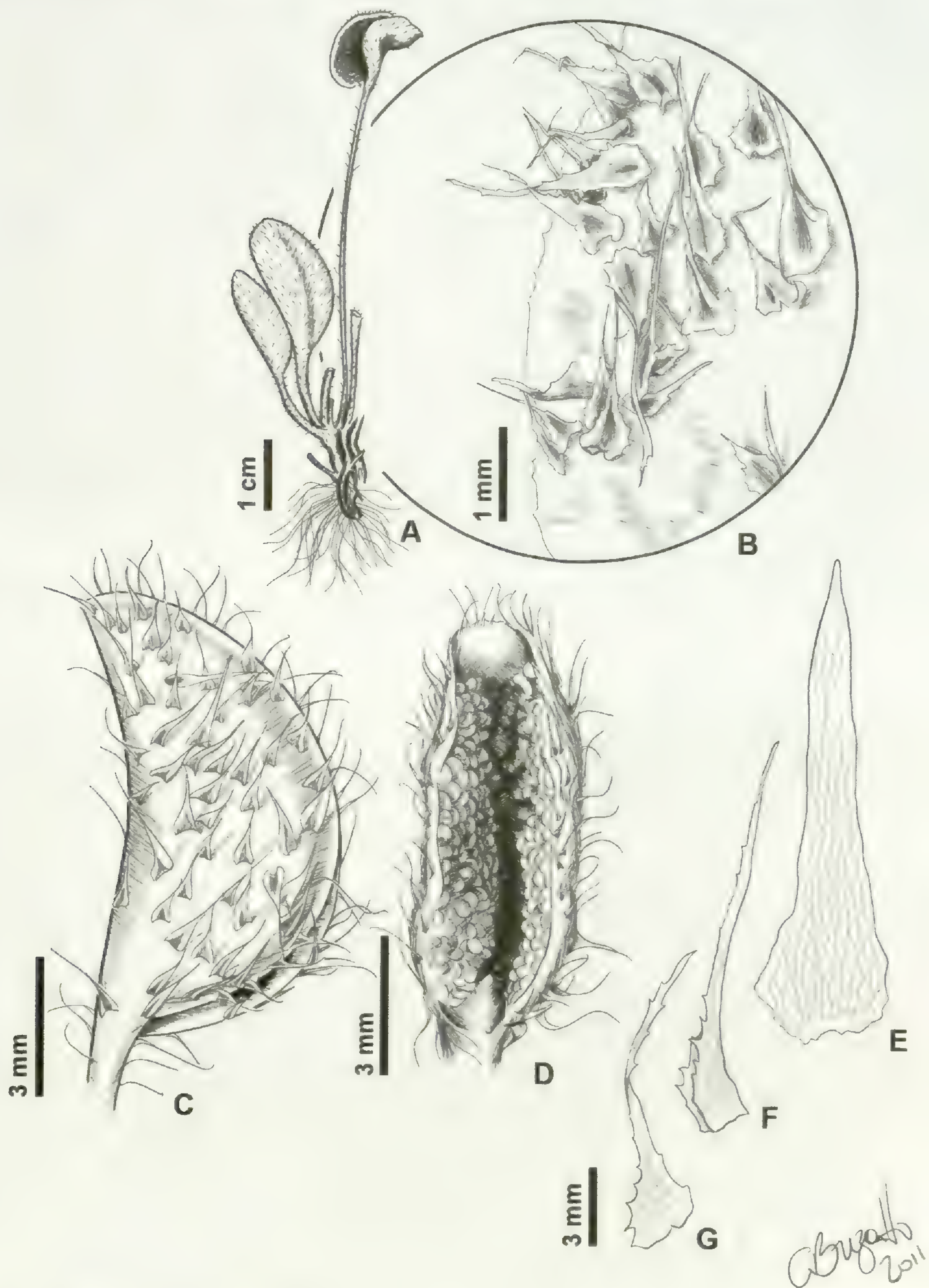


FIG. 1. *Elaphoglossum montanum*. A) Habit. B) Sterile lamina. C) Fertile lamina. D) Fertile lamina partially opened exposing sporangia. E) Rhizome scale. F–G) Laminar scales. (All Kieling-Rubio & Windisch 900 ICN).

Species *Elaphoglossum piloselloides* (C. Presl) T. Moore habitu aliquot similis, a qua frondibus fertilibus rotundatis et sporis uniformiter echinatis sine cristis differt.

Plants litophytic. Rhizomes short-ascending, 1.5–2.9 mm diam., rhizome scales 0.2–0.4 × 2.5 mm, brown, lanceolate. Fronds dimorphic, 2.8–9 cm long.

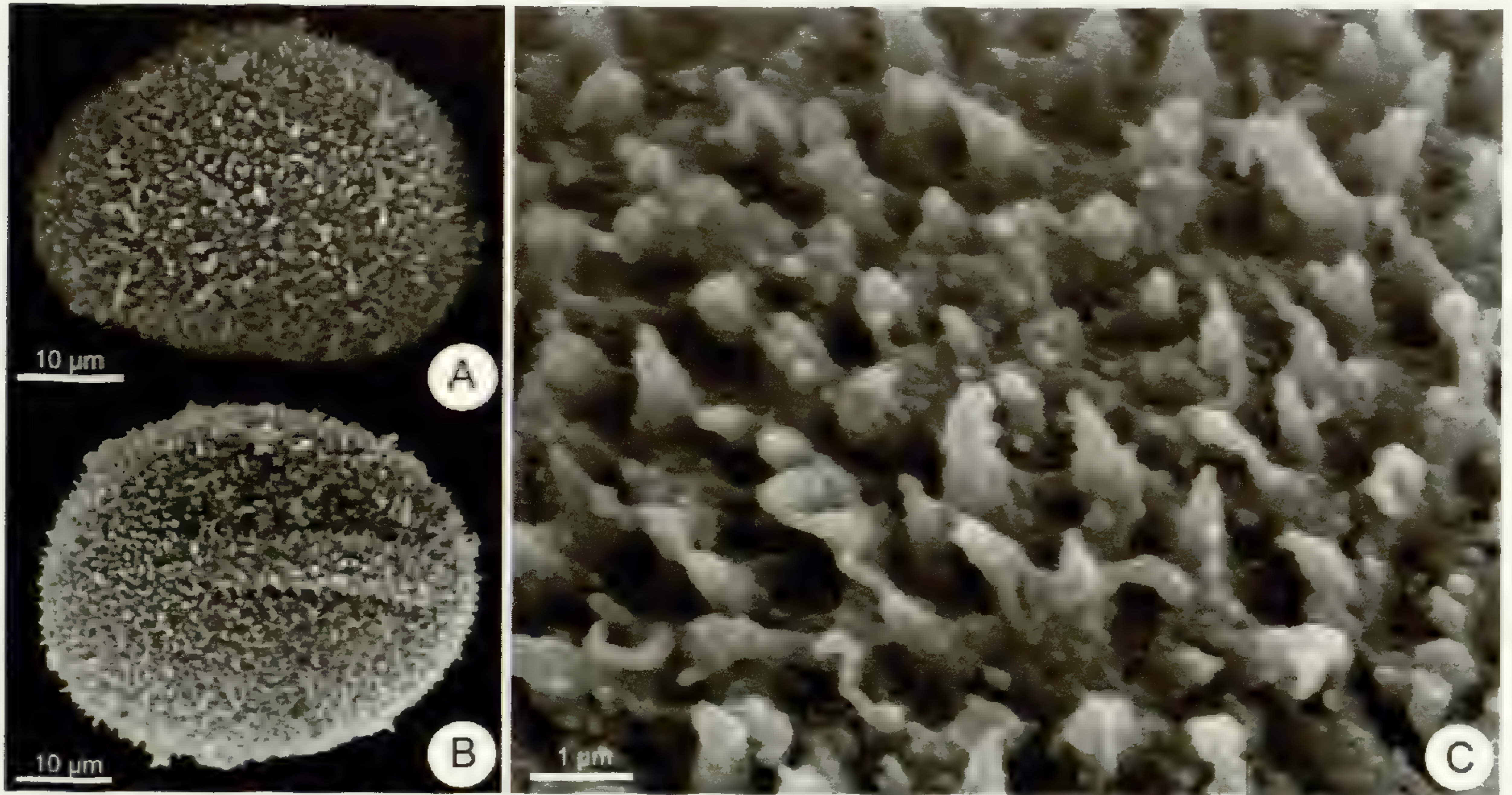


FIG. 2. A–C. Scanning electron micrographs of spore of *Elaphoglossum montanum* (Spanholi s.n. ICN). A) Distal face. B) Proximal face. C) Detail of the echinate perispore.

Sterile fronds simple, 2.8–7 cm long; stipes 1.5–4.0 cm long \times 0.5–0.8 (1.0) mm diam., light green, covered with subulate scales (bases flat or somewhat enrolled), 2–5 mm long, light brown sometimes darker at the base, with dentate margins and a hair-like apex; laminae 1.3–3.0 cm long \times 0.4–0.8 mm wide, chartaceous to coriaceous, elliptic, apices rounded, margins recurved when dry, veins barely visible, terminating in hydathodes close to the margins; laminar scales similar to those of the stipes, densely covering both surfaces when young, then glabrescent on the adaxial surface. Fertile fronds equaling or usually longer than the sterile ones; stipes 2.5–6.0 cm long \times 0.6–1.0 mm diam., brown, scales similar to those on sterile fronds; fertile laminae 0.5–3.0 cm long \times 0.4–1.0 cm wide, rounded (appearing reniform when conduplicate), base narrowly decurrent, adaxial surfaces covered with subulate scales similar to those of the sterile fronds; margins membranous. Spores monolete with a uniformly echinate perispore (Fig. 2).

DISTRIBUTION AND ECOLOGY.—*Elaphoglossum montanum* is only known from the upper montane region between the States of Rio Grande do Sul and Santa Catarina, in areas with humid forests, from 600 to ca. 1400 m. The two known populations were found on wet cliffs, in shaded places, along (and even underneath) individuals of *Gunnera manicata* Linden ex André.

ETYMOLOGY.—The specific epithet “*montanum*” refers the occurrence of the species in the mountains of the Serra Geral.

ADDITIONAL SPECIMENS EXAMINED.—BRAZIL. Rio Grande do Sul: Barracão, Rio Bernardo José com rio Pelotas, 02 Sep 2000, *Spanholi s.n.* (HAS 39037, MBM 256416); Bom Jesus, Barragem – Rio dos Touros, 09 Dec 1958, *Camargo s.n.*

(PACA 79067); São Francisco de Paula, Taimbé, 27 Feb 1959, *Sehnem s.n.* (HUCS 7292); 17 Feb 1953, *Sehnem 6328* (PACA); Taimbezinho, 30 Apr 1967, *Sehnem s.n.* (PACA 79068). Santa Catarina: Bom Jardim da Serra, Serra do Rio do Rastro, 09 Dec 1994, *Bueno 4468* (ICN); Colônia Anita Garibaldi, 1907, *Spannagel 405* (B, RB); Lages, 1906, *Spannagel 165* (HB); Urubici, Serra do Corvo Branco, 01 Jan 2009, *Buzatto 411* (ICN); Urubici, Serra do Corvo Branco, 09 Nov 2010, *Dettke 452* (ICN); Urubici, Serra do Corvo Branco, 28°03'25"S 49°21.5'41"W, 1200 m, 11 Mar 2011, *Kieling-Rubio & Windisch 915* (ICN).

Elaphoglossum montanum is similar to *E. piloselloides* (from Peru to southeastern and central-western Brazil), and *E. jamesonii* (Hook. & Grev.) T. Moore (Andean region) by presenting small fertile fronds that remain conduplicate until the full maturation of the sporangia, and by the subulate scales with dentate margins and hair like tips present on the fronds. However, *Elaphoglossum montanum* can be easily distinguished by having a more rounded fertile laminae, echinate spores, and light brown subulate scales on the adaxial surfaces of the sterile laminae. In contrast, *E. piloselloides* has narrowly and oblong fertile laminae, crested spores (Moran *et al.* 2007) and dark brown subulate scales on the adaxial surfaces of the sterile laminae. *Elaphoglossum jamesonii*, on the other hand, can be distinguished by its crested perispores (SEM of spore from the *type* at Berlin, B-200070911).

Elaphoglossum minutissimum R. C. Moran & Mickel, from Costa Rica (Moran and Mickel, 2004) is also a similar species, which differs from *E. montanum* by not having conduplicate fronds.

ACKNOWLEDGMENTS

To the Federal University of Rio Grande do Sul for the research facilities, to the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES) for the Doctoral Fellowship awarded to the first author; to the “Deutscher Akademischer Austauschdienst” (DAAD) for financial aid during the visit to the collections abroad (B, BM, K, and M), to the Brazilian Research Council (CNPq) for research grants. To the curators of the following Herbaria for the loans and assistance during our visits (B, BM, CORD, D, HAS, HB, HBG, HBR, HUCCS, ICN, K, M, MBM, MO, PACA, P, PR, R, RB, S, SJRP, SP, SPF, UPCB), especially to Dr. Brigitte Zimmer and Dr. Robert Vogt for their support at the Botanical Gardens of Berlin. Dr. David Lellinger and Dr. Robbin Moran kindly provided suggestions on a first version of the manuscript. To botanists present and past who contributed with their collections, as well as to Greta Aline Dettke for assistance in SEM and to Cristiano Roberto Buzatto for the illustrations.

LITERATURE CITED

- MICKEL, J. T. and L. ATEHORTÚA. 1980. Subdivision of the genus *Elaphoglossum*. *Am. Fern J.* 70:47–68.
- MORAN, R. C., J. GARRISON-HANKS and G. ROUHAN. 2007. Spore morphology in relation to phylogeny in the fern genus *Elaphoglossum* (Dryopteridaceae). *Int. J. of Plant Sci.* 168:905–929.
- MORAN, R. C. and J. T. MICKEL. 2004. Three new neotropical species of *Elaphoglossum* (Elaphoglossaceae) with subulate scales. *Brittonia* 56(3):200–204.

- ROUHAN, G., J. DUBUISSON, F. RAKOTONDRAINIBE, T. J. MOTLEY, J. T. MICKEL, J. LABAT and R. C. MORAN. 2004. Molecular phylogeny of the fern genus *Elaphoglossum* (Elaphoglossaceae) based on chloroplast non-coding DNA sequences: contributions of species from the Indian Ocean area. *Mol. Phyl. Evo.* 33:745–763.
- SKOG, J. E., J. T. MICKEL, R. C. MORAN, M. VOLOVSEK and E. A. ZIMMER. 2004. Molecular studies of the New World species in the fern genus *Elaphoglossum* (Dryopteridaceae) based on chloroplast DNA sequences. *Int. J. Plant Sci.* 165:1063–1075.
- THIERS, B. [continuously updated]. Index Herboriorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. Available online at <http://sweetgum.nybg.org/ih/>. Accessed september 21, 2010.
- TRYON, R. M. 1972. Endemic areas and geographic speciation in tropical American ferns. *Biotropica* 4(3):121–131.
- WINDISCH, P. G. and M. A. KIELING-RUBIO. 2010. *Elaphoglossum*. In: Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available online at <http://floradobrasil.jbrj.gov.br/2010/FB09100>. Accessed march 30, 2011.

SHORTER NOTES

First Record of *Pellaea ovata* (Pteridaceae) from Brazil.—According to the monograph of *Pellaea* sect. *Pellaea* by Alice Tryon (Ann. Missouri Bot. Gard. 44:125–193. 1957), the members of this section (about 10 species) occur primarily in the southwestern United States and Mexico and four species and their varieties (*P. ternifolia* Cav., *P. ovata* (Desv.) Weath., *P. sagittata* (Cav.) Link, and *P. myrtillifolia* Mett. ex Kuhn) are also distributed along the Andes, reaching to Argentina and Chile; only one species occurs in Africa (*P. rufa* A.F. Tryon). Alice Tryon (l.c.) also commented that Hicken (Soc. Arg. Cienc. Nat. 13:206. 1916) had reported *P. ternifolia* from Minas Gerais, Brazil, but she had not seen material of this species from Brazil during the revision of this group. Subsequent authors such as Sota (in Cabrera, Fl. Prov. Jujuy Rep. Argentina 13:109–111. 1977), Tryon and Stolze (Fieldiana, Bot., n.s. 22:2–83. 1989), Murillo-Pulido and Harker-Useche (Helechos y Pl. Afines Col.:158–159. 1990), Yatskievych (in Moran and Riba, Fl. Meso. 1:136–137. 1995), Arbeláez (in Jørgensen and León-Yáñez, Monogr. Syst. Bot. Missouri Bot. Gard. 75:168–176. 1999), Prado (in Hokche *et al.*, Nuevo Cat. Fl. Vasc. Venez.:162. 2008), and Ponce *et al.* (in Zuloaga *et al.*, Monogr. Syst. Bot. Missouri Bot. Gard. 107:161. 2008) also confirmed this pattern of distribution of these four *Pellaea* species along the Andean Cordillera, and Prado and Sylvestre (in Forzza *et al.*, Cat. Pl. and Fungi of Brazil, 1:557–558. 2010) corroborated their absence in Brazil.

However, recently, in 2010, the first author, during field work for his Master thesis, found a population of *Pellaea ovata* growing in the mountains in the center of the state of São Paulo, more precisely in the Municipality of Botucatu, Pavuna Farm, trail to the waterfall, 22° 50' 15" S, 48° 30' 40" W, 750 m, 13 Jan 2010, *Biral & Gomes 511* (BHCB, HRCB, SP). In 2011 we visited the same population of this species and another sample was collected: Pavuna Farm, Road Marechal Rondon (SP 300), km 259, between Botucatu and São Manuel, 22° 50' S, 48° 30' W, 600 m, 6 May 2011, *Prado et al. 2143* (DUKE, HRCB, MO, NY, P, SP, UC). *Pellaea ovata* belongs to the Section *Pellaea* and it can be easily recognized by the scandent habit, creeping and dichotomously branched rhizomes, flexuous and pubescent rachises, stalked and ovate to cordate segments, and lamina glabrous on both surfaces (Fig. 1). Our two collections represent the first record of *P. ovata* from Brazil.

Pellaea ovata is distributed from the southern United States (Turner *et al.*, Atlas Vasc. Pl. Texas, v.2, Sida, Bot. Misc., pg. 666. 2003) to Argentina, including Central America and Hispaniola (Tryon l.c.). At the Pavuna Farm, it grows among grasses and at the bases of *Aechmea distichantha* Lem. (Bromeliaceae) and *Praecereus euchlorus* (F.A.C. Weber) N.P. Taylor (Cactaceae). The Pavuna Farm encompasses the largest fragment of the “Mata da Pavuna” and occupies an area of 378.49 ha at an elevation of 600–761 m. The relief is steep, with slopes between 30°–90° of inclination, and soils that are shallow, dry, and sandy. There are two distinct climatic seasons in this region,

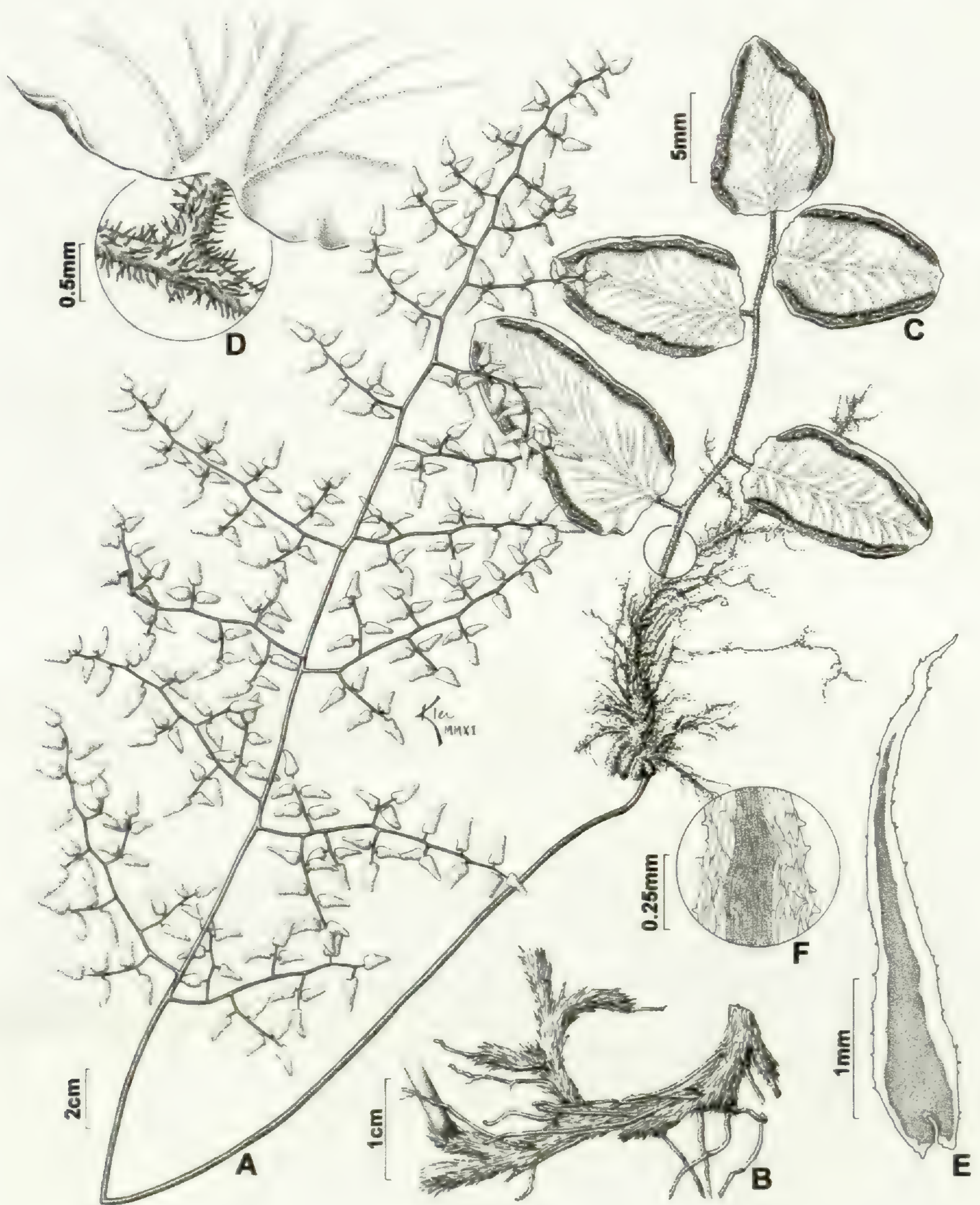


FIG. 1. A–F *Pellaea ovata*. A. Habit. B. Rhizome. C. Detail of the pinnules abaxially. D. Detail of the hairs on the segment stalk. E. Rhizome scale. F. Detail of the rhizome scale (Prado et al. 2143, SP).

the rainy summer and dry winter. Average monthly temperatures range from 12.4 C (September) to 28.1 C (January) and mean monthly rainfall from 270.15 mm (January) to 27.92 mm (August) (data graciously provided by the Meteorological Station of Experimental Farm São Manuel, Faculdade de

Ciências Agrárias, University of São Paulo State, Botucatu, SP, Brazil). The main vegetation in the area is semideciduous forest (i.e., non-Atlantic forest) and on the tops of the slopes there are some xerophitic elements among rocks, such as *Aechmea distichantha*, *Praecereus euchlorus* and *Cereus hildmannianus* K. Schum. (Cactaceae). This area belongs to the "Residual Pleistocenic Seasonal Formations Arc" in South America (Prado and Gibbs, Ann. Missouri Bot. Gard. 80:902–927. 1993). According to these authors, nowadays this arc contains remnants of the dry vegetation of that time, including the genera noted above. The arborescent component of the slopes in this area is dominated by *Aspidosperma riedelii* Müll. Arg. (Apocynaceae), a species cited by Prado and Gibbs (l.c.) as typical of dry seasonal forests of South America. *Pellaea ovata* can be added here as another example of a relictual element, because its distribution is coincident with this dry arc, especially along the line of latitudes between 15°–28°S, between Bolivia (Chuquisaca, La Paz, Tarija, Cochabamba), Argentina (Catamarca, Salta and Tucumán), and Brazil (São Paulo). It is also cited as occurring in dry vegetation in Ecuador (Wiggins, Amer. Fern J. 36(1):1–7. 1946).

This note is one of the results of the project "Vascular flora of 'Mata da Pavuna', Botucatu, SP, Brazil" developed by the first author and Julio A. Lombardi, and was supported by The National Council for Scientific and Technological Development (CNPq). We thank Klei Souza for preparing the drawings.—LEONARDO BIRAL, Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Depto. de Botânica, CEP 13506-900, Rio Claro, SP, Brazil, and JEFFERSON PRADO, Instituto de Botânica, Herbário SP, Av. Miguel Stéfano, 3687, CEP 04301-012 São Paulo, SP, Brazil.

SHORTER NOTES

***Botrychium simplex* E. Hitchcock: a New Moonwort for the Indian Himalayan Mountains.**—The genus *Botrychium* Sw., has a nearly cosmopolitan distribution in the subtropical, temperate, boreal and polar regions of the world (Copeland, *Genera Filicum*. Cronica Botanica Co. Waltham Mass. USA. 1947; Wagner Jr., *Ophioglossaceae*. In: K. Kubitzki, ed. *The Families and Genera of Vascular Plants. I. Pteridophytes and Gymnosperms* pp. 193–197. Vol. eds.: K. U. Kramer and P. S. Green, Springer-Verlag. 1990). The genus is represented by 50–60 species (Wagner and Wagner, *Ophioglossaceae* C. Agardh: Adder’s-tongue family. pp. 85–106. In: *Flora of North America* Editorial Committee, eds. *Flora of North America, north of Mexico, Part 2: Pteridophytes and gymnosperms*. Oxford University Press, New York. 1993) of which 19 occur in Asia (Sahashi, Preliminary review on Asian Botrychiaceae. pp. 54–75. In: X. -C. Zhang and K. -H. Shing, eds. *Ching Memorial Volume*. China Forestry Publishing House, Beijing. 1999). In India, the genus is well studied at national and regional levels (Beddome, *Handbook to the Ferns of British India: Ceylon and the Malay Peninsula*. pp. 469, Thacker, Spink, and Co., Calcutta, India. 1883; Clarke, *Trans. Linn. Soc. Ser. 2: Botany, Vol. 1*. 587. 1880; Panigrahi and Dixit, *Proc. Nat. Inst. Sci. India* 35 B (3): 230–266. 1969; Goswami, *Bionature*. 7:47–89. 1987; Bir and Bhusri, *Indian Fern J.* 2: 39–56. 1985; Khullar, *An illustrated fern flora of West Himalaya Vol. I*. pp. 1–15. International Book Distributors, Dehradun, India. 1994) and is included in several checklists and survey reports (Iwatsuki, *Pteridophyta*. In: *Flora of the eastern Himalaya*. pp. 166–205. In: Ohashi, ed, 3rd report. *Bull. Univ. Mus. Univ. Tokyo*. 1975; Dixit, *A Census of the Indian Pteridophytes*. pp 21–22. Botanical Survey of India. Howrah, India. 1984). Following Panigrahi and Dixit (1969), recently Fraser-Jenkins (*Taxonomic revision of three hundred Indian subcontinental Pteridophytes*. pp 21–22; 525–526. Bishen Singh Mahendra Pal Singh, Dehradun, India. 2008) recognized six species of *Botrychium* from India; these are: *Botrychium daucifolium* Wall. ex Hook. et Grev., *Botrychium lanuginosum* Wall. ex Hook. et Grev., *Botrychium lunaria* (L.) Sw., *Botrychium multifidum*(Gmel.) Rupr., *Botrychium ternatum* (Thunb.) Sw. and *Botrychium virginianum*(L.) Sw. However, he did not accept various subspecies or varieties others described from India or the Himalayas. The present author follows this treatment, because most of these taxa from the Indian region are based on abnormal specimens or on minor geographical or climatic variations. Moreover, these variations are neither stable nor found as populations. Most of the Indian *Botrychium* are distributed throughout the hilly regions, but *Botrychium lunaria*, the common moonwort, is restricted to the alpine regions of the Himalayas (Beddome, 1883; Clarke, 1880; Hope, *J. Bombay Nat. His. Soc.* 15: 108. 1903).

Botrychium simplex E. Hitchcock, the least moonwort, was described by Hitchcock in 1823 (*American Journal of Science and Arts*. 6:103. 1823). In

1821, plants initially considered to be *B. lunaria* had been discovered in Massachusetts, USA; after careful study, Hitchcock (1823) determined they were distinct enough from *B. lunaria* to warrant the status of species. Several varieties have been recognized by Eaton (Fern Bulletin 7:7–8. 1899), Clute (Our ferns in their haunts: a guide to all the native species. pp. 51–63. Frederick A. Stokes Co. New York. 1901), Clausen (Mem. Torrey Bot. Club 19:1–177. 1938) and Wherry (Amer. Fern J. 27: 58. 1937). Wagner and Wagner (1993) advocated that *B. simplex* exhibits phenotypic responses to different habitats and climates, hence almost all infraspecific taxa are environmental forms and juvenile stages of *B. simplex*; however, they accepted the alternate concept of “eastern” and “western” forms (Anderson, *Botrychium simplex* E. Hitchcock (little grape fern): a technical conservation assessment. 2006. USDA Forest Service, Rocky Mountain Region. [Online] <http://www.fs.fed.us/r2/projects/scp/assessments/botrychiumsimpler.pdf>, accessed on 3rd December 2010). Farrar (*Botrychium simplex*. In: Moonwort (*Botrychium*) Systematics. Iowa State University, Department of Ecology, Evolution and Organismal Biology, Ada Hayden Herbarium. 2005. [online] <http://www.public.iastate.edu/~herbarium/botrychium/B-simpler.pdf>, accessed on 7 August 2011) recognizes these as *B. simplex* var. *simpler* and *B. simplex* var. *compositum* (Lasch) Milde. Recent genetic analysis suggests that there is genetic variation within *B. simplex* (Hauk, Am. Fern J. 85:375–394.1995; Farrar, Population genetics of moonwort *Botrychium*. In: N. Berlin, P. Miller, J. Borovansky, U. S. Seal, and O. Byers (Eds.), Population and habitat viability assessment for the goblin fern (*Botrychium mormo*), 109–113, Final Report. The Conservation Breeding Specialist Group, Apple Valley, Minnesota, USA. 1998; Farrar, Systematics of western moonworts *Botrychium* subgenus *Botrychium*. In: Popovich, S. J. (ed.). United States Forest Service moonwort workshop. Arapaho-Roosevelt National Forests and Pawnee National Grassland, Fort Collins, Colorado, 2005).

Botrychium simplex is predominantly an American species, growing in western and eastern temperate to boreal North America and southern Greenland (Wagner and Wagner, 1993). It is rare in Iceland and according to Øllgaard (Scandinavian ferns: a natural history of the ferns, clubmosses, quillworts, and horsetails of Denmark, Norway, and Sweden. Rhodos, Copenhagen, Denmark. 1993) is distributed across Europe, where it is rare in the west, but is more abundant in eastern Europe and adjacent western Russia. Clausen (1938) cites a report of it by Nakai from Yizo, Japan. In his treatment of *Botrychium* in the Flora of Japan, Kato (Ophioglossaceae. In K. Iwatsuki, T. Yamazaki, D.E. Boufford and H. Ohba eds.: Flora of Japan. Vol. I. Pteridophyta and Gymnospermae. pp. 22–29. Kodansha, Tokyo. 1995.) placed plants previously identified *B. simplex* var. *tenebrosum* under *B. lunaria*. *Botrychium simplex* is not mentioned by Zou and Wagner (Am. Fern J. 78:122–135. 1988) as occurring in China. In his review of Asian Botrychiaceae, Sahashi (Ching Mem. Vol. 1999) did not mention the occurrence of *B. simplex* from Asia and he again advocated his previous report (Sahashi Japan. J. Jap. Bot. 58: 109–112.1983) about the merging of previously indentified *B. simplex* (Faurie’s specimen no. 5473 from Mt. Sharidake, Hokkaido in P) in to *B. lunaria*.

Furthermore, Kato (personal comm. 16-Aug. 2011) mentioned that there is no recent report of this species from Japan, and previous records of *B. simplex* from Japan (cited by Clausen 1938; and Nishida in Acta Phytotax. Geobot. 18: 39–43. 1959) are for dwarfed *B. lunaria*. In their treatment of *B. simplex* in the Flora of North America, Wagner and Wagner (1993) do not mention Asian plants. However, in Asia, *B. simplex* may have been overlooked by pteridologists because of its similarity to *B. lunaria*, and because the many cryptic species of *Botrychium* are extraordinarily difficult to distinguish from each other due to their morphological similarity (Hauk, Am. Fern J. 85: 375–394.1995).

Recently, Fraser-Jenkins (2008) found three herbarium sheets (one at PE and two at BM) of *B. simplex* within the bundles of *B. lunaria* collected by F. Kingdon-Ward from Tibet (Xizang) province of China. Because of the close proximity of the *B. simplex* Tibetan locality to the Indian frontiers, as well as climatic similarities between the regions, Fraser-Jenkins postulated that the *B. simplex* should also be present in India. He also noticed that Sino-Himalayan and American-European plants of *B. simplex* were morphologically distinct and the latter was given a new rank of subspecies as *Botrychium simplex* subsp. *kannenbergii* (Klinsm.) Fraser-Jenk. However, for the Asian plants he retained the name *Botrychium simplex* subsp. *simplex* (Syn. *Botrychium tenebrosum* A. A. Eaton, *Botrychium simplex* var. *tenebrosum* (A. A. Eaton) R. T. Clausen). Clausen (1938) placed *B. kannenbergii* in synonymy with *B. simplex* var. *typicum* Clausen, which is synonymous with *B. simplex* var. *simplex*. So, based on the text here, Sino-Himalayan and American-European plants are the same i.e., *B. simplex* var. *simplex*. Similarly, according to Clausen (1938), *B. tenebrosum* or *B. simplex* var. *tenebrosum* and *B. simplex* var. *simplex* are not synonymous, hence both are different taxa. But according to Fraser Jenkins (2008), the variations in *Botrychium simplex* var. *tenebrosum* (taller plants with fertile branched attached further up) are under the normal morphological range of *Botrychium simplex* subsp. *simplex* and are due to environmental factors and he treated them as synonyms.

While on a fern collection trip to high altitudes of North Sikkim, India, the author found some interesting *Botrychium* (*B. S. Kholia* no. 35481, 9 September 2010, BSHC) from ca. 4–5 km West of Thangu. After crossing the Thangu river via a foot bridge, and after ascending a few meters, the right foot path goes to Chopta valley and the left ascends to another beautiful valley and a Shiv temple. The plants were found growing near the mountain summit of these two valleys, on a SE facing slope hardly 5–10 m below the mountain top, ca. 4320 m elev., ca. 27° 53' 41" N, 88° 31' 23 E". These plants are small (6–13 cm tall) with somewhat deltate, shortly stalked trophophores, which are attenuate at the base and have only one or two pairs of pinnae. Pinnae are asymmetrical, spathulate or obovate deltate, cuneate and adnate to the winged rachis. The sporophore is also very short with large sessile globose sporangia arising from the rachis except the lowest pair which are short stalked and often slightly branched and bear 3–5 sporangia. These plants are markedly different from *B. lunaria*. In *B. lunaria* the trophophore is lanceolate to narrowly ovate,

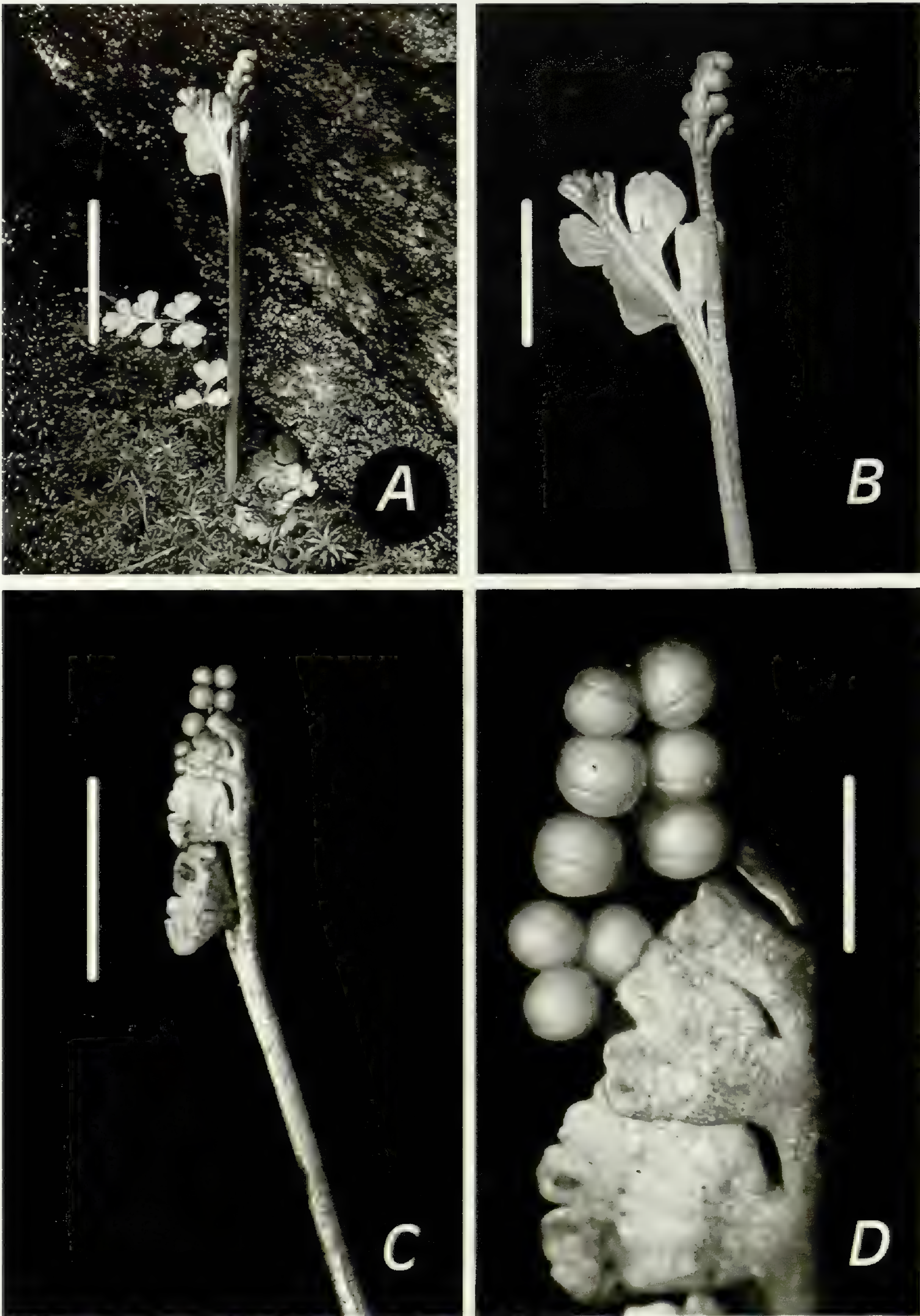


FIG. 1. *Botrychium simplex* A: Habit; B-D: Close up of trophophore and sporangiophores. (Scale bar: A= 2.5 cm, B= 1.5 cm, C = 2 cm, D = 0.5 cm).

generally long stalked, once pinnate with ca. 5–10 pairs of pinnae which are broadly fan-shaped or lunate in shape and stalkless to shortly stalked. The sporophores of *B. lunaria* are pinnate (sometimes the lower pairs are bipinnate).

During the International Symposium on Pteridophytes (November, 2010, Palampur Himachal Pradesh, India) photos of this *Botrychium* (Fig. 1 A-D) were shown to C. R. Fraser-Jenkins, who identified them as *B. simplex*. These photos were also sent to Prof. Donald Farrar, who agreed with Fraser-Jenkins' determination. Thus, this is the first record of *B. simplex* in the Indian Himalayan Mountains. This find expands the range of *B. simplex*. At present, in India, this species is known only from the locality mentioned above. More plants are likely to be found after thorough surveys in similar habitats of Indian and Sino Himalayan regions.

The author is greatly indebted to Mr. C. R. Fraser-Jenkins for identifying the specimens and his kind help throughout my studies of Himalayan ferns. Grateful thanks are also due to Prof. D. R. Farrar for encouragement. I profoundly thank Dr. M. C. Stensvold and Dr. J. M. Sharpe for their review of the previous draft and encouragements. Thanks are also due to Profs. M. Kato, S. Masuyama and N. Sahashi for the status of species in Japan. Two anonymous reviewers are also thanked for their fruitful comments. I also acknowledge Dr. D. K. Singh, Director B. S. I. and Dr. K. Das, Scientist In-charge, B.S.I. Sikkim for their help with this project.—B. S. KHOLIA, Botanical Survey of India, Sikkim Himalayan Regional Center Gangtok, Sikkim, 737 103 INDIA.

INFORMATION FOR AUTHORS

Authors are encouraged to submit manuscripts pertinent to pteridology for publication in the *American Fern Journal*. Manuscripts should be sent to the managing editor at amerfernj@hotmail.com. Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication.

Authors should adhere to the following guidelines; manuscripts not so prepared may be returned for revision prior to review. **Electronic submission is strongly encouraged**; however if it is necessary to submit hard copy, please submit one copy of the manuscript and include a review copy of illustrations and originals of illustrations. After acceptance, please submit final versions of manuscripts via FTP (contact the managing editor for instructions), email, or on diskette or CD ROM (see below for figure formatting). If submitting hard copies, use standard 8.5 by 11 inch paper of good quality, not "erasable" paper. **Double-space manuscripts throughout**, including title, author's names and full addresses, a short, informative abstract, key words, text (including heads and keys), literature cited, tables (separate from text), and figure captions (grouped as consecutive paragraphs separate from figures). Arrange parts of manuscript in order just given. Include author's name and page number in the upper right corner of every sheet, and provide an abbreviated running title. Provide margins of at least one inch (25 mm) all around on typed pages. Do not submit right-justified text, avoid footnotes, and do not break words at end of lines. Make table headings and figure captions self-explanatory. Use S.I. (metric) units for all measures (e.g., distance, elevation, weight) unless quoted or cited from another source (e.g., specimen citations). For nomenclatural matters (i.e., synonymy and typification), use one paragraph per basionym (see *Regnum Veg.* 58:39–40. 1968). Abbreviate titles according to *Botanico-Periodicum-Huntianum* (Lawrence *et al.*, 1968, Hunt Botanical Library, Pittsburgh) and its supplement (1991). References cited only as part of nomenclatural matter are not included in literature cited. For shorter notes and reviews, omit the abstract and put all references parenthetically in text. Use *Index Herbariorum* (*Regnum Veg.* 120:1–693. 1990; or <http://websun.nybg.org/bsci/ih/>) for designations of herbaria. For more detailed instructions on manuscript preparation, see <http://amerfernsoc.org/>.

Illustrations should be proportioned to fit page width (5 inches or 12.5 cm) with caption ultimately to be included on the same page. Halftone and color images should be scanned at a minimum of 300 pixels per inch (ppi). Line art should be scanned at 1200 ppi whenever possible. Please note that nearly all images that are downloaded from the Internet or that are in JPEG or GIF format will be 72 dpi and not acceptable for the printing process. Indicate the file format of the graphics. Please submit image files in TIFF (preferable) or EPS format. Provide margins of at least 25 mm on all illustrations. For continuous-tone illustrations, design originals for reproduction without reduction or by uniform amount. In composite blocks, abut edges of adjacent photographs. Avoid combining continuous-tone and line-copy in single illustrations or blocks. Coordinate sequence and numbering of figures (and tables), with order of citation in text. Explain scales and symbols in figures themselves, not in captions. Include a scale and reference to latitude and longitude in each map.

Instructions for downloading electronic proofs and reprint order forms are sent to authors by the printer. Authors should send proof corrections of corrected proofs to the editor and reprint orders to the printer. Authors will be assessed charges for extensive alterations made after type has been set.

For other matters of form or style, consult recent issues of the *American Fern Journal* and *The Chicago Manual of Style*, 14th ed. (1993, Univ. of Chicago Press, Chicago). Occasionally, departure from these guidelines may be justified. Authors are encouraged to consult the editor for assistance with any aspect of manuscript preparation.

Papers of longer than 32 printed pages may be sent to the Editor of *Pteridologia* (memoir Editor, see journal cover page 2).

PTERIDOLOGIA ISSUES IN PRINT

The following issues of *Pteridologia*, the memoir series of the American Fern Society, are available for purchase:

1. Wagner, David H. 1979. Systematics of *Polystichum* in Western North America North of Mexico. 64 pp. \$10.00 plus postage and handling.

2A. Lellinger, David B. 1989. The Ferns and Fern-allies of Costa Rica, Panama, and the Chocó (Part 1: Psilotaceae through Dicksoniaceae). 364 pp. \$32.00 plus postage and handling.

3. Lellinger, David B. 2002. A Modern Multilingual Glossary for Taxonomic Pteridology. 263 pp. \$28.00 plus postage and handling.

For orders and more information, please contact our authorized agent for sales at: Missouri Botanical Garden Press, P.O. Box 299, St. Louis, MO 63166-0299, tel. 314-577-9534 or 877-271-1930 (toll free). For online orders, visit: <http://www.mbgpress.org>.

AMERICAN FERN JOURNAL ON MICROFICHE

Volumes 1–61 of the *American Fern Journal* are available as archival quality, silver positive microfiches. Single volumes or the entire run may be purchased. The fiches are easily read with 10x or greater magnification (using a dissecting microscope and transmitted illumination or a fiche reader). Silver negative microfiches of vols. 1–50 are also available. The price is \$4.00 per volume or \$244.00 per set of 61 volumes, postpaid.

Send your inquiry or order with a check or money order to: American Fern Society, Inc., % Ecology III, 804 Salem Blvd., Berwick, PA, 18603-9801.

FIDDLEHEAD FORUM

The editor of the Bulletin of the American Fern Society welcomes contributions from members and non-members, including miscellaneous notes, offers to exchange or purchase materials, personalia, horticultural notes, and reviews of non-technical books on ferns.

SPORE EXCHANGE

Ms. Denia Mandt, 12616 Ibbetson Ave., Downey, CA 90242-5050, is Director. Spores exchanged and lists of available spores sent on request. <http://amerfernsoc.org/sporexy.html>

GIFTS AND BEQUESTS

Gifts and bequests to the Society enable it to expand its services to members and to others interested in ferns. Back issues of the Journal and cash or other gifts are always welcomed and are tax-deductible. Inquiries should be addressed to the Membership Secretary.

VISIT THE AMERICAN FERN SOCIETY'S WORLD WIDE WEB HOMEPAGE:

<http://amerfernsoc.org/>

AMERICAN FERN JOURNAL

Volume 102

Number 2

April–June 2012

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

- The Anatomy and Occurrence of Foliar Nectaries in *Cyathea* (Cyatheaceae)**
Richard A. White and Melvin D. Turner 91
- An Expanded Plastid Phylogeny of *Marsilea* with Emphasis on North American Species**
W. Mark Whitten, Colette C. Jacono, and Nathalie S. Nagalingum 114
- Effect of Habitat Modification on the Distribution of the Endangered Aquatic Fern *Ceratopteris pteridoides* (Parkeriaceae) in China**
Yuan-Huo Dong, Qing-Feng Wang, and Robert Wahiti Gituru 136
- Negative Gravitropism in Dark-Grown Gametophytes of the Fern *Ceratopteris richardii***
Hiroyuki Kamachi and Munenori Noguchi 147
- Antimicrobial and Modulatory Activity of Ethanol Extract of the Leaves from *Lygodium venustum* SW.**
M. F. B. Morais-Braga, T. M. Souza, K. K. A. Santos, J. C. Andrade, G. M. M. Guedes, S. R. Tintino, C. E. Sobral-Souza, J. G. M. Costa, I. R. A. Menezes, A. A. F. Saraiva, and H. D. M. Coutinho 154
- A New Species and a New Hybrid in the Grammitid Fern Genus *Stenogrammitis* (Polypodiaceae)**
Paulo H. Labiak 161
- Diplazium fimbriatum* (Athyriaceae), a New Species from Brazil**
Claudine M. Mynssen and Fernando B. Matos 167
- Isoetes mourabaptistae*, a New Species from Southern Brazil**
Jovani B. Pereira, Paulo G. Windisch, Maria L. Lorscheitter, and Paulo H. Labiak 174
- SHORTER NOTES**
- Agravitropic Growth of the Early Leaves of Apogamous Sporophytes of *Dryopteris tyrrhena***
Sara Magrini and Anna Scoppola 181
- Cheilanthes feei* T. Moore (Pteridaceae) and *Dryopteris erythrosora* (D.C. Eaton) Kunze (Dryopteridaceae) New for the Flora of North Carolina**
C. J. Rothfels, E. M. Sigel, and M. D. Windham 184
- REVIEWS**
- Flora de la Región del Parque Nacional Amboró, Bolivia, Vol. 1 Licofitas y Helechos, Gimnospermas**
Michael Kessler 187
- Erratum** 190

The American Fern Society

Council for 2012

MICHAEL WINDHAM, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President</i>
KATHLEEN PRYER, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President-Elect</i>
MARY STENSVOLD, USDA Forest Service, 204 Siginaka Way, Sitka, AK 99835.	<i>Secretary</i>
JAMES D. CAPONETTI, Div. of Biology, University of Tennessee, Knoxville, TN 37996-0830.	<i>Treasurer</i>
GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.	<i>Membership Secretary</i>
JAMES D. MONTGOMERY, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.	<i>Curator of Publications</i>
JENNIFER M. O. GEIGER, Dept. of Natural Sciences, Carroll College, Helena, MT 59625.	<i>Journal Editor</i>
R. JAMES HICKEY, Dept. of Botany, Miami University, Oxford, OH 45056.	<i>Memoir Editor</i>
JOAN N. E. HUDSON, Dept. of Biological Science, Sam Houston State University, Huntsville, TX 77341-2116.	
DAVID SCHWARTZ, 9715 Christey Way, Bakersfield, CA 93312-5617.	<i>Bulletin Editors</i>

American Fern Journal

EDITOR

JENNIFER M. O. GEIGER Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-4461, e-mail: jgeiger@carroll.edu

MANAGING EDITOR

JILL ANNE DILL Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-5176, e-mail: jdill@carroll.edu

ASSOCIATE EDITORS

NAN CRYSTAL ARENS Dept. of Geoscience, Hobart & William Smith Colleges, Geneva, NY 14456
JOSE MARÍA GABRIEL Y GALÁN Dept. de Biología Vegetal I, Universidad Complutense de Madrid,
Madrid, Spain 28040

GERALD J. GASTONY Dept. of Biology, Indiana University, Bloomington, IN 47405-6801

GARY K. GREER Biology Dept., Grand Valley State University, Allendale, MI 49401

CHRISTOPHER H. HAUFLER Dept. of Ecology and Evolutionary Biology, University of Kansas,
Lawrence, KS 66045-2106

R. JAMES HICKEY Dept. of Botany, Miami University, Oxford, OH 45056

PAULO LABIAK Universidade Federal do Paraná, Dept. de Botânica, 81531-980, Curitiba-PR, Brasil

ROBBIN C. MORAN New York Botanical Garden, Bronx, NY 10458-5126

NATHALIE NAGALINGUM Royal Botanical Garden Sydney, Sydney, NSW, Australia

CARL ROTHFELS Botany Dept., Duke University, Durham, NC 27708

EMILY SESSA Botany Dept., University of Wisconsin-Madison, Madison, WI 53706

MICHAEL SUNDUE Pringle Herbarium, The University of Vermont, Burlington, VT 05405

JAMES E. WATKINS, JR. Harvard University, Cambridge, MA 02138

The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, c/o Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

Claims for missing issues, made 6 months (domestic) to 12 months (foreign) after the date of issue, and orders for back issues should be addressed to Dr. James D. Montgomery, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.

Changes of address, dues, and applications for membership should be sent to the Membership Secretary.

General inquiries concerning ferns should be addressed to the Secretary.

Back volumes are available for most years as printed issues or on microfiche. Please contact the Back Issues Curator for prices and availability.

Subscriptions:

Society Membership – USA, Canada, Mexico (includes Journal and Fiddlehead Forum) \$30

Society Membership – All other countries (includes Journal and Fiddlehead Forum) \$40

Society Life Membership – \$360 (add \$120 mailing surcharge for outside USA, Canada, Mexico)

Regular Membership – USA, Canada, Mexico (includes Fiddlehead Forum) \$15

Regular Membership – All other countries (includes Fiddlehead Forum) \$22

Institutional Membership – \$40 to USA, Canada, Mexico; \$50 elsewhere (+\$3 agency fee)

POSTMASTER: Send address changes to American Fern Journal, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

The Anatomy and Occurrence of Foliar Nectaries in *Cyathea* (Cyatheaceae)

RICHARD A. WHITE* and MELVIN D. TURNER

Department of Biology, Duke University, P.O. Box 90338, Durham NC 27708

ABSTRACT.—This study reports the widespread occurrence of foliar nectaries in most New World species of the genus *Cyathea*. The anatomy of these glands and the variation in structure among the species is described. Some *Cyathea* species primitively lack glands, and the presence or absence of these glands and their structure correlate with recent molecular phylogenies.

KEY WORDS.—anatomy, *Cyathea*, glands, nectaries, phylogeny

In contrast to the widespread prominence of both floral and extrafloral nectaries in the flowering plants, nectaries have been reported in relatively few pteridophytes. Here we report the widespread occurrence of nectary-like foliar glands among many New World species of the tree fern genus *Cyathea*, and describe the anatomical structure of these glands for the first time.

Classifications of Cyatheaceae are still variable at the generic level. Some researchers include all species in *Cyathea*, while many others recognize three or more genera. DNA phylogenies have now greatly clarified the main subgroups of the scaly tree ferns (e.g., Korall *et al.*, 2007, Janssen *et al.*, 2008, and Bystriakova *et al.*, 2011). The described differences in morphological features among these major groups have chiefly involved the details of scales and spores. Here, we report on a distinctive characteristic of a large group of species within the genus *Cyathea*: the occurrence of foliar nectaries or nectary-like glands on the fronds. These nectaries are often conspicuously present on *Cyathea* pinna and pinnule bases, but previously have been little noted in the literature.

There is a long history of studies of the structure and function of nectaries, both floral and extrafloral (Zimmerman, 1932; Fahn, 1979). The major focus in studies of floral nectaries in angiosperms is primarily on their role in the pollination biology of the species; studies of extrafloral nectaries tend to consider the nectaries in relationship to insect-plant mutualism and defense strategies of plants (e.g., Agrawal, 2011; Bentley, 1977; Fahn, 1979; Bentley and Elias, 1983; Elias, 1983; Elias and Sun An-ci, 1985; Freitas *et al.*, 2001; Koptur, 1985, 1992; Rosumek *et al.*, 2009).

Detailed anatomical descriptions of nectar glands are available for numerous flowering plants (e.g., Elias, 1983; Marginson *et al.*, 1985; Durkee, 1987; McDade and Turner, 1997; Machado *et al.*, 2008; Thadeo *et al.*, 2008)). In

*Corresponding author

contrast, detailed studies of nectaries in the ferns are relatively rare. Early studies include a pioneer description of glands in *Pteridium* (Darwin, 1877). Another early study reported the presence of nectaries in *Angiopteris* and in two species of the *Cyathea* group of tree ferns (Bonnier, 1879), but provided little detail of anatomical structure. Over the many years of subsequent research additional examples of ferns with foliar nectaries have been added to the list: *Platycerium* (Dümmer, 1911); the *Aglaomorpha* and *Drynaria* group (Lüttge, 1971; Zamora and Vargas, 1974; Potes, 2010); *Polybotrya* and *Pleopeltis* (as *Polypodium*) (Koptur, 1982; Koptur *et al.*, 1998).

As in many studies of angiosperm extrafloral nectaries, research on fern nectaries has focused on the composition of the exudate (e.g., Koptur *et al.*, 1982) and the interaction between the plants and nectar-feeding ants (Tempel, 1983; Rashbrook *et al.*, 1992; Koptur *et al.*, 1998). Few of these fern nectaries have been described anatomically, with the notable exception of those of *Pteridium*, which have been described in ultrastructural detail (Power and Skog, 1987; Rumpf *et al.*, 1994). Most recently the anatomy of nectaries of *Aglaomorpha* and *Drynaria* was described (Potes, 2010).

After the initial early report of nectaries in *Cyathea* species (e.g., Bonnier, 1879), more than a century elapsed before the next significant notice of their existence. However, some careful descriptions of new *Cyathea* species described recently do refer to aerophores, and to “dark circular or oblong patches”, as well as to nodules present at the bases of pinnae (Moran, 1991). Additionally, there are examples of structures at the pinna bases that are illustrated for the new species, but not described in the text (Moran, 1991; 1995). More recently, in the description of a new species of *Cyathea*, *C. planadae*, the presence of distinctive pads of tissue on the fronds and an association of these areas with feeding activity by ants was described (Arens and Smith, 1998). The discussion here, as with *Cnemidaria* (Mickel and Beitel, 1988), focused on these areas as being associated with aerophores and on their possibly being glandular.

In the recent description of a new species of *Cyathea* in Bolivia, *Cyathea dintelmannii*, reference is made to “...a conspicuous black spot...” at the termination of the pinna with the rachis of the leaves and to “... one black (when dried) glutinous spot...” at that location (Lehnert, 2006). This ... “black spot can be regarded as homologue [*sic*] with the nectaries found in *Cyathea planadae* ... where an interaction between the fern and ants has been documented.” (Arens and Smith, 1998; Lehnert, 2006).

Our examination of numerous species of *Cyathea* in the field, as liquid-preserved material and as dried herbarium specimens, confirms that these pinna-base glands in fact characterize a large group of species within the genus. We describe the anatomy of the glands, which differs from previously described nectaries in other ferns. Comparisons of the anatomical results with recent analyses of tree fern phylogeny may help elucidate the evolutionary origin of nectaries in the tree ferns.

MATERIALS AND METHODS

The anatomy of the glands was examined in the *Cyathea* species listed in Table 1. In addition, the following non-*Cyathea* species were examined: *Sphaeropteris cooperi* (W.J. Hooker ex F. von Mueller) Tryon, *S. medullaris* (G. Forster) Bernhardt, *Alsophila firma* (Baker) Conant, and *A. polystichoides* Christ.

All vouchers are deposited in the Duke University Herbarium [DUKE] unless otherwise noted, e.g., Museo Nacional de Costa Rica [CR], University of California [UC] and Lyndon State College Herbarium [LSC].

In addition to the anatomical study of preserved materials, a survey was made of herbarium collections (UC, DUKE and CR), based on which the presence or absence of glands was described for 161 species of *Cyathea*. This represents a large proportion of the total number of *Cyathea* species, which has been estimated to be between ca. 200 (Lehnert, 2011b) and 270 species (based on Tryon, 1970). The latter number includes Tryon's estimated totals for

TABLE 1. *Cyathea* species included in the anatomical survey.

Species	Vouchers
<i>Cyathea acutidens</i> (Christ) Domin	White 200205 [DUKE]
<i>Cyathea alata</i> Copel.	P414; P419 [LSC]
<i>Cyathea andina</i> (H. Karst.) Domin	Conant 4888 [LSC]
<i>Cyathea arborea</i> (L.) Sm.	Conant 4893; Conant 4894 [LSC]
<i>Cyathea armata</i> (Sw.) Domin	Conant 4881 [LSC]
<i>Cyathea barringtonii</i> A.R. Sm. ex Lellinger	White & Lucansky 1970127 [DUKE]
<i>Cyathea borinquena</i> (Maxon) Domin	Conant 4872 [LSC]
<i>Cyathea caracasana</i> (Klotzsch) Domin	Conant 4884 [LSC]
<i>Cyathea choricarpa</i> (Maxon) Domin	White 200201 [DUKE]
<i>Cyathea cocleana</i> (Stolze) Lehnert	Wilbur 11111 [DUKE]
<i>Cyathea costaricensis</i> Domin	McAlpin 1089 [DUKE]
<i>Cyathea decomposita</i> (Karsten) Domin	White 1969238 [DUKE]
<i>Cyathea dejecta</i> (Baker) Christenh.	Beitel 8517 [UC]
<i>Cyathea delgadii</i> Sternb.	White 199906 [DUKE]
<i>Cyathea divergens</i> Kunze	Soeder 90-3 [DUKE]
<i>Cyathea fulva</i> (M. Martens & Galeotti) Fée	White 1969226 [DUKE]
<i>Cyathea furfuracea</i> Baker	Conant 4880 [LSC]
<i>Cyathea horrida</i> (L.) Sw.	Conant 4873 [LSC]
<i>Cyathea multiflora</i> Sm.	White 199907; White 200207 [DUKE]
<i>Cyathea mutica</i> (H. Christ) Domin	White 200204 [DUKE]
<i>Cyathea parvula</i> (Jenman) Domin.	Conant 4885 [LSC]
<i>Cyathea planadae</i> N.C.Arens & A.R.Sm.	Arens, s.n. [UC]
<i>Cyathea poeppigii</i> (Hook.) Domin	White 200202 [DUKE]
<i>Cyathea pungens</i> (Willd.) Domin	White 1969224 [DUKE]
<i>Cyathea schiedeana</i> (C. Presl) Domin	White 200206 [DUKE]
<i>Cyathea senilis</i> (Klotzsch) Domin	White & Lucansky 1969222 [DUKE]
<i>Cyathea squamata</i> (Klotzsch) Domin	White 1970159 [DUKE]
<i>Cyathea squamulosa</i> [I. Losch] R. C. Moran	White 200203 [DUKE]
<i>Cyathea suprastrigosa</i> (Christ) Maxon	White & Lucansky 1971035 [DUKE]
<i>Cyathea tenera</i> (J. Sm. ex Hook.) T. Moore	Conant 4869 [LSC]
<i>Cyathea trichiata</i> (Maxon)	White 199904 [DUKE]

Cyathea, *Trichipteris*, and *Cnemidaria*, and those *Cyathea* species he had assigned to *Sphaeropteris* and *Alsophila*. It also includes the species formerly classified as *Hymenophyllopsis*. Recent phylogenetic studies indicate that these groups are nested within *Cyathea* (Smith *et al.*, 2006; Smith *et al.*, 2008).

In order to study the anatomy of the glands, expanding croziers and frond axes were preserved in formalin-acetic-acid-alcohol (FAA). Samples were processed through a tertiary butyl alcohol dehydration series, embedded and sectioned in paraffin, and stained with safranin, fast green and iron hematoxylin (Johansen, 1940). In addition, fragments of dried herbarium specimens were rehydrated, embedded and sectioned.

Limited field observations were made of *Cyathea mutica*, *C. choricarpa*, *C. multiflora*, *C. delgadii*, *C. trichiata* and *C. poeppigii*. Fronds of these species were examined for the presence of glands, liquid drops and ants.

RESULTS

Foliar glands in *Cyathea* spp. occur on the abaxial surface of the frond axes at the bases of the pinnae near their points of attachment to the rachis (Figs. 1 and 2). In most species with pinna-base glands, anatomically similar but much smaller glands occur on the bases of the pinnule stalks (Fig. 3).

The characteristic anatomy of the pinna axis immediately distal to the gland is histologically similar in the main stipe and rachis: there is an epidermis which bears trichomes and small scales, a few-seriate parenchymatous outer hypodermis and an inner hypodermis of elongate fiber-like sclerified cells. The anatomy of the leaf glands described below is distinctly different: no scales or hairs occur on the gland, and the cells of the gland are organized in characteristically distinct zones, which are absent in the non glandular regions.

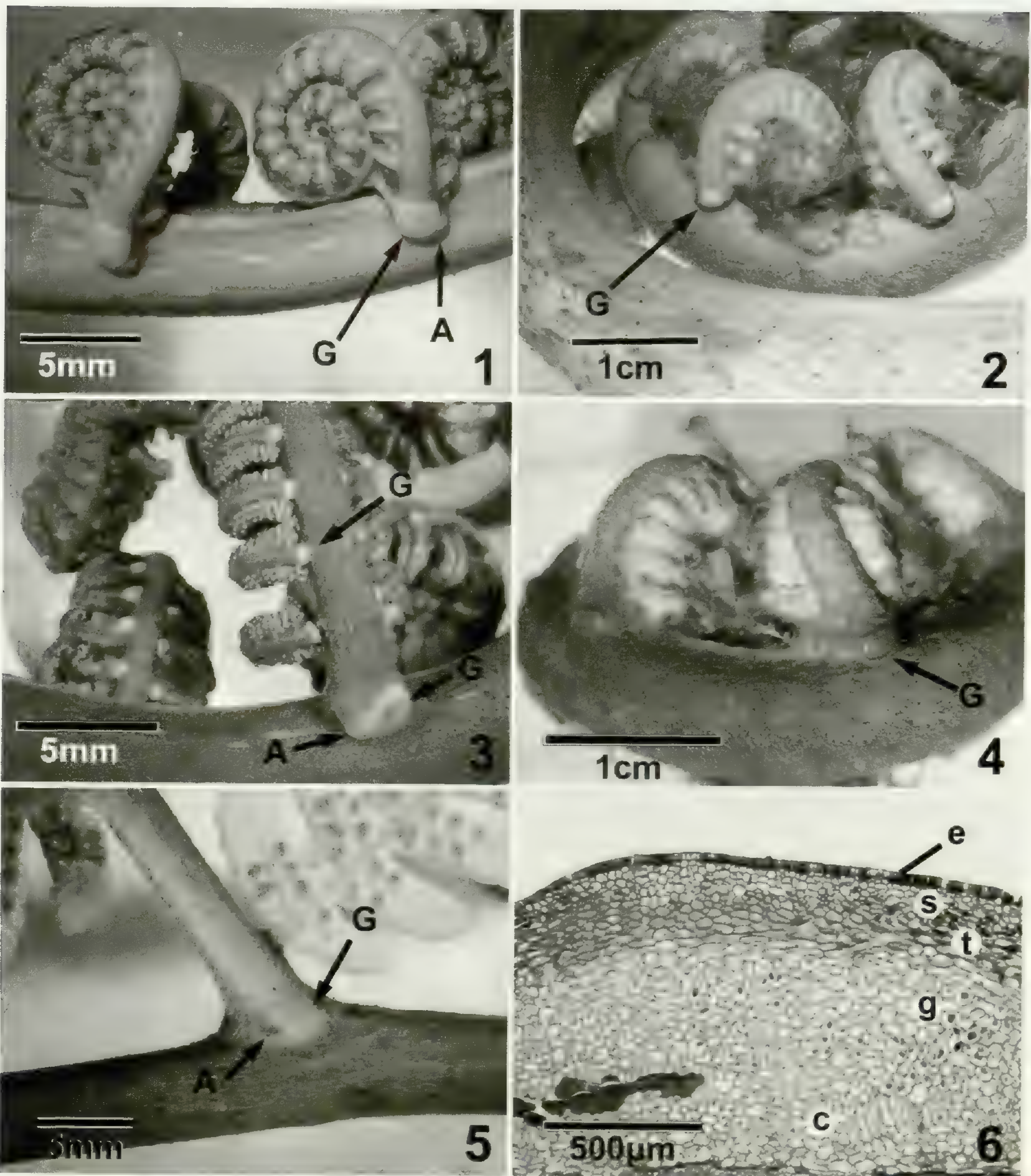
No vascular bundles extend to or into the glands, and adjacent vascular strands of the nearest parts of the rachis and pinna lack any obvious modifications.

This review of the anatomical diversity of the glands among the tree ferns that were studied suggests three broad categories of glands, which have been designated the *Cyathea delgadii* (Type I), *Cyathea multiflora* (Type II) and *Cyathea trichiata* (Type III) types as exemplified by these species (Table 2). In addition, there are *Cyathea* species that lack glands.

Cyathea delgadii (Type I)

The most commonly occurring anatomical “type” of gland among the species of *Cyathea* we studied is represented by *Cyathea delgadii*.

Organography.—The pinna bases in croziers and young fronds bear a prominent rounded green waxy-appearing gland (Figs. 2 and 4). The gland in mature leaves is less prominent (Fig. 5). An aerophore is adjacent to the gland, is oval in shape and proximal to the gland on the pinna base.



FIGS. 1-6. Type I foliar glands. 1. *Cyathea choricarpa*, expanding young frond showing prominent pinna-base gland and adjacent aerophore. 2. *C. delgadii*, crozier with some scales removed to expose glands. 3. *C. multiflora*, detail of expanding crozier with pinna-base gland adjacent to small aerophore. Small pinnule-base glands also visible. 4. *C. delgadii*, young crozier fragment, glands bare of the scurf covering the frond axes. 5. *C. delgadii*, pinna base of a mature frond with gland less raised. 6. *C. delgadii*, section of "Type I" gland showing 4 tissue zones. A= aerophore; c= rachis cortical parenchyma; e= epidermis; G= gland; g= ground tissue parenchyma of gland; s= subepidermal cell zone; t= zone of tanniferous cells.

TABLE 2. Summary of anatomical observations, and distribution of gland types.

Type I. <i>Cyathea delgadii</i> type	Gland bulging; secretory epidermis usually a single layer of anticlinally elongate cells; modest subepidermal zone; innermost parenchymatous zone prominent; fiber layer interrupted.	<i>Cyathea barringtonii</i> A.R. Sm. ex Lellinger; <i>Cyathea caracasana</i> (Klotzsch) Domin; <i>Cyathea choricarpa</i> (Maxon) Domin; <i>Cyathea cocleana</i> (Stolze) Lehnert; <i>Cyathea decomposita</i> (Karsten) Domin; <i>Cyathea delgadii</i> Sternb.; <i>Cyathea divergens</i> Kunze; <i>Cyathea fulva</i> (M. Martens & Galeotti) Fée; <i>Cyathea horrida</i> (L.) Sw.; <i>Cyathea mutica</i> (H. Christ) Domin; <i>Cyathea pungens</i> (Willd.) Domin; <i>Cyathea schiedeana</i> (C. Presl) Domin; <i>Cyathea senilis</i> (Klotzsch) Domin; <i>Cyathea squamata</i> (Klotzsch) Domin; <i>Cyathea squamulosa</i> [I. Losch] R. C. Moran; <i>Cyathea suprastrigosa</i> (Christ) Maxon; <i>Cyathea tenera</i> (J. Sm. ex Hook.) T. Moore
Type II. <i>Cyathea multiflora</i> type	Gland bulging; one or two layered secretory epidermis of anticlinally elongate cells; distinctive subepidermal layer; inner zone of numerous vacuolated more or less isodiametric parenchyma cells; fiber layer interrupted	<i>Cyathea acutidens</i> (Christ) Domin; <i>Cyathea andina</i> (H. Karst.) Domin; <i>Cyathea multiflora</i> Sm.
Type III. <i>Cyathea trichiata</i> type	Gland flat (no bulge); secretory epidermis a single layer of cells less anticlinally elongate than the previous two types; subepidermal layer not distinctive; inner parenchymatous zone lacking; fiber layer not interrupted.	<i>Cyathea armata</i> (Sw.) Domin; <i>Cyathea trichiata</i> (Maxon) Domin
Glands lacking	No parenchymatous bulge; nonsecretory epidermis with hairs and scales present; hypodermal fibrous layer not interrupted.	<i>Cyathea alata</i> Copel.; <i>Cyathea arborea</i> (L.) Sm.; <i>Cyathea costaricensis</i> Domin; <i>Cyathea parvula</i> (Jenman) Domin; <i>Cyathea poeppigii</i> (Hook.) Domin; <i>Alsophila firma</i> (Baker) D.S. Conant; <i>Alsophila polystichoides</i> H. Christ; <i>Sphaeropteris cooperi</i> (Hook. ex F. Muell.) R. Tryon; <i>Sphaeropteris medullaris</i> (G. Forster) Bernhardt.

An unusual feature seen only in *Cyathea delgadii* among the species we surveyed is an asymmetric distribution of unusually prominent glands on the pinnule bases along the pinnae. These are restricted to the distal region of each pinna and occur only on the pinnules of the acroscopic side of the pinna.

Anatomy.—Glands are prominently raised above the surrounding leaf surface (Figs. 2 and 4), and histological sections reveal that four main zones of cells compose the gland (Fig. 6). The epidermis of the gland is composed of a single layer of densely-staining anticlinally elongate cells. A thick cuticle covers the surface of the gland; no stomates are present, and scales and trichomes are absent. Subjacent to this layer is a discrete subepidermal zone of parenchyma which is 3–6 cells seriate. These cells are more or less isodiametric in shape and highly vacuolated. They are larger than the epidermal cells but smaller than the parenchyma cells which characterize the parenchyma tissue of the leaf axis. Subjacent to this zone of parenchyma is a zone of transversely-elongate cells which are usually densely stained, with granular cytoplasm. This tanniniferous layer is laterally continuous with the hypodermal fibrous layer of the pinna axis and rachis. Finally, subjacent to this tanniniferous zone is a zone of ground tissue parenchyma similar to the subjacent cortical ground tissue of the leaf axis.

Adjacent aerophores have numerous stomates and prominent intercellular spaces.

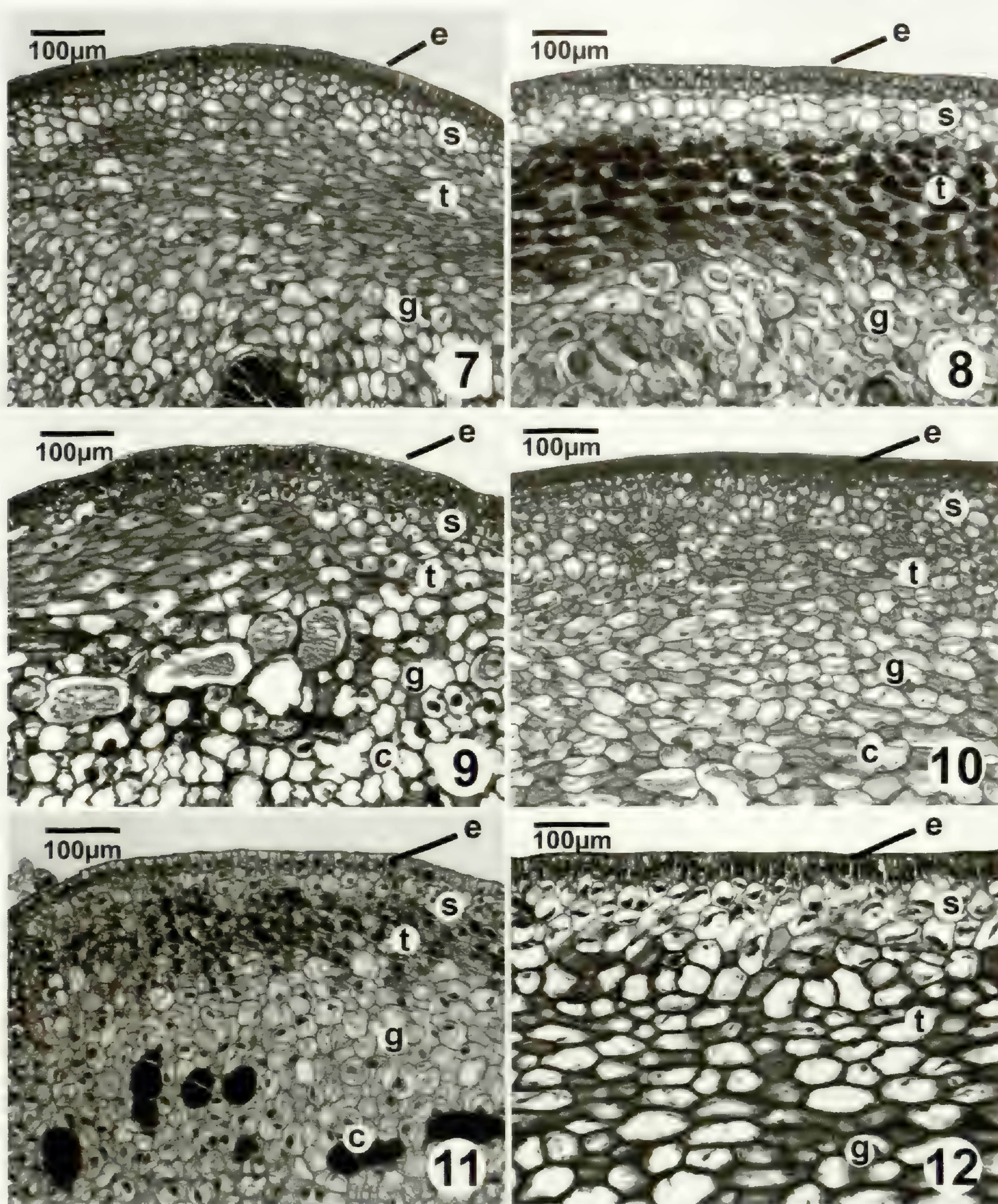
Numerous *Cyathea* species, including those formerly named *Cnemidaria* (see below), which were seen to have glands histologically similar to those of *Cyathea delgadii* include *Cyathea borinquena* (Fig. 7), *C. caracasana*, (Fig. 8), *C. decomposita* (Fig. 9), *C. divergens* (Fig. 10), *C. furfuracea* (Fig. 11), *C. senilis* (Fig. 12), *C. squamata* (Fig. 13), *C. tenera* (Figs 14 and 15). Other species included in the anatomical survey with Type I glands include *C. barringtonia*, *C. fulva*, *C. gibbosa*, *C. horrida*, *C. planadeae*, *C. pungens*, *C. schiedeana*, *C. squamulosa* and *C. suprastrigosa*.

Cyathea choricarpa (formerly *Cnemidaria*) (Type I)

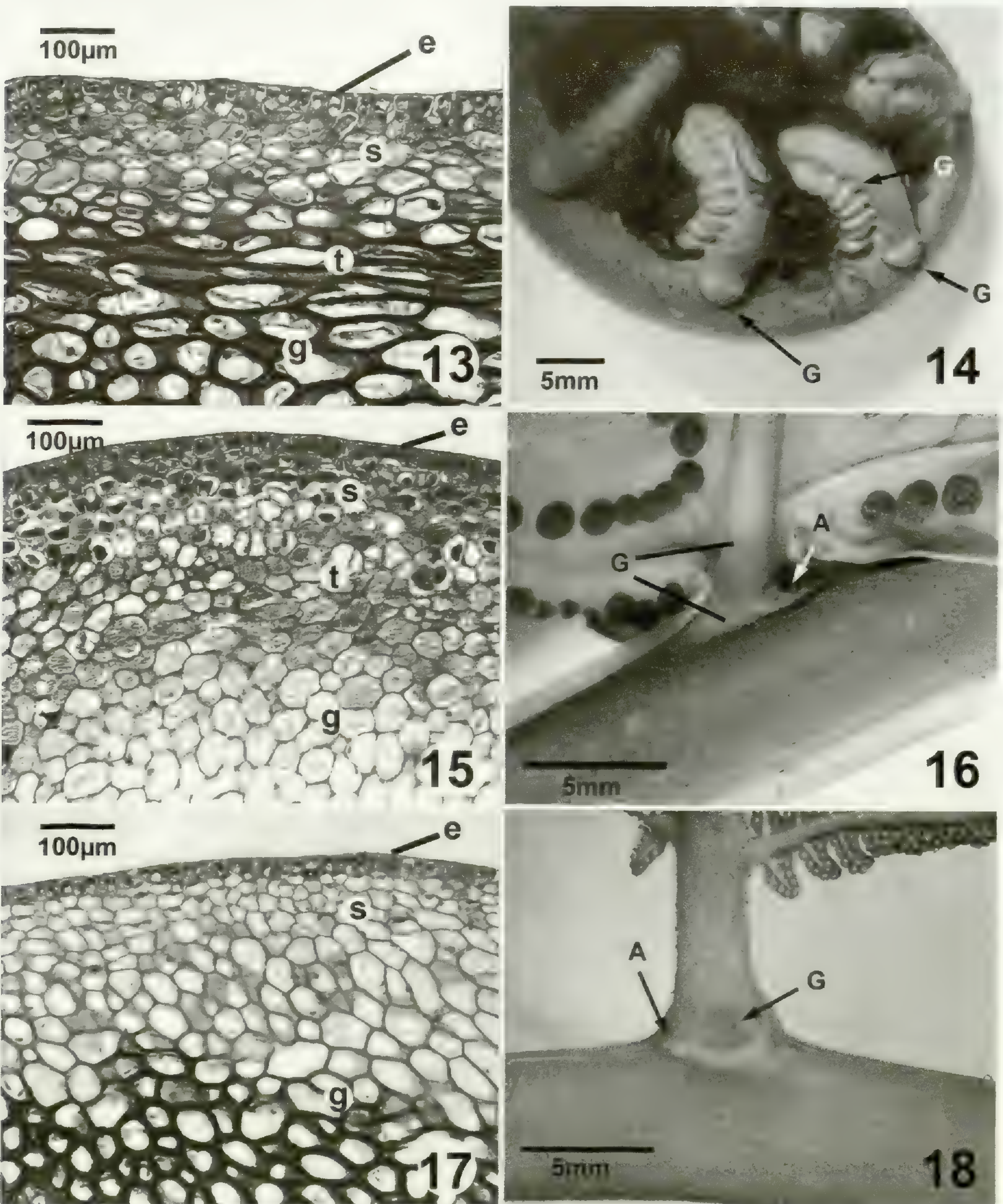
Material was examined originally as *Cnemidaria* species. These have now been incorporated into *Cyathea* (Korall *et al.*, 2007; Lehnert, 2011a). The anatomy of the glands, which is similar to that which characterizes most *Cyathea* species, is consistent with this.

Organography.—Glands in this species are recognized as bulbous waxy regions on the abaxial side of the leaflet base (Figs. 1 and 16). A bulge is present in young leaf material, but it is not as prominent in mature leaves. The gland tends to be somewhat elongate along the pinna base. A small aerophore is located immediately proximal to each gland.

Anatomy.—The epidermis of the gland is a single layer of anticlinally-elongate cells which are slightly larger than the cells which characterize the epidermis adjacent to the gland (Fig. 17). Subjacent to the secretory epidermis is a zone of isodiametric parenchyma cells 6–10 cell layers deep. There is a gradation of cell size in this zone. The cells of the 3–4 layers immediately subjacent to the epidermis are smaller and more compact than the cells of the more internal layers. Immediately subjacent to this subepidermal parenchymatous zone is a zone composed of 10–12 layers of thick-walled cells. This zone is laterally continuous with the fibrous hypodermal layer of the pinna and rachis.



FIGS. 7–12. Sections of Type I foliar glands showing tissue zonation. Figs 7–11 sections of glands from croziers; Fig 12. from a fully mature gland. 7. *Cyathea borinquena*. 8. *C. caracasana*. 9. *C. decomposita*. 10. *C. divergens*. 11. *C. furfuracea*. 12. *C. senilis*. c= rachis cortical parenchyma; e= epidermis; g= ground tissue parenchyma of gland; s= subepidermal cell zone; t= zone of tanniferous cells.



FIGS. 13-18. *Cyathea* foliar glands. 13. *Cyathea squamata*, section of mature Type I gland. 14. *C. tenera*, crozier with pinna-base and smaller but anatomically similar pinnule-base glands. 15. *C. tenera*, section of immature pinna-base gland. 16. *C. choricarpa*, pinna base of mature frond, showing less-prominent gland (bracketed by two lines from G), adjacent to aerophore (A). 17. *C. choricarpa*, section of mature gland with less pronounced zonation. 18. *Cyathea multiflora*, pinna base of almost fully-expanded young frond, showing positional relationship of aerophore to gland. A= aerophore; e= epidermis; G= gland; g= ground tissue parenchyma of gland; s= subepidermal cell zone; t= zone of tanniniferous cells.

The aerophore immediately adjacent to each gland (Fig. 16) has numerous stomata and prominent airspaces.

Three other species of *Cyathea* (formerly *Cnemidaria*) with gland anatomy similar to that of *Cyathea choricarpa* included in this survey are *C. cocleana*, *C. horrida*, and *C. mutica*.

Cyathea multiflora (Type II)

This species is characterized by the most structurally elaborate foliar glands seen in this study.

Organography.—A prominent bright green gland occurs on each pinna base on fresh croziers and expanding fronds. The glands appear as rounded or bulging, shiny, glabrous patches on the abaxial surface of the pinna base immediately distal on the pinna to its junction with the rachis (Fig. 3). Pinnule bases of this species have much smaller glands (Fig. 3), which have anatomy similar to that of the larger glands on the pinna. This is also true, for example for *Cyathea tenera* (Fig. 14). As in the other species of *Cyathea*, the glands on more mature and expanded fronds appear less prominent (Fig. 18).

In addition to the gland, there is a distinctive aerophore which tends to be a relatively small oval patch adjacent to the gland at its border with the rachis (Fig. 3; Fig. 18).

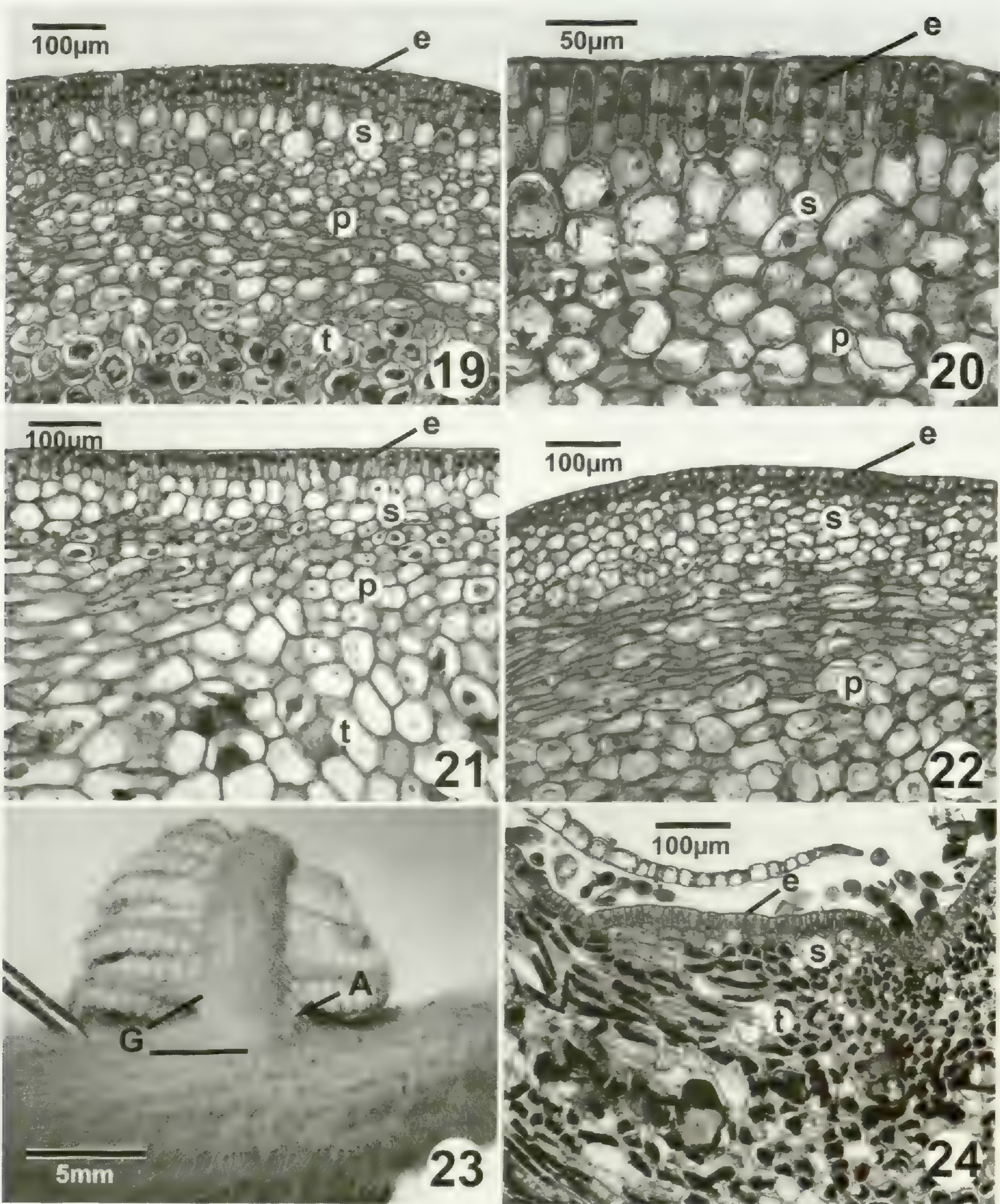
Anatomy.—The epidermal layer of the gland consists of a region of narrow anticlinally-elongate cells with densely staining cytoplasm and a thick cuticle. These cells are characterized by having prominent large nuclei and dense granular cytoplasm with small vacuoles (Fig. 20). Many of the cells in this epidermal layer are subdivided by periclinal cross walls, so that the palisade-like glandular epidermis varies locally from 1-seriate to 2-seriate. Stomata, trichomes and scales are absent. Immediately subjacent to this characteristic epidermis, is a 1- to 2-seriate zone of rounded isodiametric cells that are distinctly larger and lighter-staining than cells of surrounding layers (Figs. 19 and 20). Subjacent to this subepidermal zone, the fiber layer characteristic of the non-glandular area is interrupted. In place of hypodermal fibers, multiple layers of parenchyma cells compose a zone of cells which are smaller and more densely staining than those of the parenchymatous zone subjacent to the secretory epidermis (Fig. 19). This inner parenchymatous zone forms most of the characteristic bulge of the gland. Finally this latter zone is subtended by several layers of tanniniferous-staining cells which are similar to, and continuous with, the ground tissue of the pinna axis (Fig. 19).

Characteristic aerophores adjacent to each gland (Figs. 3 and 18) have numerous stomata and prominent air spaces.

Species in this survey characterized by gland anatomy similar to *Cyathea multiflora* include *Cyathea acutidens* (Fig. 21) and *Cyathea andina* (Fig. 22).

Cyathea trichiata (Type III)

Organography.—There is no prominently elevated glandular bulge in this species, but the glandular area is recognizable as an irregularly-shaped area of



FIGS. 19-24. Type II and Type III foliar glands. 19. *Cyathea multiflora*, section showing Type II zonation. 20. *C. multiflora*, detail showing multiseriate epidermis, and subjacent parenchyma zone. 21. *C. acutidens*, section with Type II zonation. 22. *C. andina*, multiseriate epidermis, but zonation more comparable to Type I. 23. *C. trichiata*, fragment dissected from crozier showing gland (bracketed by two lines from G) as non-elevated hairless patch on pinna base. 24. *C. trichiata*, section of Type III gland lacking extensive subepidermal layers. A = aerophore; e = 1-2-seriate epidermis; G = gland; p = multiple-layered parenchymatous zone of gland; s = subepidermal cell zone; t = zone of tanniferous cells.

the epidermis at the pinna base which lacks trichomes and scales (Fig. 23). An aerophore is associated with the pinna base, and is clearly separate from the gland. Small amounts of a liquid secretion were observed on glands in field observations of this species.

Anatomy.—The epidermis of the gland is composed of one or two rows of cells which are anticlinally elongate and larger than the cells of the single layered epidermis of the pinna adjacent to the gland (Figs. 24 and 25). The secretory epidermis has a thick cuticle, and the area has no stomata, hairs or scales. Subjacent to the epidermis is a one- to three-layered zone of isodiametric, highly vacuolated parenchyma cells. Finally, subjacent to this subepidermal zone is a multi-layered zone of densely-staining fiber cells. These cells are longitudinally elongate with respect to the pinna axis and are continuous with, though somewhat less elongate than, the fibrous layer which is characteristic of the main axis and rachis (Fig. 25). Unlike the other gland types, there is no interruption of the fibrous layer below the gland. Also, there is no extensive inner parenchymatous zone and consequently the glands are not elevated or bulging.

The characteristic aerophore has numerous stomata and prominent airspaces.

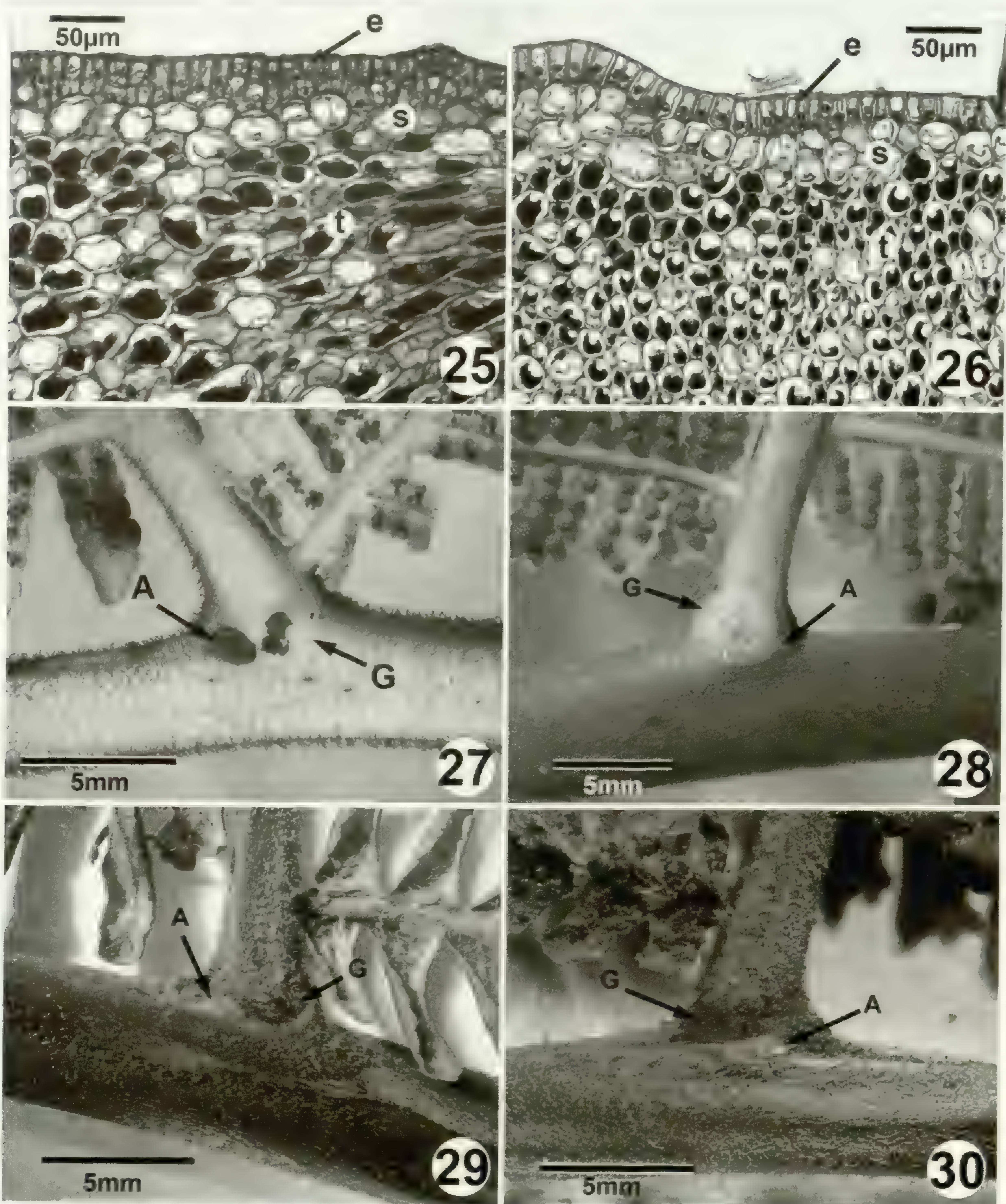
Cyathea armata (Fig. 26) has glands similar to those of *Cyathea trichiata*.

Cyathea Species Lacking Glands

The anatomical survey revealed that the following *Cyathea* species lack glands: *Cyathea alata*, *C. arborea*, *C. costaricensis*, *C. parvula*, and *C. poeppigii*. The survey of herbarium specimens confirmed the lack of glands in these species, and in addition noted the lack of glands in several other species (Table 3).

No glands were observed on the species which were examined of *Alsophila* (e.g., *Alsophila firma* and *A. polystichoides*) and *Gymnosphaera*. The species of *Sphaeropteris* that were examined anatomically (*Sphaeropteris cooperi*, and *S. medullaris*) also lacked glands. However, observations of herbarium specimens suggest that glands similar to those of *Cyathea* spp. may be present in *Sphaeropteris robinsonii* (Copel.) R. M. Tryon of the Philippines, and possibly in a few other *Sphaeropteris* species. Except for *S. robinsonii*, observations of herbarium specimens of numerous species of the other Cyatheaceous genera indicate a general lack of glands throughout Cyatheaceae other than in *Cyathea*.

Field observations have so far provided few data in regard to the function of the putative pinna-base nectaries in *Cyathea* species. In the *Cyathea multiflora* and *C. delgadii* types, the glands are large and bright green in young expanding leaves (Fig. 3; Fig. 2), but much less conspicuous in mature leaves (Fig. 18; Fig. 5). Despite their prominence, the glands on young fronds of both these species were never observed to be wet with secretions when closely examined in the field. However, small droplets of liquid were observed on the pinna-base glands of young expanded fronds of *Cyathea choricarpa*, and small amounts of secreted liquid were consistently present on glands of *Cyathea trichiata*, both



FIGS. 25-30. Foliar glands: Types III, II, and I. **25.** *Cyathea trichiata*: characteristic Type III organization. **26.** *C. armata*: Mature Type III gland. **27.** *C. acutidens*: mature pinna base showing evidence of insect damage to gland (dark wounds on gland), Type II gland. **28.** *C. multiflora*: possible evidence of insect damage to gland (small dark lesions), Type II gland. **29.** *C. delgadii* [White & Lucansky 196824 (DUKE)]: Gland of dry herbarium specimen visible as dark shrunken area adjacent to aerophore, Type I gland. **30.** *C. multiflora* [Wilbur & Luteyn 18252 (DUKE)]: Dark shriveled gland (Type II) present next to aerophore. A= aerophore; e= epidermis; G= gland; s=subepidermal cell zone; t= zone of tanniferous cells.

TABLE 3. *Cyathea* glands presence/absence based on herbarium survey.**Old World *Cyathea* species**

NO GLANDS:

Species	Representative Specimen
<i>Cyathea alata</i> Copel.	White 1969561 [DUKE]
<i>Cyathea decurrens</i> Copel.	Muzik 19006 [DUKE]
<i>Cyathea epaleata</i> (Holtum) Holtum	M. L. Grant 4233 [UC]
<i>Cyathea robertsiana</i> (F.Muell.) Domin	van der Werff & Gray 17037 [UC]

New World *Cyathea* species

NO GLANDS:

Species	Representative Specimen
<i>Cyathea arborea</i> (L.) Sm.	Crosby & Anderson 1044 [DUKE]
<i>Cyathea costaricensis</i> Domin	Hellwig & Whitaker 1437 [DUKE]
<i>Cyathea myosuroides</i> (Liebm.) Domin	Mexia 9244 [UC]
<i>Cyathea parvula</i> (Jenman) Domin	Hespenheide 957 [DUKE]
<i>Cyathea poeppigii</i> (Hook.) Domin	White & Lucansky 1968212 [DUKE]

Formerly *Hymenophyllopsis*:

<i>Cyathea asplenioides</i> (A. C. Sm.) Christenh.	Maguire & Politti 27764 [UC]
<i>Cyathea ctenitoides</i> (Lellinger) Christenh.	Wurdack 34175 [UC]
<i>Cyathea dejecta</i> (Baker) Christenh.	Beitel 8517 [UC]
<i>Cyathea hymenophylloides</i> (L. D. Gómez) Christenh.	Maguire 32895 [DUKE]
<i>Cyathea tepuiana</i> Christenh.	Liesner 25302 [UC]
<i>Cyathea trichomanoides</i> Christenh.	Beitel 85314 [UC]

GLANDS PRESENT:

<i>Cyathea acutidens</i> (Christ) Domin	Stone 2104 [DUKE]
<i>Cyathea alatissima</i> (Stolze) Lehnert	van der Werff et al. 19540 [UC]
<i>Cyathea alfonsiana</i> L. D. Gomez	Trusty 528 [DUKE]
<i>Cyathea amabilis</i> (C. V. Morton) Lehnert	Meier & Molina 9217 [UC]
<i>Cyathea amazonica</i> R. C. Moran	Øllgard 99065 [UC]
<i>Cyathea anacampta</i> Alston	White & White 197073 [DUKE]
<i>Cyathea andicola</i> Domin	Forero et al. 6803 [UC]
<i>Cyathea andina</i> (H. Karst.) Domin	White & Lucansky 1970150 [DUKE]
<i>Cyathea aristata</i> Domin	Mickel 5935 [UC]
<i>Cyathea armata</i> (Sw.) Domin	Watt 194 [UC]
<i>Cyathea aspera</i> (L.) Sw.	Nicolson 1934 [DUKE]
<i>Cyathea atahuallpa</i> (R.M. Tryon) Lellinger	Hutchinson & Wright 6922 [UC]
<i>Cyathea aterrima</i> (Hook.) Domin	van der Werff et al. 16327 [UC]
<i>Cyathea atrovirens</i> (Langsd. & Fisch.) Domin	Hatschbach 27670 [UC]
<i>Cyathea austropallescens</i> Lehnert	Smith, León & Young 13134 [DUKE]
<i>Cyathea barringtonii</i> A.R. Sm. ex Lellinger	White & Lucansky 1970126 [DUKE]
<i>Cyathea bicrenata</i> Liebm.	Hellwig 302 [DUKE]
<i>Cyathea bipinnata</i> (R.M. Tryon) R.C. Moran	van der Werff, Gray, & Tipas 12002 [UC]
<i>Cyathea bipinnatifida</i> (Baker) Domin	Beitel 85102 [UC]
<i>Cyathea boliviana</i> R. M. Tryon	Smith, Quintana & Garcia 13415 [DUKE]
<i>Cyathea borinquena</i> (Maxon) Domin	Blomquist 11740 [UC]
<i>Cyathea bradei</i> (Windisch) Lellinger	van der Werff, Vasquez & Jaramillo 10179
<i>Cyathea brevistipes</i> R. C. Moran	van der Werff & Palacios 9185 [UC]
<i>Cyathea brunnescens</i> (Barrington) R. C. Moran	Moran & Rohrbach 5297 [UC]
<i>Cyathea caracasana</i> (Klotzsch) Domin	Lehnert 998 [DUKE]
<i>Cnemidaria chocoense</i> Stolze	Lellinger & de la Sota 853 [CR]
<i>Cyathea choricarpa</i> (Maxon) Domin	Wilbur 27576 [DUKE]

TABLE 3. Continued.

<i>Cyathea cnemidaria</i> Lehnert	Metcalf & Cuatrecasas 30122 [UC]
<i>Cyathea cocleana</i> (Stolze) Lehnert	Wilbur 11111 [DUKE]
<i>Cyathea columbiana</i> Domin	Rubio, Tipaz & Taicuz 2114 [UC]
<i>Cyathea conjugata</i> (Hook.) Domin	White & White 197027 [DUKE]
<i>Cyathea corcovadensis</i> (Raddi) Domin	Mexia 4634 [UC]
<i>Cyathea cyatheoides</i> (Desv.) Kramer	Cremers 9834 [DUKE]
<i>Cyathea decomposita</i> (Karsten) Domin	White & White 1970104 [DUKE]
<i>Cyathea decorata</i> (Maxon) R.M. Tryon	Croat & Watt 70293 [UC]
<i>Cyathea decurrentiloba</i> Domin	Hodel 1466 [UC]
<i>Cyathea delgadii</i> Sternb.	Stone 2700 [DUKE]
<i>Cyathea dicromatolepis</i> (Fée) Domin	Brade 8594 [UC]
<i>Cyathea dintelmannii</i> Lehnert	van der Werff et al. 20269 [UC]
<i>Cyathea dissimilis</i> (C.V. Morton) Stolze	Liesner & Holst 20435 [UC]
<i>Cyathea divergens</i> Kunze	Hellwig 1433 [DUKE]
<i>Cyathea dudleyi</i> R. M. Tryon	Palacios & van der Werff 3748 [UC]
<i>Cyathea ebenina</i> H. Karst	van der Werff, et al. 15823 [UC]
<i>Cyathea ewanii</i> Alston	Schuettpelz 208 [DUKE]
<i>Cyathea frigida</i> (H. Karst.) Domin	van der Werff & Palacios 9425 [UC]
<i>Cyathea fulva</i> (M. Martens & Galeotti) Fée	White & Lucansky 1969237 [DUKE]
<i>Cyathea furfuracea</i> Baker	Hellwig & Whitaker 1472 [DUKE]
<i>Cyathea gibbosa</i> (Klotzsch) Domin	White & White 197017 [DUKE]
<i>Cyathea gracilis</i> Griseb.	Crosby et al. 316 [DUKE]
<i>Cyathea grandifolia</i> Willd.	Wilbur 7781 [DUKE]
<i>Cyathea grayumii</i> A. Rojas	Hodel 1486 [UC]
<i>Cyathea guentheriana</i> Lehnert	Vargas, et al. 2179 [UC]
<i>Cyathea hemiepiphytica</i> R. C. Moran	van der Werff, Gray & Tipas 11951 [UC]
<i>Cyathea herzogii</i> Rosenst.	van der Werff et al. 18570 [UC]
<i>Cyathea hirsuta</i> C. Presl	Mexia 4956 [UC]
<i>Cyathea horrida</i> (L.) Sw.	White & Lucansky 1970130 [DUKE]
<i>Cyathea iheringii</i> (Rosenst.) Domin	Brade 9842 [UC]
<i>Cyathea impar</i> R. M. Tryon	Neves, Hammel & Herrera 8529 [UC]
<i>Cyathea karsteniana</i> (Klotzsch) Domin	Meier et al. 3962 [UC]
<i>Cyathea lasiosora</i> (Kuhn) Domin	van der Werff & Palacios 10320 [DUKE]
<i>Cyathea lechleri</i> Mett.	Neill et al. 15286 [DUKE]
<i>Cyathea leucolepismata</i> Alston	Ølgaard 99065 [UC]
<i>Cyathea lindeniana</i> C. Presl	van der Werff & Rivero 8761 [UC]
<i>Cyathea lindigii</i> (Baker) Domin	van der Werff & Ortiz 5681 [UC]
<i>Cyathea lockwoodiana</i> (P.G. Windisch) Lellinger	White & White 197047 [DUKE]
<i>Cyathea macrocarpa</i> (C. Presl) Domin	Boudrie MB-3024 [UC]
<i>Cyathea macrosora</i> (Baker) Domin	van der Werff et al. 18171 [UC]
<i>Cyathea marginalis</i> (Klotzsch) Domin	Liesner 19544 [UC]
<i>Cyathea maxonii</i> Underw. ex Maxon	Wilbur & Luteyn 18370 [DUKE]
<i>Cyathea meridensis</i> H. Karst.	Duno & Riina 1469 [UC]
<i>Cyathea mexiae</i> Copel.	Mexia 5175 [UC]
<i>Cyathea microdonta</i> (Desv.) Domin	Grayum 3069 [DUKE]
<i>Cyathea moranii</i> Lehnert	Lehnert 1076 [UC]
<i>Cyathea mucilaginata</i> R. C. Moran	van der Werff et al. 13295 [UC]
<i>Cyathea multiflora</i> Sm.	Wilbur & Luteyn 18252 [DUKE]
<i>Cyathea mutica</i> (H. Christ) Domin	Lellinger 1281 [DUKE]
<i>Cyathea neblinae</i> A. R. Sm.	Stergios 11892 [UC]
<i>Cyathea nephela</i> Lehnert	van der Werff, et al. 8562 [UC]
<i>Cyathea nervosa</i> (Maxon) Lehnert	Mexia 6291 [UC]

TABLE 3. Continued.

<i>Cyathea nesiotica</i> (Maxon) Domin	Trusty 527 [DUKE]
<i>Cyathea nigripes</i> (C. Chr.) Domin	Forero & Jaramillo 5315 [DUKE]
<i>Cyathea nodulifera</i> R. C. Moran	Grayum, Herrera & Santana 7800 [CR]
<i>Cyathea notabilis</i> Domin	Trusty 533 [DUKE]
<i>Cyathea obnoxia</i> Lehnert	Lehnert 802 [UC]
<i>Cyathea onusta</i> Christ	Wilson & Wilson 69-26 [UC]
<i>Cyathea oxapampana</i> Lehnert	Mellado & Monteagudo 0464 [UC]
<i>Cyathea palaciosii</i> R.C. Moran	Neill, et al. 14437 [UC]
<i>Cyathea panamensis</i> Domin	Lellinger & de la Sota 374 [CR]
<i>Cyathea parianensis</i> (P. G. Windisch) Lellinger	Meier & Elsner 6647 [UC]
<i>Cyathea parvifolia</i> Sodiro	van der Werff & Palacios 9185 [UC]
<i>Cyathea patens</i> H. Karst.	Lehnert 950 [DUKE]
<i>Cyathea pauciflora</i> (Kuhn) Lellinger	White & Lucansky 1970125 [DUKE]
<i>Cyathea petiolata</i> (Hook.) R. M. Tryon	Croat 4299 [DUKE]
<i>Cyathea phalerata</i> Mart.	White & White 197046 [DUKE]
<i>Cyathea pilozana</i> Murillo & Murillo	Betancur et al. 3213 [UC]
<i>Cyathea pinnula</i> (H. Christ) Domin	Croat 66590 [DUKE]
<i>Cyathea planadae</i> N.C.Arens & A.R.Sm.	Palacios & Freine 4988 [UC]
<i>Cyathea platylepis</i> (Hook.) Domin	Liesner, Steyermark & Holst 20873 [UC]
<i>Cyathea plicata</i> Lehnert	Lehnert 844 [UC]
<i>Cyathea pseudonanna</i> (L.D. Gómez) Lellinger	Moran 4013 [UC]
<i>Cyathea punctata</i> R.C.Moran & B.Øllg.	Lehnert 1581 [UC]
<i>Cyathea pungens</i> (Willd.) Domin	White & Lucansky 1969224 [DUKE]
<i>Cyathea purpurea</i> C.V. Morton	Huber 11879 [UC]
<i>Cyathea roraimensis</i> Domin	Liesner & Stannard 16919 [UC]
<i>Cyathea rufa</i> (Fée) Lellinger	Rodin 8856 [UC]
<i>Cyathea ruiziana</i> Klotzsch	van der Werff et al. 21185 [UC]
<i>Cyathea sagittifolia</i> (Hook.) Domin.	Hekking 1438 [DUKE]
<i>Cyathea schiedeana</i> (C. Presl) Domin	Morton 7588 [DUKE]
<i>Cyathea schlimii</i> (Mett. ex Kuhn) Domin	MacDougal et al. 4023 [UC]
<i>Cyathea senilis</i> (Klotzsch) Domin	White & Lucansky 1969222 [DUKE]
<i>Cyathea serpens</i> (R. M. Tryon) Lehnert	Mellado & Monteagudo 464 [UC]
<i>Cyathea sipapoensis</i> (R.M. Tryon) Lellinger	Maguire & Politi 28765 [UC]
<i>Cyathea speciosa</i> Willd.	White & Lucansky 1969223 [DUKE]
<i>Cyathea spectabilis</i> (Kunze) Domin	Fendler 25 [UC]
<i>Cyathea squamata</i> (Klotzsch) Domin	White & White 197016 [DUKE]
<i>Cyathea squamipes</i> H. Karst.	Gentry et al. 55093 [UC]
<i>Cyathea squamulosa</i> [I. Losch] R. C. Moran	Wilbur & Stone 8914 [DUKE]
<i>Cyathea squarrosa</i> (Rosenst) Domin	Brade 405 [UC]
<i>Cyathea stipularis</i> (H. Christ) Domin	Wilbur & Luteyn 18299 [DUKE]
<i>Cyathea stolzei</i> A.R. Sm. ex Lellinger	Kennedy 2750 [DUKE]
<i>Cyathea straminea</i> H. Karst.	Palacios & Tirado 12952 [UC]
<i>Cyathea subincisa</i> (Kunze) Domin.	Wood 14938 [UC]
<i>Cyathea suprapilosa</i> Lehnert	Croat 79065 [UC]
<i>Cyathea suprastrigosa</i> (Christ) Maxon	Stone 2023 [DUKE]
<i>Cyathea surinamensis</i> (Miq.) Domin	McDowell 4225 [DUKE]
<i>Cyathea tenera</i> (J. Sm. ex Hook.) T. Moore	Morton 5492 [DUKE]
<i>Cyathea thelypteroides</i> A. R. Smith	van der Werff, et al. 16323 [UC]
<i>Cyathea thysanolepis</i> (Barrington) A. R. Sm.	Boom & Weitzman 5825 [UC]
<i>Cyathea tortuosa</i> R. C. Moran	van der Werff, et al. 16542 [UC]
<i>Cyathea trailii</i> (Desv.) K. U. Kramer	van der Werff, et al. 19965 [UC]
<i>Cyathea trichiata</i> (Maxon) Domin	Wilbur & Luteyn 18225 [DUKE]

TABLE 3. Continued.

<i>Cyathea tryonorum</i> (Riba) Lellinger	White & White 197049 [DUKE]
<i>Cyathea tuerckheimii</i> Maxon	Hallberg 1531 [UC]
<i>Cyathea tungurahuae</i> Sodiro	Wilson et al. 2770 [UC]
<i>Cyathea uleana</i> (A. Samp.) Lehnert	Kessler et al. 7289 [UC]
<i>Cyathea ulei</i> (H. Christ) Domin	Anderson 13415 [DUKE]
<i>Cyathea ursina</i> (Maxon) Lellinger	Hammel 8710 [DUKE]
<i>Cnemidaria varians</i> R. C. Moran	Valdespino & Aranda 139 [UC]
<i>Cyathea venezuelensis</i> A. R. Smith	Steyermark et al. 21547 [UC]
<i>Cyathea villosa</i> Humb.& Bonpl. ex Willd.	Hatschbach 29880 [UC]
<i>Cyathea weatherbyana</i> (C.V. Morton) C.V. Morton	Mears & Adersen 5390 [UC]
<i>Cyathea wendlandii</i> (Mett. ex. Kuhn) Domin	White & Lucansky 1968185 [DUKE]
<i>Cyathea werffii</i> R.C. Moran	van der Werff & Gudino 11386
<i>Cyathea williamsii</i> (Maxon) Domin	Foster & Kennedy 1878 [DUKE]
<i>Cyathea windischiana</i> A. R. Smith	van der Werff, et al. 16207
<i>Cyathea xenoxyla</i> Lehnert	Kessler et al. 7220 [UC]

in the field and in the greenhouse. A small number of ants was observed on fronds of some plants of *Cyathea choricarpa*, but ants were not observed visiting the glands. Gland surfaces in some species (e.g. *C. acutidens*, Fig. 27; *C. multiflora*, Fig. 28) were frequently observed to be damaged and scarred, possibly due to feeding by ants or other insects. The glands of *Cyathea acutidens* were heavily damaged in this way, including the small pinnule-base glands.

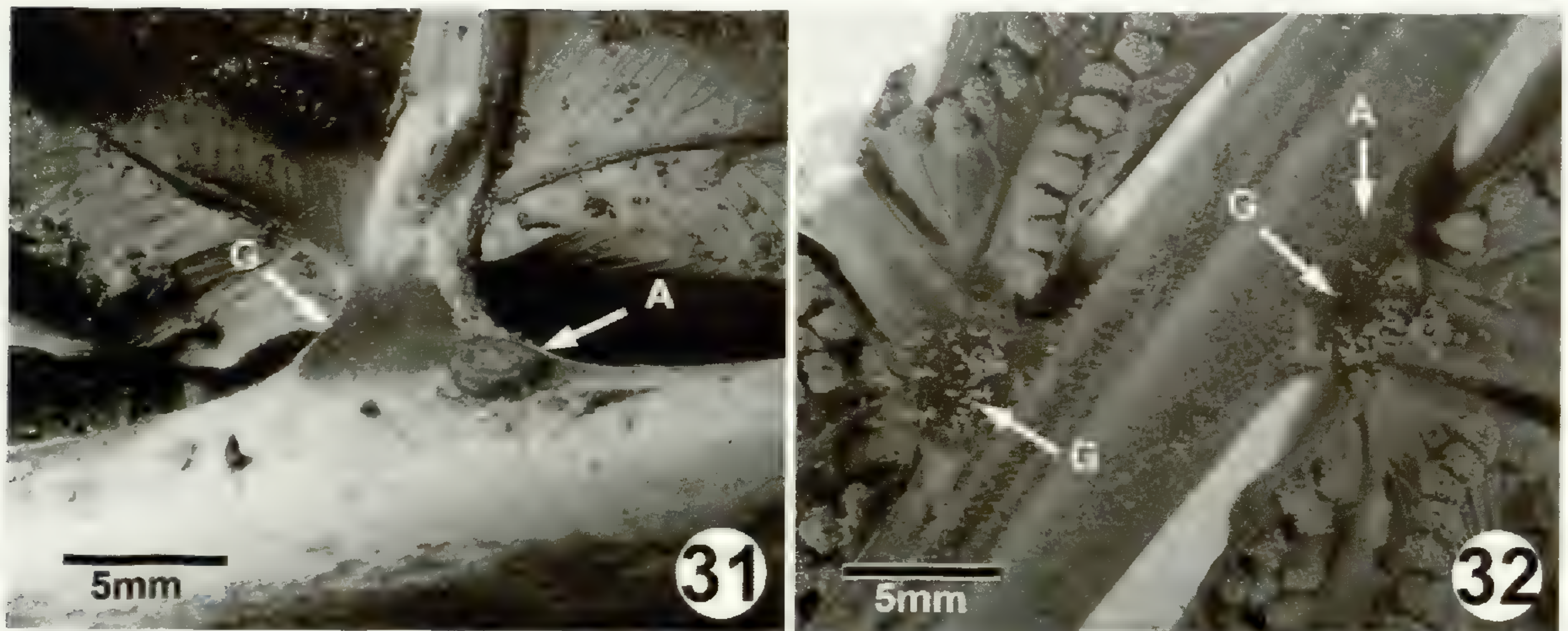
The review of selected herbarium sheets indicates that indeed glands can be identified on the dried leaves of *Cyathea* species. These structures appear as dark, often shiny and shriveled spots, and are located where glands are seen to occur in live plants (*Cyathea delgadii*, Fig. 29; *C. multiflora*, Fig. 30; *Cyathea horrida*, Fig. 31). Some of these spots appear to have visible surface deposits of dried secretion, and others appear to be sticky, with spores and sporangia attached to the gland areas (e.g., *Cyathea cocleana* (Fig. 32).

Table 3 summarizes our herbarium survey of *Cyathea* foliar glands. For each species cited, a single representative specimen is listed. The four species we examined of Old World *Cyatheas* (the *Cyathea decurrens* group) lack glands. Among the New World species surveyed, there were five species which were observed to lack glands, in addition to six glandless species formerly members of the genus *Hymenophyllopsis*. Glands were observed to be present in specimens of the remaining 146 *Cyathea* species surveyed.

Where species were included in both the anatomical survey and the herbarium survey, the observations are consistent. Of the 15 *Cyathea* species in the herbarium survey that lack nectaries, the absence of glands was confirmed anatomically for five of them. Similarly, of the 146 gland-bearing species in the herbarium survey, the glands were studied anatomically for 25 species.

DISCUSSION

The anatomy of the foliar glands in the species of *Cyathea* which were examined is distinctly different from the foliar nectaries that have been



FIGS. 31–32. Foliar glands on dried herbarium specimens. **31.** *Cyathea horrida* [White & Lucansky 1970130 (DUKE)], with large dark gland adjacent to prominent pinna-base aerophore. **32.** *C. cocleana* [Wilbur, et al. 11111 (DUKE)], showing numerous loose sporangia apparently stuck to dried secretions on gland surfaces. A= aerophore; G= gland.

previously described for a few other species of ferns (e.g., Bonnier, 1879; Power and Skog, 1967; Potes, 2010). We have identified three distinctive anatomical features which characterize *Cyathea* glands: (1) the cells of the epidermis of the gland can be distinguished from the epidermal cells adjacent to the glands. The secretory epidermal cells of the gland are densely staining, anticlinally elongate, have a thick cuticle, and scales, scurf and trichomes are absent; (2) subjacent to the epidermis there is a subepidermal zone of a few to several layers of larger more highly vacuolated parenchyma cells; and (3) subjacent to this latter zone is an inner zone of parenchyma and an interruption of the fibrous hypodermal zone (except for Type III) characteristic of adjacent areas of the pinna axis and rachis. We have observed no modification or specialization of vascular tissue associated with nectaries in any *Cyathea* species.

The glands of the *Cyathea* species we reviewed can be organized into three general groups. This characterization of gland “types” is not intended to be rigid, however. The detailed anatomical structure of the glands in *Cyathea* varies from the more standard and general form which is widely distributed among the species we examined (Type I) to a more elaborate and distinctive type found in only a few species (Type II), to a form which is far less elaborate in anatomy compared to the other types, with less modified epidermal cells and fewer cells composing the subjacent parenchyma zones. These most simple glands are not associated with an interruption of the fibrous layer, and do not form an elevated bulge on the leaf surface (Type III).

The presence of nectaries varies among the species in *Cyathea*. As noted, in addition to species with nectaries, there are *Cyathea* species which lack them. The presence and absence of glands among species of a given genus has been reported in other genera (e.g., *Pleopeltis* as *Polypodium*: *P. plesiosorus* Kunze and *P. furfuraceum* Schldl. & Cham. lack glands (Koptur *et al.*, 1998). In this

survey, as noted earlier, *Alsophila* and *Gymnosphaera* consistently lack glands. Although nearly all species of *Sphaeropteris* we examined lacked glands, a very few species appear to have them (e.g., *S. robinsonii*).

Based on these results compared with recent phylogenetic analyses, the presence and structure of the foliar glands appear to have potential value as markers of major clades within *Cyathea*. The members of the earliest diverging branch of the genus, the Old World *Cyathea decurrens* group (e.g., Conant *et al.*, 1995, 1996; Korall *et al.*, 2007; Janssen *et al.*, 2008; Bystriakova *et al.*, 2011), lack these glands, as do the other genera of *Cyatheaceae* (viz. *Alsophila*, *Gymnosphaera* and some species of *Sphaeropteris*). Within another early branch of the *Cyathea* clade, which includes the *C. armata* group of Korall *et al.* (2007), several species were observed to lack glands, including *C. arborea*, *C. parvula* and *C. poeppigii*. However, within this group there is a distinctive sub-group, the *Trichipteris armata* group of Barrington (1978) and Gastony (1979), including *C. armata*, *C. trichiata*, *C. stipularis* and *C. nesiotica*, which do have glands. It is noteworthy that the glands of *C. armata* and *C. trichiata* are of the simplest anatomical type we have seen (Type III).

A large clade of New World *Cyathea* species comprising the *Cnemidaria* group, the *Cyathea gibbosa* group, and the *Cyathea divergens* group (sensu Conant *et al.*, 1995, 1996; Korall *et al.*, 2007) includes most species of this genus. The members of this large group that we have examined all possess well-developed foliar glands of Type I or Type II. Although we made only a modest anatomical survey of species, the review of herbarium specimens has identified the presence of foliar glands in numerous other species of *Cyathea* throughout these groups. Thus, these more elaborate, more prominent foliar glands appear to be a shared derived characteristic which marks the largest clade within New World *Cyathea*.

The presence of the group of *Cyathea* species with Type III glands (e.g., *C. armata*, *C. trichiata*, and *C. stipularis*) within a clade that otherwise lacks glands (e.g., *C. arborea*, *C. parvula* and *C. poeppigii*) is problematic. Possible explanations include: (1) the common ancestor of this clade with the *Cnemidaria-C. gibbosa-C. divergens* group had glands, and the glandless *C. armata* group members have lost glands; (2) the *C. armata/C. trichiata* gland-bearing subgroup represents an independent, parallel or convergent origin of glands; or (3) it is possible that an ancient hybridization between an ancestor of the *C. armata/C. trichiata* species group and an early member of the gland-bearing *Cnemidaria-C. gibbosa-C. divergens* group would explain the presence of glands in this group. The glandless *Cyathea arborea* is known to form hybrids with different gland-bearing *Cyathea* species today (Conant, 1975; Caluff, 2002). Early members of the two groups might similarly have been able to hybridize.

The group of *Cyathea* species that was formerly considered the genus *Hymenophyllopsis* has recently been reclassified as subgenus *Hymenophyllopsis* of *Cyathea* (Christenhusz, 2009). This group has been weakly supported as the sister group to all other New World *Cyathea* species (Korall *et al.*, 2007), or sister to a large group corresponding to the *Cnemidaria-C. gibbosa-C. divergens* group (Janssen *et al.*, 2008; Bystriakova *et al.*, 2011). The observed

lack of pinna-base glands in *Cyathea* (*Hymenophyllopsis*) *dejecta* may be consistent with either position. On the other hand, the absence of glands might be expected from its great reduction in size and complexity, even if *Hymenophyllopsis* was derived from gland-bearing *Cyathea* ancestors.

The presence of nectaries marks a large group of *Cyathea* species and the shared presence of nectaries helps unify the group which contains most New World *Cyathea* species. This includes the species formerly included in the genus *Cnemidaria* (Korall *et al.*, 2007; Lehnert, 2011a), *Trichipteris* (Lellinger, 1987) and a group of *Cyathea* species formerly included in *Sphaeropteris* (Tryon, 1970; Windisch, 1977, 1978).

The comparatively simple morphology and anatomy of the glands in the *C.armata/C. trichiata* group may well represent the most primitive state of the gland seen in extant gland-bearing *Cyathea*.

As mentioned earlier, although all the *Sphaeropteris* species we examined anatomically lack glands, herbarium specimens of *S. robinsonii* of the Phillipines (e.g., Ramos & Edanō, Bureau of Sci. 47350 [UC]) do have dark areas similar in appearance and position to the pinna-base glands of *Cyathea* species. An anatomical examination of this and related *Sphaeropteris* species is needed. If pinna-base nectaries are indeed present in this one or a few *Sphaeropteris* species, most likely they will have evolved independently from the *Cyathea* nectaries reported here.

In previous tree fern literature, reference is made to observations of glands and aerophores at the base of fern pinnae. Ants have been observed on “glabrous pads at the base of primary pinnae” (Arens and Smith, 1998), “swollen dark glossy aerophores” have been described (Holttum and Edwards, 1983), dark spots have been observed on herbarium specimens (Arens and Smith, 1998) and reference has been made to the need to clarify “the dark spots at the base of each pinna...regarding its fundamental origin as a possible nectary or aerophore” (Mickel and Beitel, 1988; Mickel and Smith, 2004; Lehnert, 2006).

It is clear that there has been some confusion in the literature in distinguishing between pinna-base glands and aerophores. Our observations confirm that these glands have a distinctive anatomy and that they exist as structures independent of the adjacent aerophores. Functionally, they have been shown to have exudate of interest to ants (Arens and Smith, 1998), and to have fungi associated with them, reflecting the likely presence of sugary exudate. The dark spots on herbarium specimens have been useful in identifying the presence and location of nectaries. All of this is to say that careful observations are necessary in order to describe the presence of nectaries and to distinguish these from aerophores.

Field observations have so far provided few data in regard to the function of the pinna-base glands in *Cyathea* species. In *Cyathea multiflora*, and *C. delgadii*, the glands are large and bright green in young expanding leaves, but much less conspicuous in mature leaves. Despite their prominence, the glands on young fronds of both these species were never observed to be wet with secretions when examined closely in the field. *Cyathea* leaf glands are not vascularized, and this may explain the very low secretion rates that we

observed. However, droplets of liquid were observed on the pinna-base glands of young expanded fronds of *Cyathea choricarpa*, and small amounts of secreted liquid were consistently present on glands of *Cyathea trichiata*, both in the field and in the greenhouse. A small number of ants was observed on the fronds of some plants of *Cyathea choricarpa*, but were not observed in association with the glands. The surfaces of glands in some species, for example in *Cyathea acutidens*, were frequently observed to be damaged and scarred, possibly due to feeding by ants or other insects.

On the basis of these anatomical studies, their location on the leaves, and the modest observations of droplets, and insect activity, we have concluded that these glands are indeed nectaries. In support of this conclusion there is an obvious need for more extensive field observations and greenhouse studies as well as chemical analyses of the exudate. The presence or absence of glands and the variation in gland anatomy may well be useful as markers in analyses of species relationships within a genus and among fern families. A broader survey of the presence or absence of fern glands, their distribution and their anatomical structure is fully warranted.

ACKNOWLEDGMENTS

We thank the following people for their advice, materials and technical assistance: David Conant, Marcus Lehnert, Robin Moran, Kathleen Pryer, Amber Ratchford, and Alan Smith. We also acknowledge the support of the herbarium curators of CR, DUKE, LSC and UC.

LITERATURE CITED

- AGRAWAL, A. A. 2011. Current trends in evolutionary ecology of plant defences. *Functional Ecology* 25:420–432.
- ARENS, N. C. and A. R. SMITH. 1998. *Cyathea planadae*, a remarkable new creeping tree fern from Colombia, South America. *Amer. Fern J.* 88:49–59.
- BARRINGTON, D. S. 1978. A Revision of the Genus *Trichipteris*. *Contrib. Gray Herbarium. Harvard Univ.* 208:3–93.
- BENTLEY, B. L. 1977. Extrafloral nectaries and protection by pugnacious bodyguards. *Ann. Rev. Ecol. Syst.* 8:407–427.
- BENTLEY, B. L. and T. ELIAS. (eds). 1983. *The Biology of Nectaries*. Columbia Univ. Press, N.Y.
- BONNIER, G. 1879. Les nectaries: étude critique, anatomique et physiologique. *Ann. Sci. Nat.* [Ser. VI] 8:5–212.
- BYSTRIAKOVA, N., H. SCHNEIDER and D. COOMES. 2011. Evolution of the climatic niche in scaly tree ferns (Cyatheaceae, Polypodopsida). *Bot. J. Linn. Soc.* 165:1–19.
- CALUFF, M. G. 2002. *xCyathidaria*, a new nothogenus in the Cyatheaceae (Pteridophyta). *Willdenowia* 32:281–283.
- CHRISTENHUSZ, M. J. M. 2009. New combinations and an overview of *Cyathea* subg. *Hymenophyllopsis* (Cyatheaceae). *Phytotaxa* 1:37–42.
- CONANT, D. S. 1975. Hybrids in American Cyatheaceae. *Rhodora* 77:441–455.
- CONANT, D. S., L. A. RAUBESON, D. K. ATTWOOD and D. B. STEIN. 1995. The relationships of Papuanian Cyatheaceae to New World tree ferns. *Amer. Fern J.* 85:328–340.
- CONANT, D. S., L. A. RAUBESON, D. K. ATTWOOD, S. PERERA, E. A. ZIMMER, J. A. SWEERE and D. B. STEIN. 1996. Phylogenetic and evolutionary implications of combined analysis of DNA and morphology in the Cyatheaceae. Pp. 231–248, in *Pteridology in Perspective*, J. M. Camus, M. Gibby and R. J. Johns, eds, Royal Botanic Gardens, Kew.

- DARWIN, F. 1877. On the nectar-glands of the common brakefern. *J. Linn. Soc. Bot.* 15:407–409.
- DÜMMER, R. 1911. Grape sugar as an excretion in *Platyserium*. *Ann. Bot.* 25:1205–1206.
- DURKEE, L. T. 1987. Ultrastructure of extrafloral nectaries in *Aphelandra* spp. (Acanthaceae). *Proc. Iowa Acad. Sci.* 94:78–83.
- ELIAS, T. S. 1983. Extrafloral nectaries: their structure and distribution, Pp. 175–203, in *The Biology of Nectaries*, B. L. Bentley and T. S. Elias, eds, Columbia University Press, New York.
- ELIAS, T. S. and SUN AN-CI. 1985. Morphology and anatomy of foliar nectaries and associated leaves in *Mallotus* (Euphorbiaceae). *Aliso* 11:17–25.
- FAHN, A. 1979. *Secretory Tissues in Plants*. Academic Press, London; New York.
- FREITUS, L., G. BERNARDILLO, L. GALETTO and A. A. S. PAOLI. 2001. Nectaries and reproductive biology in *Croton sarcopetalus* (Euphorbiaceae). *Bot. J. Linn. Soc.* 136:267–277.
- GASTONY, G. J. 1979. Spore morphology in the Cyatheaceae III. The Genus *Trichipteris*. *Amer. J. Bot.* 66:1238–1260.
- HOLTUM, R. E. and P. J. EDWARDS. 1983. The tree ferns of Mount Roraima and neighboring areas of the Guayana Highlands with comments on the family Cyatheaceae. *Kew Bull.* 38:155–188.
- JANSSEN, T., N. BYSTRIAKOVA, F. RAKOTONDRAINIBE, D. COOMES, J. N. LABAT and H. SCHNEIDER. 2008. Neoendemism in Madagascan scaly tree ferns results from recent coincident diversification bursts. *Evolution* 62:1876–1889.
- JOHANSEN, D. A. 1940. *Plant Microtechnique*. McGraw-Hill Book Co., New York.
- KOPTUR, S., A. R. SMITH and I. BAKER. 1982. Nectaries in some neotropical species of *Polypodium* (Polypodiaceae): Preliminary observations and analyses. *Biotropica* 14:108–113.
- KOPTUR, S. 1985. Alternative defenses against herbivores in *Inga* (Fabaceae, Mimosoideae) over an elevational gradient. *Ecology* 66:1639–1650.
- KOPTUR, S. 1992. Extrafloral nectary-mediated interactions between insects and plants. Pp. 81–129, in *Insect-Plant Interactions vol. IV*, E. A. Bernays, ed, CRC Press, Boca Raton, FL, USA.
- KOPTUR, S., V. RICO-GRAY and M. PALACIOS-RIOS. 1998. Ant protection of the nectaried fern *Polypodium plebium* in central Mexico. *Amer. J. Bot.* 85:736–739.
- KORALL, P., D. S. CONANT, J. S. METZGAR, H. A. SCHNEIDER and K. M. PRYER. 2007. A molecular phylogeny of scaly tree ferns. *Amer. J. Bot.* 94:873–886.
- LEHNERT, M. 2006. The Cyatheaceae and Dicksoniaceae (Pteridophyta) of Bolivia. *Brittonia* 58:229–244.
- LEHNERT, M. 2011a. The Cyatheaceae (Polypodopsida) of Peru. *Brittonia* 63:11–45.
- LEHNERT, M. 2011b. Species of *Cyathea* in America related to the western Pacific species *C. decurrens*. *Phytotaxa* 26:39–59.
- LELLINGER, D. B. 1987. The disposition of *Trichopteris* (Cyatheaceae). *Amer. Fern J.* 77:90–94.
- LÜTTGE, V. 1971. Structure and function of plant glands. *Ann. Rev. Pl. Physiol.* 22:23–44.
- MACHADO, S. R., L. P. C. MORELLATO, M. G. SAJO and P. S. OLIVEIRA. 2008. Morphological patterns of extrafloral nectaries in woody plant species of the Brazilian cerrado. *Plant Biology* 10:660–673.
- MARGINSON, R., M. SEDGLEY and R. B. KNOX. 1985. Structure and histochemistry of the extrafloral nectary of *Acacia terminalis* (Salisb). McBride (Leguminosae, Mimosoideae). *Protoplasma*. 127:21–30.
- MCDADE, L. A. and M. D. TURNER. 1997. Structure and development of bracteal nectary glands in *Aphelandra* (Acanthaceae). *Amer. J. Bot.* 84:1–15.
- MICKEL, J. T. and J. BEITEL. 1988. *Pteridophyte Flora of Oaxaca Mexico*. New York Botanical Gardens, New York.
- MICKEL, J. T. and A. R. SMITH. 2004. The Pteridophytes of Mexico. *Mem. N.Y. Bot Garden* 88:216.
- MORAN, R. C. 1991. Eight new species of tree ferns (*Cyathea*, Cyatheaceae) from the American tropics and three new combinations. *Novon* 1:88–104.
- MORAN, R. C. 1995. Five new species and two new combinations of ferns (Polypodiopsida) from Ecuador. *Nordic Jour. Bot.* 15:49–58.
- POTES, A. 2010. Comparative anatomy of the nectaries of *Agloamorpha* and *Drynaria* (Polypodiaceae). *Amer. Fern J.* 100:80–92.
- POWER, M. S. and J. E. SKOG. 1987. Ultrastructure of the extrafloral nectaries of *Pteridium aquilinum*. *Amer. Fern J.* 77:1–15.

- RASHBROOK, V. K., S. G. COMPTON and J. H. LAWTON. 1992. Ant-herbivore interactions: reasons for the absence of benefits to a fern with foliar nectaries. *Ecology* 73:2167–2174.
- ROSUMEK, F. B., F. A. O. SILVEIRA, F. D. NEVES, N. P. D. BARBOSA, L. DINIZ, Y. OKI, F. PEZZINI, G. W. FERNANDES and T. CORNELISSEN. 2009. Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. *Oecologia* 160:537–549.
- RUMPF, S. M., M. CROMEY and C. J. WEBB. 1994. Ultrastructure and function of the nectaries of New Zealand bracken (*Pteridium esculentum* (Forst. f.) Cockayne). *New Zealand J. Bot.* 32:487–496.
- 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 and P. WOLF. 2008. Fern Classification. Pp. 417–467, in *Biological Evolution of Ferns and Lycophytes*, T. A. Ranker and C. H. Haufler, eds, Cambridge Univ. Press, New York.
- TEMPEL, A. S. 1983. Bracken fern (*Pteridium aquilinum*) and nectar-feeding ants: a non-mutualistic interaction. *Ecology* 64:1411–1422.
- THADEO, M., M. F. CASSINO, N. H. VITARELLI, A. A. AZEVEDO, J. M. ARAUJO, V. M. M. VALENTE and R. M. S. A. MEIRA. 2008. Anatomical and histochemical characterization of extrafloral nectaries of *Prockia crucis* (Salicaceae). *Amer. J. Bot.* 95:1515–1522.
- TRYON, R. M. 1970. The classification of the Cyatheaceae. *Contrib. from the Gray Herb.* 200:3–50.
- WINDISCH, P. G. 1977. Synopsis of the genus *Sphaeropteris* with a revision of the neotropical exindusiate species. *Bot. Jahr. für Systematic Pflanzengeschichte und Pflanzengeographie* 92:176–198.
- WINDISCH, P. G. 1978. The systematics of the group of *Sphaeropteris hirsuta* (Cyatheaceae). In *The Botany of the Guayana Highland*, B. Maguire, ed. *Mem. N.Y. Bot. Gard.* 29:2–22.
- ZAMORA, P. M. and N. S. VARGA. 1974. Nectary-costule association in Philippine drynarioid ferns. *The Philipp. Agric.* 57:72–88.
- ZIMMERMAN, J. 1932. Über die extrafloralen Nektarien der Angiospermen. *Beih. Bot. Zentralbl.* 49:99–196.

An Expanded Plastid Phylogeny of *Marsilea* with Emphasis on North American Species

W. MARK WHITTEN

Florida Museum of Natural History, 385 Dickinson Hall, Gainesville, FL 32611-7800, email: whitten@flmnh.ufl.edu

COLETTE C. JACONO

Center for Aquatic and Invasive Plants, University of Florida, 7922 NW 71st Street, Gainesville, FL 32653-3701, email: Colette.C.Jacono@aphis.usda.gov

NATHALIE S. NAGALINGUM

Royal Botanic Garden Sydney, National Herbarium of New South Wales, Mrs Macquaries Road, Sydney, NSW, Australia, 2000, email: nathalie.nagalingum@rbgsyd.nsw.gov.au

ABSTRACT.—Ferns of the genus *Marsilea* (water clover) are potentially invasive aquatic and wetland plants. They are difficult to identify to species because of subtle diagnostic characters, the sterile condition of many specimens, and unresolved taxonomic problems. We sequenced four plastid regions (*rbcL*, *rps4*, *rps4-trnS* spacer, and *trnL-F* spacer) from 223 accessions across ca. 38 species. Our goals were to: 1) attempt to identify problematic *Marsilea* specimens from the southeastern U.S., and 2) assess species delimitation using molecular data. Florida specimens previously identified as *M. aff. oligospora* do not match true *M. oligospora* (native to the western USA), and might represent an undescribed native species. The molecular data fail to resolve many species as monophyletic within the New World *Marsilea* section *Nodorhizae*. The data reveal two strongly supported clades within section *Nodorhizae*: 1) A western U.S. /Mexican clade; and 2) A U.S. Gulf coastal plain/Florida/Caribbean clade. This DNA/morphology discordance suggests that these taxa either may have hybridized extensively or that the number of *Marsilea* species within these clades may be overestimated. Either case warrants the addition of nuclear data sets and reevaluation of the species boundaries within the genus.

KEY WORDS.—*Marsilea*, phylogenetics, plastid, species delimitations

Marsilea L. (ca. 50 spp.) occur worldwide as two ecological types: 1) true aquatic species with glabrous leaves and fleshy rhizomes that inhabit more permanent water bodies, and 2) semi-aquatic species with hairy leaves and tough, fibrous rhizomes that prefer fluctuating wetland habitats and prevail through seasonal extremes in wet and dry periods (Jacono and Johnson, 2006). *Marsilea* have few dependable morphological characters on which to base species-level identifications. Phenotypic plasticity is widespread, and sporocarps, which contain many characters used for species delimitation, are commonly absent in field populations. Because identification of *Marsilea* based upon morphology is so difficult, molecular data might provide more reliable tools for identification.

The impetus for this study was an applied resource management need to clarify the identity of three western North American species of *Marsilea* in Florida (Jacono and Johnson, 2006). *Marsilea vestita* Hook. & Grev. and *M. macropoda* Engelm. ex A. Braun have been regarded as introduced to eastern

North America based on their disjunct and widely scattered populations at ruderal sites in Gulf coastal Alabama and Florida. A third species, centered on three central Florida counties, was tentatively identified as *M. aff. oligospora* Goodd. (Jacono and Johnson, 2006) based on sporocarp morphology; however, *Marsilea oligospora* is a semi-aquatic North American species otherwise endemic to the northern fringe of the Great Basin. Variation was noted between the Florida and the Great Basin material and it was difficult for the authors to speculate how a geographically restricted plant with no known economic value might have become established in central Florida over 100 years ago. The great difference in climate between northwestern U.S. and Florida added to our suspicion that these were two different taxa. These Florida *M. aff. oligospora* were first collected in the early 1890s near Eustis, Florida, and their determination has vacillated from *M. vestita*, an introduction from the western U.S. (Ward and Hall, 1976) to *M. ancylopoda* A.Braun, a rare and potentially extinct native species (FNA, 1993).

Here we use DNA sequences of four plastid regions (*rbcL*, *rps4*, the *rps4-trnS* spacer, and the *trnL-F* spacer) to expand upon the recent molecular phylogeny of *Marsilea* (Nagalingum *et al.*, 2007), using a greater sampling of North American specimens. Our first objective was to determine the status of the Florida plants assigned to *M. aff. oligospora*. We surveyed all known populations of *Marsilea* within Florida and compared them to all U.S., Mexican, and Caribbean species, as well as *Marsilea* species common in the aquatic plant trade that are established in the southeastern U.S. These data will provide a baseline for evaluating *M. aff. oligospora* in Florida and for distinguishing future introductions of *Marsilea*. Our second objective is to assess species monophyly using multiple accessions of each species, particularly for the North American specimens assigned to *Marsilea* sect. *Nodorhizae*.

MATERIALS AND METHODS

Thirty-three samples were included from Nagalingum *et al.* (2007), and are distinguished by the GenBank prefix DQ; the remainder were generated in this study (Table 1). Because Florida collections of *M. oligospora* were hypothesized to be introductions from the western U.S. (Jacono and Johnson, 2006), we included as many specimens as possible from western states. Species not present in the Nagalingum *et al.* (2007) study include *M. coromandelina* Willd., *M. costulifera* D.L.Jones, *M. crenulata* Desv., *M. deflexa* A.Braun, *M. exarata* A.Braun, *M. fournieri* C.Chr., *M. hirsuta* R.Br., *M. mexicana* A.Braun, *M. mucronata* A.Braun, *M. scalaripes* D.M. Johnson, *M. tenuifolia* Engelm. ex Kunze, and *M. uncinata* A.Braun.

Samples were taken from herbarium specimens. Leaf samples (ca. 25 mm²) were ground using a tissue mill and extracted using a modified version of the 2× CTAB procedure of Doyle and Doyle (1987) with exclusion of beta-mercaptoethanol and inclusion of 5 units of proteinase K. Primers for *rbcL* were designed to allow amplification and sequencing in two overlapping

TABLE 1. Specimens sampled for DNA analysis in this study and corresponding GenBank numbers. DNA numbers (column 1) correspond to three-digit numbers following taxon names in Fig. 1. DNA numbers 256–280 are data from Nagalingum *et al.* (2007); the remainder were generated in this study. USA localities include state abbreviation followed by county.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition		Voucher	Locality
					FLAS	deposition		
005	<i>Marsilea aencylopoda</i> A. Braun	FJ533947	FJ533854	FJ534040	FLAS	E. Lott 3321	Mexico: Jalisco	
006	<i>Marsilea hirsuta</i> R. Br.	FJ533948	FJ533855	FJ534041	FLAS	Beckner 2619	USA: FL: Pinellas	
007	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533956	FJ533864	na	FLAS	Ward 7623	USA: TX: San Patricio	
008	<i>Marsilea hirsuta</i> R. Br.	FJ533953	FJ533861	FJ534046	FLAS	Jacono 257	USA: FL: Hillsborough	
009	<i>Marsilea hirsuta</i> R. Br.	FJ533954	FJ533862	FJ534047	FLAS	Jacono 235	USA: FL: Pinellas	
010	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533957	FJ533865	FJ534049	FLAS	Burkhalter 8846	USA: AL: Mobile	
011	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533958	FJ533866	FJ534050	FLAS	Burkhalter 5672	USA: AL: Mobile	
012	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533959	FJ533867	FJ534051	FLAS	McAlpin s.n.	USA: FL: Sarasota	
013	<i>Marsilea aencylopoda</i> A. Braun	HQ631073	HQ631266	HQ631171	FLAS	E. Lott 3321	Mexico: Jalisco	
014	<i>Marsilea minuta</i> L.	FJ533965	FJ533873	FJ534057	FLAS	J. Allison 8001	USA: GA: Cobb	
015	<i>Marsilea minuta</i> L.	FJ533966	FJ533874	FJ534058	FLAS	J. Germann s.n.	USA: GA: McDuffie	
016	<i>Marsilea minuta</i> L.	FJ533967	FJ533875	FJ534059	FLAS	Jacono 394	USA: FL: Escambia	
017	<i>Marsilea minuta</i> L.	FJ533968	FJ533876	FJ534060	FLAS	Burkhalter 13304	USA: FL: Escambia	
018	<i>Marsilea minuta</i> L.	FJ533969	FJ533877	FJ534061	FLAS	Jacono 498	USA: FL: St. Lucie	
020	<i>Marsilea mutica</i> Mett.	HQ631074	HQ631267	FJ534075	FLAS	Jacono 503	USA: OK: Hughes	
021	<i>Marsilea mutica</i> Mett.	FJ533983	FJ533891	FJ534076	FLAS	Jacono 362	USA: GA: Dekalb	
022	<i>Marsilea mutica</i> Mett.	FJ533984	FJ533892	FJ534077	FLAS	Cooper s.n.	USA: GA: Fulton	
023	<i>Marsilea mutica</i> Mett.	FJ533985	FJ533893	FJ534078	FLAS	Jacono 361	USA: GA: Spalding	
024	<i>Marsilea mutica</i> Mett.	HQ631075	HQ631268	HQ631172	FLAS	Kirk s.n.	USA: MS: Madison	
025	<i>Marsilea mutica</i> Mett.	FJ533986	FJ533894	FJ534079	FLAS	Davis 1208	USA: FL: Alachua	
026	<i>Marsilea mutica</i> Mett.	FJ533987	FJ533895	FJ534080	FLAS	Rodgers s.n.	USA: FL: Hillsborough	
027	<i>Marsilea mutica</i> Mett.	FJ533988	FJ533896	FJ534081	FLAS	Greene s.n.	USA: AL: Marion	
028	<i>Marsilea mutica</i> Mett.	FJ533989	FJ533897	FJ534082	FLAS	Jacono 502	USA: AL: Marion	
030	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533993	FJ533901	FJ534086	FLAS	Jacono 508	USA: FL: Lake	
031	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533994	FJ533902	FJ534087	FLAS	Jacono 510	USA: FL: Lake	
032	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533995	FJ533903	FJ534088	FLAS	Jacono 511	USA: FL: Lake	
033	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533996	FJ533904	FJ534089	FLAS	Jacono 571	USA: FL: Lake	
034	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533997	FJ533905	FJ534090	FLAS	Jacono 572	USA: FL: Lake	

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of				Voucher	Locality
		rbcL	trnL-F	rps4	deposition		
035	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	FJ533998	FJ533906	FJ534091	FLAS	Brinson s.n.	USA: FL: Seminole
036	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	HQ631076	HQ631269	FJ534092	FLAS	Jacono 177	USA: FL: Seminole
037	<i>Marsilea</i> aff. <i>oligospora</i> Goodd.	HQ631077	FJ533907	FJ534093	FLAS	Meisenburg s.n.	USA: FL: Volusia
038	<i>Marsilea vestita</i> Hook. & Grev.	FJ534005	FJ533913	FJ534098	FLAS	Raymond & Painter 81	USA: LA: Caldwell
039	<i>Marsilea vestita</i> Hook. & Grev.	FJ534028	FJ533935	FJ534122	FLAS	Thomas 114,754	USA: LA: Caldwell
040	<i>Marsilea vestita</i> Hook. & Grev.	FJ534007	FJ533915	FJ534100	FLAS	Brodie s.n.	USA: FL: Collier
041	<i>Marsilea vestita</i> Hook. & Grev.	FJ534008	FJ533916	FJ534101	FLAS	Jacono 619	USA: FL: Collier
042	<i>Marsilea vestita</i> Hook. & Grev.	FJ534009	FJ533917	FJ534102	FLAS	Anderson 7356	USA: FL: Franklin
044	<i>Marsilea vestita</i> Hook. & Grev.	FJ534010	FJ533918	FJ534103	FLAS	Jacono 183	USA: FL: Franklin
045	<i>Marsilea vestita</i> Hook. & Grev.	FJ534011	FJ533919	FJ534104	FLAS	Jacono 504	USA: FL: Franklin
046	<i>Marsilea vestita</i> Hook. & Grev.	HQ631078	HQ631270	FJ534105	FLAS	Hall 414	USA: FL: Hillsborough
047	<i>Marsilea vestita</i> Hook. & Grev.	HQ631079	FJ533920	FJ534106	TENN	D. Thomas & E. Sundell 167474	USA: AR: Lincoln
048	<i>Marsilea vestita</i> Hook. & Grev.	FJ534013	FJ533921	FJ534107	TENN	D. Thomas 114172	USA: LA: Caldwell
049	<i>Marsilea vestita</i> Hook. & Grev.	FJ534014	FJ533922	FJ534108	TENN	D. Thomas & C. M. Allen	USA: LA: St. James
050	<i>Marsilea vestita</i> Hook. & Grev.	FJ534015	FJ533923	FJ534109	TENN	J. Taylor 29759	USA: OK: Johnston
051	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533960	FJ533868	na	TENN	G. Landry & S. Holder 7832	USA: LA: Orleans
052	<i>Marsilea minuta</i> L.	FJ533970	FJ533878	HQ631173	TENN	J.T. Beck 3867	USA: TN: Hamilton
053	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533961	FJ533869	FJ534052	USF	K. Bradley 1858	USA: FL: Collier
054	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533962	FJ533870	FJ534053	USF	B. McAlpin sn	USA: FL: Sarasota
055	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533963	FJ533871	FJ534054	USF	B. McAlpin sn	USA: FL: Collier
056	<i>Marsilea vestita</i> Hook. & Grev.	FJ534016	HQ631271	HQ631174	USF	A. Gholson et al. 11253	USA: FL: Franklin
057	<i>Marsilea minuta</i> L.	FJ533971	FJ533879	HQ631175	USF	J. Burkhalter 13304	USA: FL: Escambia
058	<i>Marsilea minuta</i> L.	FJ533972	FJ533880	FJ534063	USF	Jacono 667	USA: FL: Brevard
059	<i>Marsilea hirsuta</i> R. Br.	HQ631080	HQ631272	FJ534048	USF	J. Beckner 2619	USA: FL: Pinellas
060	<i>Marsilea hirsuta</i> R. Br.	FJ533955	FJ533863	HQ631176	USF	R. Wunderlin et al. 10365	USA: FL: Pinellas
063	<i>Marsilea mutica</i> Mett.	FJ533990	FJ533898	FJ534083	ASU	D. Damrel 2429	USA: AZ: Maricopa
064	<i>Marsilea hirsuta</i> R. Br.	FJ533991	FJ533899	FJ534084	ASU	L. Makings 2090	USA: AZ: Maricopa
066	<i>Marsilea vestita</i> Hook. & Grev.	FJ534017	FJ533924	FJ534110	ASU	R. Felger 85-484	Mexico: Sonora

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of							Locality
		rbcl	trnL-F	rps4	deposition	Voucher			
067	<i>Marsilea deflexa</i> A. Braun	FJ534018	FJ533925	FJ534111	ASU	Steinman et al. 93-373		Mexico: Sonora	
068	<i>Marsilea vestita</i> Hook. & Grev.	FJ534019	FJ533926	FJ534112	ASU	J. Rebman & H. Lopez 2608		Mexico: Baja California	
069	<i>Marsilea vestita</i> Hook. & Grev.	FJ534020	FJ533927	FJ534113	ASU	S. Friedman & K. Johnson 456-94		Mexico: Sonora	
070	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533976	FJ533884	FJ534067	ASU	G. Ferguson 118		Mexico: Chihuahua	
071	<i>Marsilea mexicana</i> A. Braun	HQ631081	HQ631273	FJ534056	ASU	J. Hernandez s.n.		Mexico: Zacatecas	
072	<i>Marsilea fournieri</i> C. Chr.	FJ533952	FJ533860	FJ534045	ASU	J. Rebman & C. Davis 1684		Mexico: Baja California Sur	
073	<i>Marsilea vestita</i> Hook. & Grev.	FJ534021	FJ533928	FJ534114	ASU	E. Wise 1716		USA: CA: San Luis Obispo	
074	<i>Marsilea vestita</i> Hook. & Grev.	FJ534022	FJ533929	FJ534115	ASU	R. Worthington 21894		USA: NM: Luna	
075	<i>Marsilea vestita</i> Hook. & Grev.	FJ534023	FJ533930	FJ534116	ASU	D. Keil 18024		USA: CA: San Luis Obispo	
076	<i>Marsilea vestita</i> Hook. & Grev.	FJ534024	FJ533931	FJ534117	ASU	L. McGill 7203		USA: AZ: Cochise	
077	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631082	HQ631274	FJ534118	ASU	W. T. Johnson s.n.		USA: AZ: Coconino	
078	<i>Marsilea vestita</i> Hook. & Grev.	FJ534025	FJ533932	FJ534119	ASU	G. Marrs-Smith 1211		USA: AZ: Cochise	
079	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ534026	FJ533933	FJ534120	ASU	D. Damrel 627-A		USA: AZ: Gila	
080	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ534027	FJ533934	FJ534121	ASU	T. Wright & M. Baker 93-102		USA: AZ: Yaupai	
081	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631083	HQ631275	FJ534068	ASU	J. Collins s.n.		USA: AZ: Cochise	
082	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533977	FJ533885	FJ534069	ASU	E. Lehto 24541		USA: AZ: Coconino	
083	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533978	FJ533886	FJ534070	ASU	M. Baker 8595		USA: AZ: Coconino	
084	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533979	FJ533887	FJ534071	ASU	L. McGill 6860		USA: AZ: Cochise	
085	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533980	FJ533888	FJ534072	ASU	M. Windham 0114D		USA: AZ: Coconino	
086	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533981	FJ533889	FJ534073	ASU	D.J. Pinkava et al. s.n.		USA: AZ: Coconino	
087	<i>Marsilea vestita</i> Hook. & Grev.	FJ534006	FJ533914	FJ534099	VPI	R.D. Thomas 114754		USA: LA: Caldwell	
088	<i>Marsilea mutica</i> Mett.	FJ533992	FJ533900	FJ534085	VPI	M. Robinette sn		USA: VA: Patrick	
089	<i>Marsilea vestita</i> Hook. & Grev.	FJ534029	FJ533936	FJ534123	ID	C.R. Bjork 6868		USA: ID: Nez Perce	
090	<i>Marsilea vestita</i> Hook. & Grev.	FJ534030	FJ533937	FJ534124	ID	C.R. Bjork s.n.		USA: ID: Nez Perce	
091	<i>Marsilea vestita</i> Hook. & Grev.	FJ534031	FJ533938	FJ534125	ID	A. Tiehm 15267		USA: NV: Washoe	

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of				Voucher	Locality
		rbcL	trnL-F	rps4	deposition		
092	<i>Marsilea vestita</i> Hook. & Grev.	FJ534032	FJ533939	FJ534126	ID	C.R. Bjork 2404	USA: OR: Malheur
093	<i>Marsilea oligospora</i> Goodd.	FJ534000	FJ533908	FJ534094	ID	C.R. Bjork 3916	USA: ID: Owyhee
094	<i>Marsilea oligospora</i> Goodd.	FJ534001	FJ533909	FJ534095	ID	F.D. Johnson sn	USA: ID: Idaho
095	<i>Marsilea polycarpa</i> Hook. & Grev.	FJ534002	FJ533910	FJ534096	MO	S.R. Hall & L. Phillippe 28868	Dominica: St. Andrews
096	<i>Marsilea vestita</i> Hook. & Grev.	FJ534033	FJ533940	FJ534127	UTC	N. & P. Holmgren	USA: NV: Elko
097	<i>Marsilea vestita</i> Hook. & Grev.	FJ534034	FJ533941	FJ534128	UTC	A. Tiehm 14569	USA: NV: Elko
098	<i>Marsilea vestita</i> Hook. & Grev.	FJ534035	FJ533942	FJ534129	UTC	R.J. Shaw 3708	USA: UT: Millard
099	<i>Marsilea vestita</i> Hook. & Grev.	FJ534036	FJ533943	FJ534130	UTC	Weber & Wittmann 18558	USA: CO: Baca
100	<i>Marsilea vestita</i> Hook. & Grev.	FJ534037	FJ533944	FJ534131	UTC	R.J. & M. Shaw 4986	USA: MT: Lake
101	<i>Marsilea minuta</i> L.	FJ533973	FJ533881	FJ534064	MISS	A.R. Diamond 14269	USA: AL: Pike
102	<i>Marsilea minuta</i> L.	FJ533974	FJ533882	FJ534065	MICH	D.M. Johnson 800	Trinidad: Nariva Cocal
103	<i>Marsilea deflexa</i> A. Braun	FJ533951	FJ533859	FJ534044	MICH	D.M. Johnson 794	Venezuela: Guarico
104	<i>Marsilea ancylopoda</i> A. Braun	FJ533949	FJ533856	FJ534042	MICH	D.M. Johnson 773	Argentina: Corrientes
105	<i>Marsilea ancylopoda</i> A. Braun	HQ631084	FJ533857	HQ631177	MICH	E. Lott & A. Sanders 3987	Mexico: Jalisco: Quemaro
106	<i>Marsilea minuta</i> L.	FJ533975	FJ533883	FJ534066	MICH	M. Dyer 173	Nigeria: Kano: Acha Lafia
107	<i>Marsilea polycarpa</i> Hook. & Grev.	FJ534003	FJ533911	HQ631178	MICH	W. Wagner	Puerto Rico: Loiza
108	<i>Marsilea polycarpa</i> Hook. & Grev.	FJ534004	FJ533912	FJ534097	MICH	D.M. Johnson 793	Venezuela: Apure
109	<i>Marsilea mollis</i> B.L. Rob. & Fernald	FJ533982	FJ533890	FJ534074	MICH	N. Murray & D.M. Johnson 1404	Mexico: Chiapas
110	<i>Marsilea vestita</i> Hook. & Grev.	FJ534038	FJ533945	FJ534132	MICH	B. Ertter et al. 8131	USA: CA: Contra Costa
111	<i>Marsilea macropoda</i> Engelm. ex A. Braun	FJ533964	FJ533872	FJ534055	MICH	D.M. Johnson 721	USA: TX: Aransas
112	<i>Marsilea coromandelina</i> Willd.	FJ533950	FJ533858	FJ534043	MICH	M. Dyer 172	Nigeria: Kano: Acha Lafia
116	<i>Marsilea mutica</i> Mett.	HQ631085	FJ533946	FJ534133	VPI	R. Page s.n.	USA: VA: Hanover
119	<i>Marsilea nashii</i> Underw.	HQ631086	HQ631276	HQ631179	FLAS	R.J. Abbott 8678	USA: FL: Dade
121	<i>Marsilea quadrifolia</i> L.	HQ631087	HQ631277	HQ631180	FLAS	C. Jacono 622	USA: PA: Bradford
122	<i>Marsilea polycarpa</i> Hook. & Grev.	HQ631088	HQ631278	HQ631181	FLAS	R. Dressler 6004	Panama: Panama

TABLE 1. Continued.

DNA sample number	Taxon	rbcl	trnL-F	rps4	Herbarium of deposition		Voucher	Locality
					FLAS	BISH		
124	<i>Marsilea costulifera</i> D.L. Jones	HQ631089	HQ631279	HQ631182	FLAS	M. Whitten	3756	Australia: NSW
125	<i>Marsilea villosa</i> Kaulf.	HQ631090	HQ631280	HQ631183	BISH	Marian Chau	002	USA: HI: Oahu
127	<i>Marsilea villosa</i> Kaulf.	HQ631091	HQ631281	HQ631184	BISH	Marian Chau	029	USA: HI: Oahu
128	<i>Marsilea villosa</i> Kaulf.	HQ631092	HQ631282	HQ631185	BISH	Marian Chau	022	USA: HI: Moloka'i
129	<i>Marsilea villosa</i> Kaulf.	HQ631093	HQ631283	HQ631186	BISH	Marian Chau	015	USA: HI: Moloka'i
130	<i>Marsilea angustifolia</i> R. Br.	HQ631094	HQ631284	HQ631187	MEL	L.D. Cowie	9345	Australia: NT
131	<i>Marsilea crenata</i> C. Presl	HQ631095	HQ631285	HQ631188	MEL	G. Wightman	1458	Australia: NT
132	<i>Marsilea costulifera</i> D.L. Jones	HQ631096	HQ631286	HQ631189	MEL	I. Tankard	s.n.	Australia: UC
133	<i>Marsilea exarata</i> A. Braun	HQ631097	HQ631287	HQ631190	MEL	P.K. Latz	11823	Australia: NT
134	<i>Marsilea exarata</i> A. Braun	HQ631098	HQ631288	HQ631191	MEL	M.E. Trudgen	15576	Australia: WA
135	<i>Marsilea exarata</i> A. Braun	HQ631099	HQ631289	HQ631192	MEL	P.I. Forster	20421	Australia: QLD
136	<i>Marsilea hirsuta</i> R. Br.	HQ631100	HQ631290	HQ631193	MEL	K. Alcock	s.n.	Australia: SA
137	<i>Marsilea hirsuta</i> R. Br.	HQ631101	HQ631291	HQ631194	MEL	P.I. Forster	20681	Australia: QLD
138	<i>Marsilea hirsuta</i> R. Br.	HQ631102	HQ631292	HQ631195	MEL	I.D. Cowie	9590	Australia: NT
139	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631103	HQ631293	HQ631196	CAS	M.L. Arreguin	589	Mexico
141	<i>Marsilea minuta</i> L.	HQ631104	HQ631294	HQ631197	CAS	D.M. Johnson	800	Trinidad: Nariva Cocal
144	<i>Marsilea ancylopoda</i> A. Braun	HQ631105	HQ631295	HQ631198	CAS	E.J. Lott	3987 with A.C. Sanders	Mexico: Jalisco: Mpio La Huerta
145	<i>Marsilea mucronata</i> A. Braun	HQ631106	HQ631296	HQ631199	CAS	B. Ertter	3894 with J. Strachan	USA: CA: Plumas
146	<i>Marsilea vestita</i> Hook. & Grev.	HQ631107	HQ631297	HQ631200	CAS	A. Day	83-59	USA: CA: Merced
147	<i>Marsilea vestita</i> Hook. & Grev.	HQ631108	HQ631298	HQ631201	CAS	L. Ahart	4404	USA: CA: Sutter
148	<i>Marsilea vestita</i> Hook. & Grev.	HQ631109	HQ631299	HQ631202	CAS	A. Tiehm	11938	USA: NV: Elko
149	<i>Marsilea quadrifolia</i> L.	na	HQ631300	HQ631203	CAS	B. Bartholomew et al.	Guizhou Bot. Exped. 2380	China: Guizhou
150	<i>Marsilea polycarpa</i> Hook. & Grev.	na	HQ631301	HQ631204	CAS	W.H. Wagner	82018	Puerto Rico: Loiza
151	<i>Marsilea oligospora</i> Goodd.	HQ631110	HQ631302	HQ631205	CAS	J.T. Howell	36949	USA: CA: Plumas
152	<i>Marsilea oligospora</i> Goodd.	HQ631111	HQ631303	HQ631206	CAS	A. Tiehm	13199 with G. Schoolcraft	USA: NV: Washoe

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of deposition				Voucher	Locality
		rbcl	trnL-F	rps4	deposition		
154	<i>Marsilea oligospora</i> Goodd.	HQ631112	HQ631304	HQ631207	JEPS	V.H. Oswald & L. Ahart 9591	USA: CA: Modoc
155	<i>Marsilea oligospora</i> Goodd.	HQ631113	HQ631305	HQ631208	JEPS	V.H. Oswald & L. Ahart 9597	USA: CA: Modoc
156	<i>Marsilea vestita</i> Hook. & Grev.	HQ631114	HQ631306	HQ631209	JEPS	L. Ahart 13088	USA: CA: Butte
157	<i>Marsilea vestita</i> Hook. & Grev.	HQ631115	HQ631307	HQ631210	JEPS	L. Ahart 14483	USA: CA: Butte
158	<i>Marsilea vestita</i> Hook. & Grev.	HQ631116	HQ631308	HQ631211	JEPS	L. Ahart 14579	USA: CA: Yuba
159	<i>Marsilea vestita</i> Hook. & Grev.	HQ631117	HQ631309	HQ631212	JEPS	L. Ahart 9320	USA: CA: Plumas
160	<i>Marsilea vestita</i> Hook. & Grev.	HQ631118	HQ631310	HQ631213	UC	B. Ertter 9696	USA: CA: Amador
161	<i>Marsilea oligospora</i> Goodd.	HQ631119	HQ631311	HQ631214	UC	V.H. Oswald & L. Ahart 4837	USA: CA: Butte
162	<i>Marsilea oligospora</i> Goodd.	HQ631120	HQ631312	HQ631215	UC	V.H. Oswald & L. Ahart 5126	USA: CA: Lassen
165	<i>Marsilea ancylopoda</i> A. Braun	HQ631121	HQ631313	HQ631216	UC	R. B. Hayward et al. 56	Venezuela: Zulia
167	<i>Marsilea costulifera</i> D.L. Jones	HQ631122	HQ631314	HQ631217	UC	R.G. Coveny et al. 12653	Australia: NSW
169	<i>Marsilea ancylopoda</i> A. Braun	HQ631123	HQ631315	HQ631218	UC	D.M. Johnson 769	Argentina: Corrientes
170	<i>Marsilea ancylopoda</i> A. Braun	HQ631124	HQ631316	HQ631219	UC	E.J. Lott 3987 with A.C. Sanders	Mexico: Jalisco
171	<i>Marsilea vestita</i> Hook. & Grev.	HQ631125	HQ631317	HQ631220	UC	A.C. Sanders et al. 13527	Mexico: Sonora
172	<i>Marsilea quadrifolia</i> L.	HQ631126	HQ631318	HQ631221	UC	B. Dickore 13374	Pakistan
173	<i>Marsilea vestita</i> Hook. & Grev.	HQ631127	HQ631319	HQ631222	UC	A. Tiehm 12601	USA: NV: Washoe
174	<i>Marsilea ancylopoda</i> A. Braun	HQ631128	HQ631320	HQ631223	UC	M. Lehnert 745	Bolivia: Tarija
175	<i>Marsilea deflexa</i> A. Braun	HQ631129	HQ631321	HQ631224	UC	R. Rueda & R. Dolmus 1221	Nicaragua
176	<i>Marsilea vestita</i> Hook. & Grev.	HQ631130	HQ631322	HQ631225	CICY	J.L. Tapia et al. 1597	Mexico: Yucatan
177	<i>Marsilea ancylopoda</i> A. Braun	HQ631131	HQ631323	HQ631226	F (US)	S. Llatas & Quiroz 2401	Peru: San Nicolas
179	<i>Marsilea vestita</i> Hook. & Grev.	HQ631132	HQ631324	HQ631227	US	J. Pruski 3743	USA: LA: Orleans
182	<i>Marsilea vestita</i> Hook. & Grev.	HQ631133	HQ631325	HQ631228	US	H. van der Werff 8708	USA: CA: San Diego
186	<i>Marsilea exarata</i> A. Braun	HQ631134	HQ631326	HQ631229	US	A. Faden 1/91	Australia: QLD
187	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631135	HQ631327	HQ631230	QCA	Terneus & Gonzalez 347	Ecuador: Imbabura: Lago San Pablo
188	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631136	HQ631328	HQ631231	GH	R. Moran 28429	Mexico: Baja California
189	<i>Marsilea vestita</i> Hook. & Grev.	HQ631137	HQ631329	HQ631232	GH	P.H. Raven 16601	USA: CA: Stanislaus
190	<i>Marsilea vestita</i> Hook. & Grev.	HQ631138	HQ631330	HQ631233	GH	G. Carnevali et al. 6740	Mexico: Yucatan

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of				Voucher	Locality
		rbcL	trnL-F	rps4	deposition		
192	<i>Marsilea aencylopoda</i> A. Braun	HQ631139	HQ631331 na		GH	V. Solis Neffa 001	Argentina: Corrientes
194	<i>Marsilea tenuifolia</i> Engelm. ex Kunze	HQ631140	HQ631332	HQ631235	OWU	D.M. Johnson 2124	USA: TX: Mason
195	<i>Marsilea vestita</i> Hook. & Grev.	HQ631141	HQ631333	HQ631236	OWU	D.M. Johnson 2123	USA: LA: LaSalle Parish
196	<i>Marsilea aencylopoda</i> A. Braun	HQ631142	HQ631334	HQ631237	NY	N.A. Murray & D.M. Johnson 1458	Mexico: Nayarit
197	<i>Marsilea vestita</i> Hook. & Grev.	HQ631143	HQ631335	HQ631238	NY	W.T. Barker 1836	USA: KS: Kiowa
199	<i>Marsilea oligospora</i> Goodd.	HQ631144	HQ631336	HQ631239	NY	D. Lytjen 131	USA: OR: Malheur
200	<i>Marsilea vestita</i> Hook. & Grev.	HQ631145	HQ631337	HQ631240	NY	K. Thorne & S. Goodrich 3550	USA: UT: Uintah
201	<i>Marsilea vestita</i> Hook. & Grev.	HQ631146	HQ631338	HQ631241	NY	A. Tiehm 11766	USA: NV: Pershing
203	<i>Marsilea vestita</i> Hook. & Grev.	HQ631147	HQ631339	HQ631242	NY	N.H. & P.K. Holmgren 9585	USA: OR: Lake
204	<i>Marsilea polycarpa</i> Hook. & Grev.	HQ631148	HQ631340	HQ631243	NY	M. Boudrie & S. Gonzalez 4191	French Guyana
205	<i>Marsilea minuta</i> L.	HQ631149	HQ631341	HQ631244	NY	D.M. Johnson 798	Trinidad
207	<i>Marsilea exarata</i> A. Braun	HQ631150	HQ631342	HQ631245	NY	F.J. Badman 6949	Australia: SA
208	<i>Marsilea hirsuta</i> R. Br.	HQ631151	HQ631343	HQ631246	NY	C.R. Michell & D.S. Calliss 994	Australia: NT
211	<i>Marsilea crenulata</i> Desv.	HQ631152	HQ631344	HQ631247	NY	J.E. Madsen 6013	Burkina Faso: Oudalan
216	<i>Marsilea vestita</i> Hook. & Grev.	HQ631153	HQ631345	HQ631248	NY	L. Ahart 2376	USA: CA: Butte
217	<i>Marsilea vestita</i> Hook. & Grev.	HQ631154	HQ631346	HQ631249	NY	N.H. & P.K. Holmgren 14745	USA: NV: Elko
220	<i>Marsilea vestita</i> Hook. & Grev.	HQ631155	HQ631347	HQ631250	NY	R. Moran 28429	Mexico: BC
222	<i>Marsilea vestita</i> Hook. & Grev.	HQ631156	HQ631348	HQ631251	NY	Bisse et al. 32886	Cuba
224	<i>Marsilea uncinata</i> A. Braun	HQ631157	HQ631349	HQ631252	NY	S.W. Leonard et al. 8495	USA: FL: Franklin
225	<i>Marsilea vestita</i> Hook. & Grev.	HQ631158	HQ631350	HQ631253	NY	A. Tave 4957	USA: UT: Piute
227	<i>Marsilea mollis</i> B.L. Rob. & Fernald	HQ631159	HQ631351	HQ631254	NY	R. McVaugh 16917	Mexico: Jalisco
230	<i>Marsilea deflexa</i> A. Braun	HQ631160	HQ631352	HQ631255	NY	J.T. Mickel 2856	Costa Rica: Guanacaste
231	<i>Marsilea deflexa</i> A. Braun	HQ631161	HQ631353	HQ631256	NY	R. McVaugh 19287	Mexico: Nayarit
233	<i>Marsilea vestita</i> Hook. & Grev.	HQ631162	HQ631354	HQ631257	TX	W.R. Carr 25313	USA: TX: McMullen
234	<i>Marsilea vestita</i> Hook. & Grev.	HQ631163	HQ631355	HQ631258	TX	L.L. Hansen 5163	USA: TX: Corvell
235	<i>Marsilea macropoda</i> Engelm. ex A. Braun	HQ631164	HQ631356	HQ631259	TX	W.R. Carr 24546	USA: TX: Goliad
239	<i>Marsilea ephippiocarpa</i> Alston	HQ631165	HQ631357	HQ631260	AA	PRE 99559	Namibia

TABLE 1. Continued.

DNA sample number	Taxon	Herbarium of deposition				Voucher	Locality
		rbcl	trnL-F	rps4			
240	<i>Marsilea crenata</i> C. Presl	HQ631166	HQ631358	HQ631261	FLAS	Whitten 3765	Thailand
241	<i>Marsilea scalaripes</i> D.M. Johnson	HQ631167	HQ631359	HQ631262	FLAS	Whitten 3766	Thailand
242	<i>Marsilea vestita</i> Hook. & Grev.	HQ631168	HQ631360	HQ631263	MEXU	R.S. Felger 85-588	Mexico: Sonora
243	<i>Marsilea vestita</i> Hook. & Grev.	HQ631169	HQ631361	HQ631264	MEXU	R.S. Felger 01-746	Mexico: Sonora
244	<i>Marsilea vestita</i> Hook. & Grev.	HQ631170	HQ631362	HQ631265	MEXU	R.S. Felger 85-1118	Mexico: Sonora
256	<i>Marsilea aegyptica</i> Willd.	DQ643291	DQ643359	DQ536323	BM	Smith 3623	Namibia
257	<i>Marsilea ancylopoda</i> A. Braun	DQ643292	DQ643360	DQ536324	F	Pryor et al. 963	Puerto Rico
258	<i>Marsilea angustifolia</i> R. Br.	DQ643293	DQ643361	DQ536325	UC	Hoshizaki 1250	Australia
259	<i>Marsilea botryocarpa</i> Ballard	DQ643294	DQ643362	DQ536326	UC	Faden s.n.	Kenya
260	<i>Marsilea capensis</i> A. Braun	DQ643295	DQ643363	DQ536327	DUKE	Ramberg s.n.	Botswana
261	<i>Marsilea crenata</i> C. Presl	DQ643296	DQ643364	DQ536328	DUKE	Kato J-38	Indonesia
262	<i>Marsilea crenata</i> C. Presl	DQ643297	DQ643365	DQ536329	DUKE	Kato s.n.	Thailand
263	<i>Marsilea crotophora</i> D.M. Johnson	DQ643298	na	DQ536330	H	Ritter et al. 4561	Bolivia
264	<i>Marsilea distorta</i> A. Braun	na	na	DQ536331	BM	Kornas 6271	Nigeria
265	<i>Marsilea drummondii</i> A. Braun	DQ643299	DQ643366	DQ536332	UC	Hoshizaki 577	Australia
266	<i>Marsilea ephippiocarpa</i> Alston	DQ643300	na	na	UC	Chase 2255	Zimbabwe
267	<i>Marsilea fadeniana</i> Launert	DQ643301	DQ643367	DQ536333	US	Evans and Maikweki 55	Kenya
268	<i>Marsilea farinosa</i> Launert	DQ643302	DQ643368	DQ536334	US	Faden 70/902	Kenya
269	<i>Marsilea gibba</i> A. Braun	DQ643303	DQ643369	DQ536335	US	Faden and Ng'weno 87/33	Kenya
270	<i>Marsilea macrocarpa</i> C. Presl	DQ643304	DQ643370	DQ536336	UC	Hoshizaki 236	South Africa
271	<i>Marsilea macropoda</i> Engelm. ex A. Braun	DQ643305	DQ643371	DQ536337	DUKE	Hoshizaki 1458	USA: TX
272	<i>Marsilea minuta</i> L.	DQ643306	DQ643372	DQ536338	DUKE	Shimozono s.n.	Burma
273	<i>Marsilea minuta</i> L.	DQ643307	na	DQ536339	DUKE	Rajesh 87938	India
274	<i>Marsilea minuta</i> L.	DQ643308	DQ643373	DQ536340	DUKE	Hoshizaki 237	Africa
275	<i>Marsilea mollis</i> B.L. Rob. & Fernald	na	na	DQ536341	F	Johnson s.n.	Mexico
276	<i>Marsilea mutica</i> Mett.	DQ643309	DQ643374	DQ536342	DUKE	Hoshizaki 840	New Caledonia
277	<i>Marsilea nashii</i> Underw.	DQ643311	DQ643376	DQ536344	DUKE	Correll s.n.	West Indies
278	<i>Marsilea nashii</i> Underw.	DQ643310	DQ643375	DQ536343	F	Correll 46631	Turks & Caicos

TABLE 1. Continued.

DNA sample number	Taxon	DNA				Herbarium of deposition			Voucher	Locality
		rbcl	trnL-F	rps4						
279	<i>Marsilea nubica</i> var. <i>gymnocarpa</i> (A. Braun) Launert	DQ643312 na		DQ536345	BM	Smith 1988			Botswana	
280	<i>Marsilea nubica</i> var. <i>gymnocarpa</i> (A. Braun) Launert	DQ643313 na		DQ536346	BM	Kornas 6379			Nigeria	
281	<i>Marsilea oligospora</i> Goodd.	DQ643314	DQ643377	DQ536347	UC	Tiehm 13199			USA: NV	
282	<i>Marsilea polycarpa</i> Hook. & Grev.	DQ643315	DQ643378	DQ536348	DUKE	Pryer 960			Puerto Rico	
283	<i>Marsilea quadrifolia</i> L.	DQ643316	DQ643379	DQ536349	DUKE	Anno s.n.			Japan	
284	<i>Marsilea schelpeana</i> Launert	DQ643317	DQ643380	DQ536350	DUKE	Honshizaki 742			South Africa	
285	<i>Marsilea vera</i> Launert	DQ643318 na		DQ536351	BM	Burrows 3716			Botswana	
286	<i>Marsilea vestita</i> Hook. & Grev.	DQ643319	DQ643381	DQ536352	US	Howell 47460			USA: CA	
287	<i>Marsilea villifolia</i> Brem. & Oberm. ex Alston & Schelpe	DQ643320 na		DQ536353	BM	Hansen 3232			Botswana	
288	<i>Marsilea villosa</i> Kaulf.	DQ643321	DQ643382	DQ536354	US	Degener 9049			USA: HI	
289	<i>Pilularia americana</i> A. Braun	DQ643288	DQ643383	DQ536355	DUKE	Pryer 978			USA: GA	

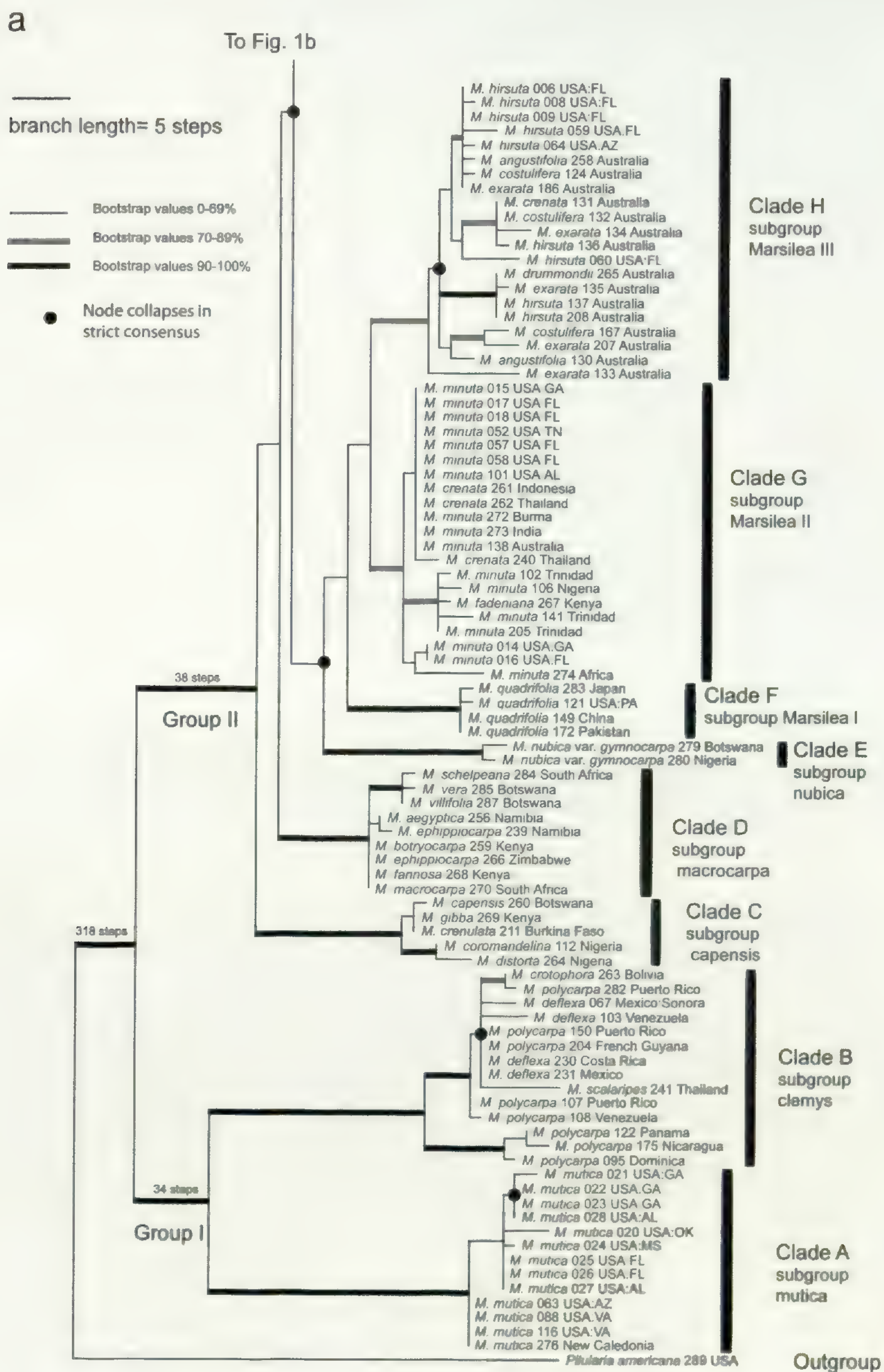


FIG. 1. (A-C). A single randomly-chosen shortest tree from maximum parsimony analysis of *Marsilea* combined plastid DNA data matrix (*rbcl*, *rps4*, *rps4-trnS* spacer, and *trnL-F* spacer). Branch lengths are indicated by scale bars (except for longer branches of Fig. 1a); bootstrap support is indicated by branch thickness/grayscale. Nodes that collapse in the strict consensus are marked with a black dot. Tree length = 743; consistency index (CI) = 0.80; retention index (RI) = 0.96. Major clades are labeled A-L; the informal clade names (groups and subgroups) correspond to those used in Nagalingum *et al.* (2007).

b

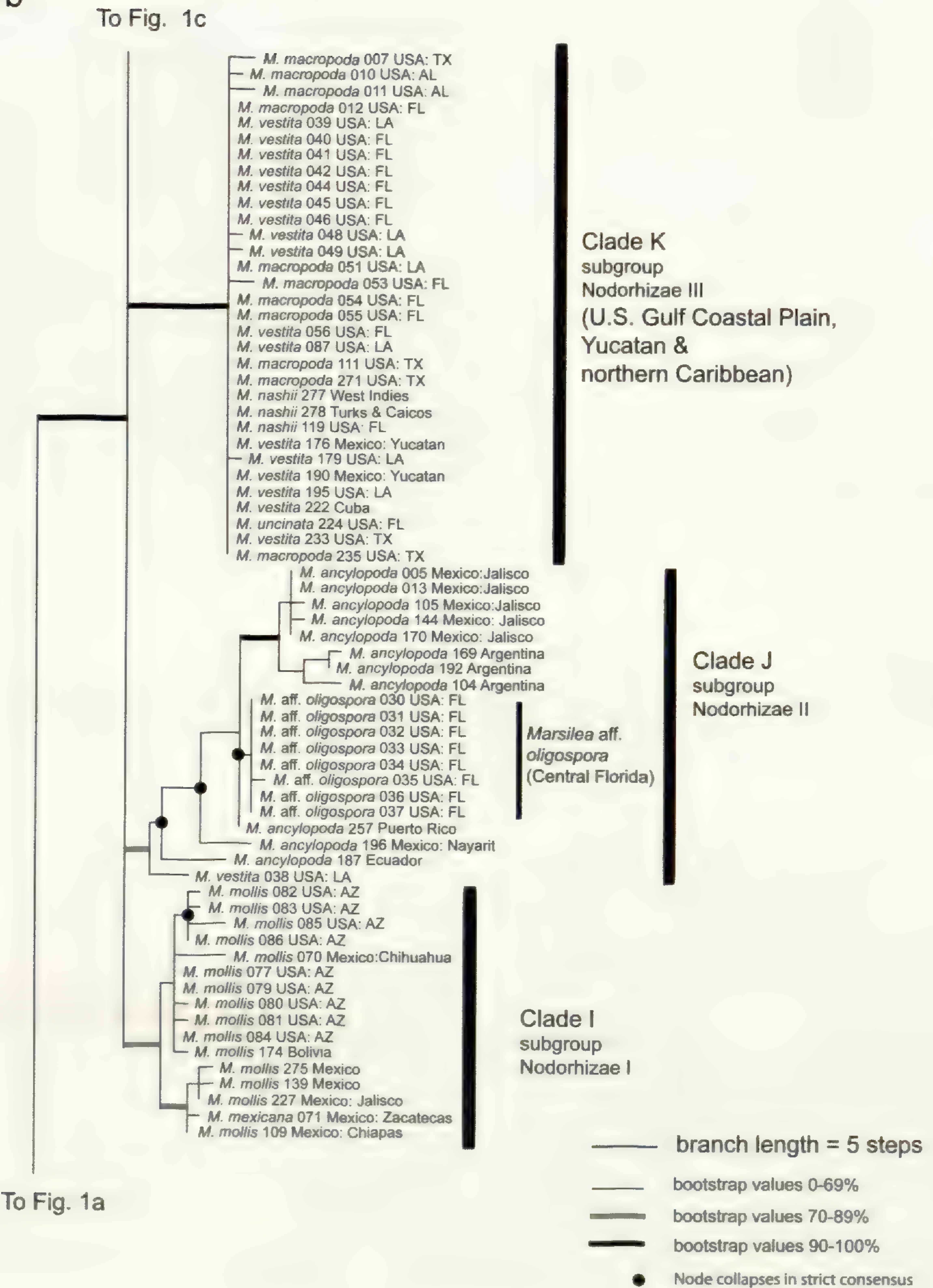


FIG. 1 Continued.

pieces, facilitating amplification from degraded total DNAs. Primers for *rbcL* are: *rbcLF* ATGTCACCACAAACAGAGACTAAAGC; *rbcL* intF TGAGAACG-TAAACTCCCAACCATTCA; *rbcL* intR CTGTCTATCGATAACAGCATGCAT; and *rbcLR* GCAGCAGCTAGTTCCGGGCTCCA. The *rps4* exon and the adjacent

C



FIG. 1 Continued.

rps4-trnS spacer were amplified in one piece using the primers *rps4F* ATGTCCCGTTATCGAGGACCT and *rps4R* TACCGAGGGTTCGAATC; problematic samples were amplified in two pieces using the internal primers *rps4* intF TGCCAAACGAGAATCTATGG and *rps4* intR CGATGGGTTGT-TAGTTGTTAG. Primers for the *trnL-F* spacer (primers E&F) were those of Nagalingum *et al.* (2007). All amplifications utilized Sigma Jumpstart *Taq* polymerase and reagents (Sigma-Aldrich, Inc., St. Louis, MO, USA) in 25 μ l reactions with 3.0 mM MgCl₂. Thermocycler conditions were: 94 °C for

3 minutes followed by 37 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, with a final extension of 3 min at 72 °C. Problematic taxa were amplified using Phusion polymerase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocols. PCR products were sequenced in both directions using the Big Dye Terminator reagents on an 3130 automated sequencer following manufacturer's protocols (Applied Biosystems, Inc.). Electropherograms were edited and assembled using Sequencher 4.10 (GeneCodes Inc., Ann Arbor, MI, USA), and the resulting sequences were aligned manually using SE-AL (Rambaut, 1996). All sequences were deposited in GenBank (Table 1). A 25 bp portion of the *rps4-trnS* spacer contained a homopolymer region of ambiguous alignment; this region was excluded from analyses. We analyzed the data using maximum parsimony rather than maximum likelihood because the number of steps in the resulting trees more clearly represents the number of base pair differences among accessions. Analyses were performed using PAUP* version 4.0 b10 (Swofford, 2003) with Fitch parsimony (equal weights, unordered characters, ACCTRAN optimization and gaps treated as missing data). Heuristic searches consisted of 1000 random taxon addition replicates of subtree-pruning-regrafting (SPR) and "keeping multiple trees" (MULTREES) with the number of trees limited to 10 per replicate to minimize extensive swapping on islands with many suboptimal trees; 10,000 shortest trees were saved. Support was estimated by 1000 bootstrap (BS) replicates, saving only 5 trees per replicate and ten trees per bootstrap replicate. The data matrix is available from the senior author or at <ftp://ftp.flmnh.ufl.edu/Public/Marsilea/>.

RESULTS

In total, our dataset comprised 2629 characters for a total of 223 ingroup accessions, plus *Pilularia americana* A.Braun. We used existing sequence data for 33 accessions from 26 species and newly sequenced data for an additional 190 accessions from 12 species (Table 1).

Figure 1 (a, b, c) presents a single randomly-chosen maximum parsimony (MP) phylogram out of 10,000 shortest trees saved. Tree length = 743; consistency index (CI) = 0.80; retention index (RI) = 0.96; ACCTRAN optimization. BS values are indicated by line thickness and shading of branches.

The DNA data revealed that several specimens sampled in this study were misdetermined (based upon their anomalous placement in the tree and reexamination of the voucher specimens). DNA data were especially effective in clarifying the identification of sterile specimens of both North American and introduced origin.

The cladogram is distinguished by a basal dichotomy separating two strongly supported clades, earlier designated informally as Groups I and II (Schneider and Pryer, 2001; Nagalingum *et al.*, 2007). Group I comprises informal subgroups "mutica"/A and "clemys"/B, and Group II includes subgroups "capensis", "macrocarpa", "nubica", "marsilea I–III", and "nodorhizae I–IV", here designated Clades C through H, respectively. Clades A

and C–H are Old World (Launert, 1968) and Clades A–G have glabrous leaves. Clade H includes hairy-leafed species from Australia. Clades I, J, K, and L are New World, have hairy leaves typical of the semi-aquatic ecotype, and include the majority of the specimens sequenced in this study. These latter four clades are united by high BS support into a single clade that corresponds to Johnson's *Marsilea* sect. *Nodorhizae* (Johnson, 1986; Nagalingum *et al.*, 2007), which includes six species (plus many names that Johnson synonymized).

Clade A is monotypic, consisting only of *M. mutica* Mett. It is clearly distinct from all other taxa in terms of DNA sequence and morphology, with its two-toned leaflets and petioles inflated at the apex to function as air bladders for floating leaves. This species has elliptical sporocarps that lack a transverse vein, are borne at the base of the petiole, and are either solitary or in clusters of 2–4 on branched pedicels. Indigenous to Australia and New Caledonia, *M. mutica* may be the most popular species in the water garden trade. The southeastern U.S. specimens plus one from Oklahoma are genetically distinct from specimens from Arizona and Virginia, a result suggestive of at least two distinct geographic origins for material introduced into the U.S.

Clade B includes several species that share the distinctive feature of linear rows of globose sporocarps borne on the petiole and a transverse sporocarp veining; this clade corresponds to *Marsilea* sect. *Clemys* (Johnson, 1986, 1988). The inclusion of *M. scalaripes* and *M. deflexa* in this clade confirms their hypothesized placement in the *clemys* subgroup (Nagalingum *et al.*, 2007). However, these plastid data do not resolve the sampled taxa into monophyletic species. There are two well-supported (between 90–100% BS) clades, both of which include samples of *M. polycarpa* Hook. & Grev. and *M. deflexa*. The non-monophyly of species in this clade and Johnson's (1986) putative designation of hybrids of these species may warrant a reexamination of determinations of these specimens and/or species concepts. Sample #175 from Nicaragua is sterile and its determination as *M. deflexa* is tentative.

Clade C contains five African species: *M. capensis* A.Braun, *M. gibba* A.Braun, *M. crenulata* Desv., *M. distorta*, and *M. coromandelina*, which as described by Launert (1968) are all of the glabrous leaflet type. Although this clade is strongly supported (100% BS), the plastid data fail to fully resolve relationships among these species.

Clade D contains eight African species: *M. schelpeana* Launert, *M. aegyptica* Willd., *M. botryocarpa* Ballard, *M. ephippiocarpa* Alston, *M. farinosa* Launert, and *M. macrocarpa* C.Presl, and partial plastid data also place *M. vera* Launert and *M. villifolia* Brem. & Oberm. ex Alston & Schelpe in this clade. In contrast to Clade C, all eight species of Clade D are of the hairy leaflet type (Launert, 1968).

Clade E consists of two samples of *M. nubica* A.Braun, a glabrous species from Africa that forms abundant colonies (Launert, 1968).

Clade F consists entirely of *M. quadrifolia* L., the type species of the genus, the only glabrous species from a cool-temperate climate, and a protected species in Europe. Four accessions from different continents, both native and introduced in range, display little sequence variation.

Clade G is moderately supported (84% BS) and includes a single accession of the African species *M. fadeniana* Launert, several Asian accessions of *M. crenata* C.Presl, and numerous accessions of *M. minuta* L., including several from introduced populations in the southeastern U.S. and Trinidad. The *M. crenata* – *M. minuta* complex is one of the largest and most variable groups within the genus (Launert, 1968). Earlier molecular data showed that *M. crenata* was nested within *M. minuta* (Nagalingum *et al.*, 2007), and the addition of more accessions provides additional evidence that the two taxa are likely conspecific. Three samples from Trinidad (introduced) form a moderately supported clade with samples from Kenya and Nigeria. A single accession (#138) originally determined as *M. hirsuta* was probably misdetermined, but was not available for examination.

Clade H includes Australian hairy-leaved species: *M. drummondii* A.Braun, *M. exarata*, *M. hirsuta* R.Br., *M. angustifolia* R.Br., and *M. costulifera*. There are several subclades resolved, but only one has high (90–100%) bootstrap support. None of the species within this clade are resolved as monophyletic. DNA data fail to distinguish *M. hirsuta* from *M. angustifolia*. Morphologically, *M. angustifolia* differs from *M. hirsuta* in having smaller and more elongated leaves and smaller sporocarps (Aston, 1973). These characters, however, are typically considered insufficient for species distinction within the genus (Launert, 1968). This clade includes a single specimen (#131) determined as *M. crenata*; it is probably misdetermined, as all other specimens of *M. crenata* fall in Clade G.

The majority of the specimens sampled are in Clades I, J, K, and L; these form a highly supported group that include all species native to North and South America. Species within each clade are poorly resolved due to low sequence divergence. Both clades K and L include members of a complex of mainly North American species related to *M. vestita* Hook. & Grev. and *M. oligospora*. Although they receive moderate to high bootstrap support, clades K and L correlate strongly with geographic origin (K=U.S. Gulf coastal plain, Yucatan, Mexico, and the northern Caribbean; L= Mexico, western U.S., and Hawaii), but not with accepted species concepts.

Clade I consists primarily of accessions of *M. mollis* B.L.Rob. & Fernald from north central Mexico, Arizona, and one from Bolivia. One specimen from Zacatecas, Mexico is determined as *M. mexicana*; the molecular data do not distinguish it from *M. mollis*.

Clade J has partially resolved but unsupported internal structure and includes *M. aff. oligospora* from Florida, *M. ancylopoda* from west-central Mexico, Puerto Rico and northeastern Argentina, plus one sterile sample (#187) originally determined as *M. mollis* from Andean Ecuador (Lago San Pablo). Johnson (1986) cited three sterile collections of *M. mollis* from this same lake and suggested that many sterile Andean collections above 1500 m are probably referable to *M. mollis*. Our molecular data indicate these Ecuadorian collections are not *M. mollis*, but instead belong to this clade that includes *M. ancylopoda*.

Sample #38 (*M. vestita* from Louisiana) is sister to all other taxa in this clade in the strict consensus of all trees; its anomalous placement caused us to resequence this specimen, but the second sequence was identical to the first.

Clade K includes specimens of *M. vestita*, *M. macropoda*, and one of *M. uncinata* from the Gulf coastal plain of the southeastern U.S., together with several accessions of *M. nashii* from Yucatan and the northern Caribbean. Johnson (1986) regarded *M. uncinata* as a synonym of *M. vestita*, but considered *M. nashii* to be a valid species distinguished by its strongly nodding sporocarps (vs. slightly nodding to ascending in *M. vestita*), a feature which we have found to vary greatly across and within species, presumably in response to the microenvironment under which sporocarps develop. The molecular data provide no resolution within this clade.

Clade L consists mostly of specimens of *M. vestita* and *M. oligospora* from central Texas through the western United States and northwestern Mexico, plus a specimen of *M. mucronata* A.Braun from California, which Johnson (1986) regarded as a synonym of *M. vestita*. It also includes two specimens of *M. ancylopoda* from Venezuela and Peru, but they are not resolved as sister taxa. The clade also includes several accessions of *M. villosa* Kaulf., an endangered Hawaiian endemic, which form a weakly supported clade with *M. vestita* and *M. fournieri*, both from Baja California, Mexico. Johnson (1986) considered *M. fournieri* C.Chr. to be a small-leaved form of *M. vestita*. This tree is consistent with the hypothesis that *M. villosa* arose via long-distance dispersal of *M. vestita* from western Mexico to the dry lowlands of Moloka'i, Ni'ihau, and O'ahu where seasonal flooding of shallow depressions offers restricted habitats (Wester, 1994). This clade also includes samples of *M. oligospora* from northern California and Idaho; the type locality of this species is in Wyoming (see discussion of *M. aff. oligospora* in Florida in clade J). The ten samples of *M. oligospora* are not monophyletic and are scattered throughout this clade, but without resolution or support.

DISCUSSION

Evaluation of Florida *Marsilea* aff. *oligospora*

Based on our phylogenetic trees, the eight accessions of *M. aff. oligospora* from central Florida (samples 30–37) fall within Clade J; these plants form a weakly supported clade distinct from all others and are sister to *M. ancylopoda* from Mexico and Argentina. These eight plants also share a four basepair insertion in the *trnL-F* spacer that is absent in all other *Marsilea*; this indel is an unambiguous synapomorphy that distinguishes these Florida plants. Jacono and Johnson (2006) tentatively identified these populations as *M. aff. oligospora*, although noting subtle morphological differences from western U.S. *M. oligospora*, and they regarded the Florida populations as introductions from the western U.S. Our data contradict their hypothesis; “true” *M. oligospora* (e.g., samples 93 and 94, from near the type locality in the western U.S.; Jackson Hole, Wyoming) fall in clade L, and our data clearly distinguish

the Florida populations from all other taxa. The molecular data indicate that these Florida populations are nested within *M. ancylopoda* from Mexico, Puerto Rico, Argentina, and Ecuador (based on current sampling).

According to Johnson's (1986) morphological concept of *M. ancylopoda*, the species includes considerable variation in sporocarp morphology, but the sporocarps always lack a superior tooth. The Florida populations of *M. aff. oligospora* (sensu Jacono and Johnson, 2006) bear sporocarps with prominent tooth. The presence of toothed and toothless taxa together in Clade J indicate that this character may be homoplasious and may not provide reliable characters for diagnosis of species, at least within this species complex.

The data show that central Florida specimens of *M. aff. oligospora* (samples 30–37) are distinct from all other sampled *Marsilea* and might represent an undescribed species or a morphological and molecular variant of *M. ancylopoda*. We are unable to match them with *Marsilea* from any other geographic locality. Our sampling of the Caribbean, Central America, and northern South America is poor, and more extensive sampling might provide a match for the Florida populations. The type of *M. ancylopoda* is from coastal arid lowlands just north of the Gulf of Guayaquil, Ecuador. Future studies should include material from the type locality. Although our sampling does not include material from the type locality of *M. ancylopoda*, it does include Peruvian material from similar low-lying habitats along the arid west coastal strip of South America. This specimen (#177, *Llatas & Quiroz 2401*), is in Clade L where it groups weakly with *M. vestita* from the desert regions of New Mexico and Arizona. Additional sampling from low elevation neotropical localities is also needed to seek matches for *M. ancylopoda* from west-central Mexico and northeastern Argentina, as included in Clade J of this study. Until further sampling yields a match for the Florida plants, we suggest that the populations should be regarded as endemic and given protected status by vegetation managers until its status as native or alien is resolved more definitively.

Evaluation of Morphological Species Concepts in *Marsilea* Section *Nodorhizae*

These plastid data provide an independent dataset with which to evaluate morphological species concepts in *Marsilea*, especially for the North American species that were heavily sampled. The failure of the plastid data to resolve specimens into clades that correspond to morphospecies is most obvious in *Marsilea* sect. *Nodorhizae* (*M. oligospora*, *M. mollis*, *M. villosa*, *M. vestita*, *M. macropoda*, *M. nashii*, and *M. ancylopoda*). Instead, plastid data group these seven species into four distinct clades with strong geographic structure that correspond to climactic and habitat zones: Clade L includes western North American accessions from ephemeral ponds in arid climates; Clade K includes plants from humid, seasonally influenced low elevation floodplains and wet depressions of the Gulf coastal plain, Florida, and the northern Caribbean; Clade I consists only of *M. mollis* from Arizona to Bolivia; Clade J includes *M. ancylopoda* (from Mexico and Argentina), the central Florida material (*M. aff.*

ancylopoda) and nearby Puerto Rico, plus a geographically disparate accession from the montane highlands of north central Ecuador and an aberrant sterile specimen from Louisiana (#038).

The incongruence of these plastid trees and the currently accepted species of *Marsilea* may have several explanations, which we discuss below. Extensive hybridization among *Marsilea* species might have led to chloroplast capture of a single plastid type among many species resulting in plastid trees that do not accurately reflect phylogenetic relationships. Johnson (1986) cited several specimens as putative interspecific hybrids, based solely on interpretation of subtle morphological characters. To our knowledge, no one has created artificial *Marsilea* hybrids, nor used molecular data to demonstrate the parental origin of putative hybrids. Additionally, the non-monophyly of species may be due to incomplete lineage sorting. However, we did not examine the individual gene trees to determine if this could be the cause of non-monophyly.

The absence of monophyletic species may also be due to the presence of cryptic species. This is exemplified by our finding that the plants originally identified as *M. aff. oligospora* are a potentially undescribed species (see above). These plants display subtle morphological differences compared to all other known *Marsilea*, and molecular data indicate that they have a unique molecular signature as well. Therefore, it is possible that through more intense sampling and reassessment of morphology, the non-monophyletic species may reveal the presence of underlying cryptic species.

Through our analyses we discovered several accessions that were misidentified, and it is possible that some of polyphyletic species are due to identification errors. However, given the extent of polyphyletic species (and that many specimens were annotated by D.M. Johnson), we suggest that this is unlikely.

A final explanation for failure of the existing alpha-taxonomy could be an inflated number of species within *Marsilea* sect. *Nodorhizae* (clades J, K, L). Many species of *Marsilea* are based upon subtle morphological traits that are phenotypically plastic or that represent homoplasious local adaptations to environmental conditions. We found that plant size, leaflet size, extent of leaflet hairiness, the angle and extent of sporocarp nodding, and the curvature of the peduncle demonstrated variability that might preclude their taxonomic utility for species delimitation.

It was beyond the scope of this project to re-examine all of the specimens used in this study. However, we suggest that future work include re-examination of the morphology of multiple accessions within a phylogenetic framework to ascertain the reliability of the existing characters for species delimitation and to determine if cryptic species are present. There is also the need for more extensive sampling and sequencing of more variable plastid regions, to be contrasted with nuclear gene data sets, which will provide a better framework to settle questions of hybridization and incomplete lineage sorting as well as provide greater resolution and support for the relationship among species.

Conclusions

Using the extensively sampled phylogeny, we found that Florida plants earlier identified as *M. aff. oligospora* possess a unique molecular signature (an insertion in the *trnL-F* spacer) but morphological characters that distinguish it from other taxa in *M. sect. Nodorhizae* are subtle and require more detailed analyses (Jacono and Johnson, 2006). It is possible that these plants represent an undescribed, cryptic species endemic to Florida, or a geographically restricted variant of an existing species. Our plastid trees reveal the same major clades as the previous study by Nagalingum *et al.* (2007). Although our increased taxon sampling reveals no conflicts, many species are not resolved as monophyletic within these informally named clades. We were unable to determine if this is due to hybridization, incomplete lineage sorting, misidentification of specimens, the presence of cryptic species, and/or inappropriate morphological characters for species delimitation—the present data are inadequate to resolve these large taxonomic questions. We advise that the existing alpha-taxonomic classification and circumscription of species in *Marsilea*, especially *M. sect. Nodorhizae*, should be treated with caution.

ACKNOWLEDGMENTS

This work was funded by Florida Dept. of Environmental Protection Bureau of Invasive Plant Management Grant #6770 and by The St. Johns Water Management District Contract #74607. We thank curators of many herbaria for permission to destructively sample specimens; we are especially grateful to Kent Perkins (FLAS) for management of loans. We thank Marian Chau, Robert Gibson, and Siriporn Zungontiporn for specimens.

LITERATURE CITED

- ASTON, H. 1973. Aquatic Plants of Australia: A guide to the identification of the aquatic ferns and flowering plants of Australia, both native and naturalized. National Herbarium of Victoria, Melbourne University Press, Melbourne.
- DOYLE, J. J. and J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bull.* 19:11–15.
- (FNA) FLORA OF NORTH AMERICA EDITORIAL COMMITTEE (eds.). 1993. Flora of North America North of Mexico. Volume 2. Pteridophytes and Gymnosperms. Oxford University Press, New York.
- JACONO, C. C. and D. M. JOHNSON. 2006. Water-clover Ferns, *Marsilea*, in the southeastern United States. *Castanea* 71(1):1–14.
- JOHNSON, D. M. 1986. Systematics of the new world species of *Marsilea* (Marsileaceae). *Syst. Bot. Monographs* 11:1–87.
- JOHNSON, D. M. 1988. *Marsilea scalaripes*, a new member of *Marsilea* section *Clemys* from the Asian tropics. *Amer. Fern J.* 78:68–71.
- LAUNERT, E. 1968. A monographic survey of the genus *Marsilea* Linnaeus: I. The species of Africa and Madagascar. *Senckenbergiana Biologica* 49:273–315.
- NAGALINGUM, N. S., H. SCHNEIDER and K. M. PRYER. 2007. Molecular phylogenetic relationships and morphological evolution in the heterosporous fern genus *Marsilea*. *Syst. Bot.* 32:16–25.
- RAMBAUT, A. 1996. Se-AL: sequence alignment editor, Ver. 2.0a11. Website <http://tree.bio.ed.ac.uk/software/seal/> [accessed August 2010]
- SCHNEIDER, H. and K. M. PRYER. 2001. Structure and function of spores in the aquatic heterosporous fern family Marsileaceae. *Int. J. Plant Sci.* 163:485–505.

- SWOFFORD, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4 beta 10. Sinauer Associates, Sunderland, Massachusetts.
- WARD, D. B. and D. W. HALL. 1976. Re-introduction of *Marsilea vestita* into Florida. Amer. Fern J. 66:113–115.
- WESTER, L. 1994. Weed management and the habitat protection of rare species: a case study of the endemic Hawaiian fern, *Marsilea villosa*. Biol. Conserv. 68:1–9.

Effect of Habitat Modification on the Distribution of the Endangered Aquatic Fern *Ceratopteris pteridoides* (Parkeriaceae) in China

YUAN-HUO DONG*

Department of Biologic Technology, College of Life Sciences, Jiangnan University,
Wuhan 430056, China

State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography &
Limnology, Chinese Academy of Sciences, Nanjing 210008, China

QING-FENG WANG

Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China, email:
qfwang@wbcas.cn

ROBERT WAHITI GITURU

Botany Department, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-
00200, Nairobi, Kenya, email: wahitigituru@yahoo.com

ABSTRACT.—Sixteen sites in China where *Ceratopteris pteridoides* occurs based on historical records and/or from observations were surveyed during preliminary field surveys. Eight previously recorded populations were found to have been extirpated. Decline in natural populations of *C. pteridoides* has resulted from the destruction or complete loss of its primary habitat. Analysis of 17 parameters of water quality indicated that differences in pH and dissolved oxygen might be principal factors determining the distribution and occurrence of *C. pteridoides*. The sites of the extirpated populations had higher water pH values than those of the sites of the extant populations ($P < 0.05$). The value of dissolved oxygen concentration at the sites of the extirpated populations was lower than at the sites of the extant populations ($P < 0.05$). The degeneration of primary habitats, a decline in the area of wetland coverage and deterioration of water quality caused by human activities are identified as the likely key factors responsible for the reduction in *C. pteridoides* populations. Because the habitat and population characteristics of eleven remaining populations were different, different sites should adopt different conservation methods as appropriate. Some small populations could be conserved by establishing conservation areas; other relatively large populations could be conserved by establishing nature reserves.

KEY WORDS.—*Ceratopteris pteridoides*, endanger, habitat modification, distribution, conservation

Ceratopteris pteridoides (Hook.) Hieron. (Parkeriaceae), an annual diploid ($n = 39$), is an aquatic, homosporous, floating fern. The species displays clonal growth by means of numerous marginal leaf buds that rapidly develop into plantlets (Hickok *et al.*, 1987). Both spores and the plantlets from marginal leaf buds are dispersed mainly by water flow. *Ceratopteris pteridoides* mainly grows in ponds, lakes, rivers, and ditches (Lloyd, 1974). The species has principally been identified in Central and South America, Southeastern Asia, Eastern India, and China (Diao, 1990; Hickok *et al.*, 1995). In China, *C.*

* Corresponding author: E-mail: dongyh2008@yahoo.com.cn; Tel: +86-27-84230422

pteridoides is mainly distributed in central, eastern, and southern China (Diao, 1990). Although *C. pteridoides* was widely distributed in China, in recent decades the species has declined rapidly in the numbers and sizes of populations, and has even disappeared from many locations (Yu, 1999; Dong *et al.*, 2007). *Ceratopteris pteridoides* is now considered endangered and is listed in the second category of the National Key Protected Wild Plants in China (Yu, 1999). Several factors, including the degeneration of primary habitats, the decline in area of coverage of wetlands, and the deterioration of water quality due to human activities, have been identified as being responsible for the reduction in *C. pteridoides* populations (see Dong *et al.*, 2007). However, no data have been provided to support these assertions.

Earlier studies on *Ceratopteris pteridoides* have mainly focused on taxonomy and morphology (Hickok *et al.*, 1995; Fan and Dai, 1999; Carquist and Schneider, 2000). In recent years, Dong *et al.* (2007, 2010), using RAPD and ISSR data, revealed low levels of genetic diversity (the percentage of polymorphic bands (PPB): RAPD, 33.6%; ISSR, 25.2%) and high levels of gene flow between the remaining *C. pteridoides* populations in China. Tao *et al.* (2008) reported that bensulfuron-methyl inhibits gametophyte growth and sex organ differentiation of *C. pteridoides* at low concentration and may pose a risk to sexual reproduction of *C. pteridoides* in the field. However, the information available on the conservation biology of *C. pteridoides* in China is limited in comparison with that for the more widely studied closely related species *Ceratopteris thalictroides* (L.) Brongn. This latter species is now also considered to be endangered in China (Yu, 1999).

It is probable that additional studies on the various aspects of the conservation biology of *Ceratopteris pteridoides* will provide information that will justify more stringent conservation practices for this rare plant. The important objective in the present study was to report the current distribution of *C. pteridoides* in China, its habitats, and population characteristics. Another aim was to investigate the natural distribution of *C. pteridoides* in China in relation to habitat characteristics, especially wetland and water chemistry parameters, in order to contribute to the available information on the biology of this endangered species.

MATERIALS AND METHODS

From August of 2003 to October of 2006, sixteen sites throughout the historic geographic distribution of *Ceratopteris pteridoides* in China were investigated (Fig. 1). The sites were identified on the basis of records on labels of herbarium specimens and/or observations during field surveys. The sixteen sites surveyed are located in Hubei, Jiangxi, Anhui, Zhejiang, Jiangsu, Fujian and Shandong provinces in Mainland China. At each sampling station elevation, latitude, and longitude were measured by Global Positioning System (GPS). The habitat characteristics of *C. pteridoides* were recorded (Table 1). The population characteristics of the species including population numbers and

TABLE 1. Geographic distribution, habitat and populations characteristics of extant and extinct populations of *Ceratopteris pteridoidea* in China.

Location	Population code	Extant/ extinct population	Elevation (m)	Latitude(N)/ Longitude(E)		Habitats	Population area (m ²)		Population size
				Latitude(N)	Longitude(E)		Population area (m ²)	Population size	
Xiaogang, Hubei province	HB-1	Extant	24	29°55'	113°13'	Ditch, Fishpond	700-800	6000-10000	
Jiayu, Hubei province	HB-2	Extant	19	29°54'	114°01'	Lake	40-50	200-500	
Liangzihu, Hubei province	HB-3	Extant	15	30°15'	114°34'	Lake	400-500	10000-15000	
Yangxin, Hubei province	HB-4	Extant	14	29°55'	113°59'	Fishpond	1800-2000	50000-60000	
Jiayu, Hubei province	HB-5	Extant	19	29°55'	113°59'	Pond	30-50	500-1000	
Ruichang, Jiangxi province	JX-1	Extant	21	29°48'	115°44'	Lake	200-300	20-50	
Huzhou, Zhejiang province	ZJ-1	Extant	-	30°51'	120°04'	Wetland, pond	40-50	500-1000	
Wuyishan, Fujian province	FJ-1	Extant	-	27°46'	118°01'	Ditch	3-5	3-5	
Nansihu, Shandong province	SD-1	Extinct	10	34°40'	117°15'	Lake			
Taibaihu, Hubei province	HB-6	Extinct	14	29°58'	115°37'	Lake			
Haikouhu, Hubei province	HB-7	Extinct	18	30°02'	115°17'	Lake			
Honghu, Hubei province	HB-8	Extinct	24	29°55'	113°13'	Lake			
Changhu, Hubei province	HB-9	Extinct	19	30°44'	112°12'	Lake			
Mulanhu, Hubei province	HB-10	Extinct	-	31°06'	112°12'	Reservoir			
Futouhu, Hubei province	HB-11	Extinct	20	30°01'	114°17'	Lake			
Dongliu, Anhui province	AH-1	Extinct	16	30°12'	116°54'	Lake lost			

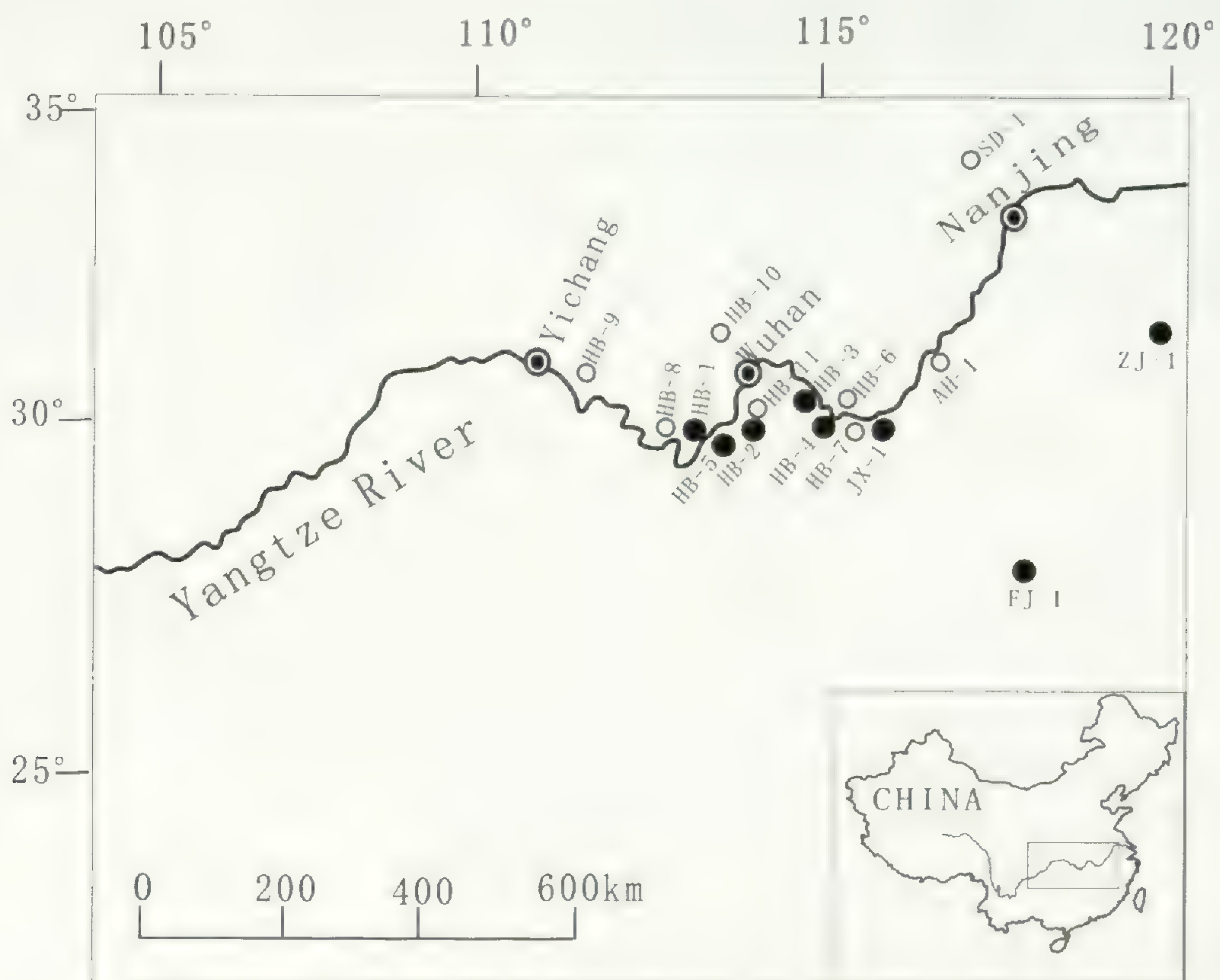


FIG. 1. Distribution map and water sampling sites of *Ceratopteris pteridoides* in China. ● Sites of extant populations. ○ Sites extirpated populations. Eleven water sampling sites including HB-1, HB-2, HB-3, HB-4, HB-6, HB-7, HB-9, HB-10, HB-11, JX-1, and SD-1. Codes and names of populations see Table 1.

population area, population sizes (numbers of individuals), and companion species were investigated (Table 1).

Eleven sites of the sixteen sites were investigated in 2003. Thus, in order to decrease statistical error, the water chemistry parameters of only the 11 sites surveyed from August to September of 2003 were analyzed (Fig 1). Five sites which still have populations of *Ceratopteris pteridoides* were designated as type A sites while six sites from which the species has been extirpated were designated as type B sites (Table 2). Seventeen water parameters were measured at seventeen sites (Table 2). The chemical parameters, including $\text{NO}_3\text{-N}$, $\text{NH}_3\text{-N}$, PO_4^{3-} , total Cl, Ca, Mg, Fe, Cu, Zn, Mo, and Cr^{6+} , were measured from the sample with a multi-parameter ion-specific photometer (C200, Hanna Co., Italy) in the laboratory using a 500 ml water sample collected in a plastic bottle in eleven sites respectively.

pH was measured with a portable meter (HI 98107, Hanna Co., Italy), as was conductivity (HI 983004, Hanna Co., Italy). Dissolved oxygen, dissolved carbon dioxide, alkalinity, and hardness were measured in the field with a portable test kit (HI 3814, Hanna Co., Italy). The water temperature of the water sample sites were about 25–30°C from August to September.

The mean values of the 17 chemistry parameters of water at the type A and type B sites were compared statistically using a one-way ANOVA when the

TABLE 2. Significance tests of water chemical parameters at the sample sites in 2003. Significance level for all comparisons: * $P < 0.05$; Type A: Sites of extant populations; Type B: Sites extirpated populations.

Parameters	Type A ($\bar{X} \pm \text{sd } n=5$)	Type B ($\bar{X} \pm \text{sd } n = 6$)	Statistical Tests	
			One-way ANOVA (F-test)	Rank sum test (U-value)
Cl (mg/L)	0.23 ± 0.03	0.24 ± 0.07	0.02	—
Cu (mg/L)	0.27 ± 0.11	0.45 ± 0.27	1.93	—
Ca (mg/L)	0.17 ± 0.22	0.20 ± 0.20	0.07	—
Mg (mg/L)	0.38 ± 0.16	0.40 ± 0.17	0.03	—
Zn (mg/L)	0.11 ± 0.11	0.20 ± 0.33	0.28	—
Mo (mg/L)	1.28 ± 1.32	1.31 ± 0.95	0.002	—
PO ₄ ³⁻ (mg/L)	0.26 ± 0.25	0.56 ± 0.34	2.68	—
Dissolved carbon dioxide (mg/L)	22.60 ± 9.71	24.83 ± 13.67	0.09	—
pH	7.54 ± 0.22	7.97 ± 0.27	7.90*	—
Dissolved oxygen (mg/L)	7.34 ± 1.11	5.05 ± 1.15	11.57*	—
Alkalinity (mgCaCO ₃ /L)	97.80 ± 28.11	109.50 ± 50.33	0.21	—
Hardness (mgCaCO ₃ /L)	93.00 ± 26.07	136.00 ± 79.41	—	25.00
NH ₃ -N (mg/L)	0.12 ± 0.04	0.19 ± 0.14	—	24.50
Fe (mg/L)	0.28 ± 0.45	0.06 ± 0.08	—	32.34
Cr ⁶⁺ (ug/L)	2.80 ± 6.26	9.83 ± 23.12	—	28.00
NO ₃ -N (mg/L)	0.60 ± 0.82	0.19 ± 0.24	—	32.00
Conductivity (ms/cm)	0.22 ± 0.08	0.36 ± 0.27	—	26.00

variance was homoscedastic and using a rank sum test when the variance was heteroscedastic. The homogeneity of variance of all factors was assessed using the Bartlett test (Li, 2002). Any factors that showed significant difference between the type A and type B sites were evaluated further by analysis of multiple comparison using Least Significant Difference. Level of significance was set at $P < 0.05$.

RESULTS

A total of eight extant populations of *Ceratopteris pteridoides* were found in the 16 sites surveyed from August of 2003 to October of 2006 across the natural geographic distribution range of *C. pteridoides* in Mainland China (Fig. 1, Table 1). These extant populations of *C. pteridoides* were found growing in ponds, lakes, rivers, and ditches. These extant populations are located mainly in the middle and lower reaches of the Yangtze River, which is also the site of thousands of shallow lakes most of which are interconnected to the main artery of the Yangtze River. With the exception of five populations (Jx-1, FJ-1, HB-2, ZJ-1 and HB-5) that had fewer than 1000 individuals, the rest of the populations (HB-1, HB-3, and HB-4) had more than 1000 *C. pteridoides* individuals per population. Most of the individuals were floating. *Ceratopteris pteridoides* mainly grows together with *Nelumbo nucifera* Gaertn., *Hydrocharis dubia* (Blume.) Backer., *Phragmites communis* Trin., *Alternanthera philoxeroides* (Mart.) Griseb., *Phalaris arundinacea* Linn., *Trapa bispinosa*

Roxb., and *Potamogeton distinctus* A. Benn. *Ceratopteris pteridoides* was the dominant species in three of the extant populations including (HB-1, HB-4, HB-5). The habitats at the sites of the extinct populations of *C. pteridoides* have been greatly modified. At some stations, including at the Nansi, Taibai, Haikou, Honghu, and Changhu Lakes, water pollution was clearly evident, while some lakes and wetlands including Dongliu Lake (AH-1 population) in Anhui province have vanished (Table 1). Area of coverage of wetland in site of ZJ-1 population was reduced due to uncontrolled real estate development.

Analysis of the 17 parameters of water quality indicated that type B sites had significantly higher mean pH ($P < 0.05$) and lower dissolved oxygen ($P < 0.05$) than type A sites (Table 2). None of the remaining chemical variances differed significantly between type A sites and type B sites.

DISCUSSION

Various kinds of human activities can bring about changes of wetlands and aquatic habitats (Carrier, 1991). Loss of habitat is the single most important cause of extinction of species (Primack, 1993). The accelerated loss of habitat of *Ceratopteris pteridoides* in China, together with a decline in the wetland surface area might have put the species at the risk of becoming extirpated in this expansive region and resulted in the extinction of *C. pteridoides* population at some sites. In recent decades, *C. pteridoides* in Mainland China have declined rapidly in number of individuals and populations, and plants have disappeared from many locations. For instance, loss of Dongliu Lake, which was part of the Yangtze River system in Anhui province undoubtedly led to the disappearance of this species from the site. Excessive aquaculture and water pollution have been identified as the most likely cause for the extinction of *C. pteridoides* at Haikou, Taibai, and Honghu Lakes (Jian *et al.*, 2001; Lu and Jiang, 2003).

Five of the eight extant populations of *Ceratopteris pteridoides* in Hubei province of central China are located in an area which was occupied by a large wetland known as the Yunmeng marshland in ancient times. In 239 B.C. the Yunmeng marshland was reputed to have a surface area spanning more than six million ha and plants of the genus *Ceratopteris* were recorded there (Liu, 1984; Shi *et al.*, 1989; Diao, 1990). Over a period in excess of two thousands years, the surface coverage of the wetland has continued to decline at different rates in different historical periods, mainly due to overexploitation, irrigation and tourism activities. As a result, the number of lakes in Hubei Province, which is known in China as "The province of a thousand lakes", decreased from 305 in the 1950s to 217 currently. Compared with the 1950s, the total area of lakes in Hubei Province was reduced by 66% to 2438.6 km² at present (Wang *et al.*, 2009). In addition, due to overexploitation and uncontrolled real estate development (Wan *et al.*, 2004), the number of urban lakes in Wuhan of Hubei Province was reduced from 89 in 1949 to 38, within which *C. pteridoides* only exists in one lake (HB-3 population) in this study (Table 1). Our own field investigations also have indicated that population ZJ-1 is in imminent danger of extirpation due to rapid expansion of Huzhou city. The current body of evidence

gathered in several studies, including our present study, shows that the progressive reduction in the numbers and sizes of *C. pteridoides* populations is likely attributable to sedimentation from the upper reaches of the Yangtze River (Shi *et al.*, 1989) and to human activities including farming, excessive aquaculture, overexploitation of water bodies, building of irrigations works, reclamation of land from lakes, tourism activities, uncontrolled real estate development and run-off water pollution (Shi *et al.*, 1989; Jian *et al.*, 2001; Lu and Jiang, 2003; Dong *et al.*, 2007).

Potamic and lacustrine water chemical properties are among the principal factors determining the kind, number, and distribution of aquatic plants (Shi *et al.*, 1989; Yang and Ye, 2001; Liu *et al.*, 2003). Hydrobiological communities are greatly threatened by water pollution (Moyle and leidy, 1992). Analysis of 17 parameters of water quality indicated that type B sites had significantly higher mean pH ($P < 0.05$) than type A sites (Table 2) in 2003. The mean pH of type A was 7.16 ± 0.49 in August of 2011, and lower than that of type B sites, which was consistent with the results in 2003. At the same stations, some sites of extant populations had higher pH values than the recorded primary habitat indicated on the labels of herbarium specimens. For example, the mean water pH value at Haikou Lake site has risen from 6.3 recorded in 2001 to 7.9 (Jian *et al.*, 2001). While the pH value at both type A and Type B sites indicated a progressive rise in the recent past, the proportional increase is markedly higher for the type B sites. Significant variability of pH at different sites might be largely due to different levels of pollution at the sites. Excessive aquaculture and land usage (Ivahanenko *et al.*, 1988; Downey *et al.*, 1994) could have a profound influence on pH variation in wetlands.

Significant changes in water pH may interfere with physiological activities of aquatic plants (Tang *et al.*, 2002). Water pH affects the bioavailability of Ca, Fe, Mn and Zn to rooted aquatic macrophytes (Jackson *et al.*, 1993). Furthermore, variation of pH may upset the subtle ionic balance in the environment; For example, pH variability affects the ionic balance between ammonium and ammonia in water (Körner *et al.*, 2001). pH variation has been shown to be the principal factor affecting the distribution of *Ceratopteris thalictroides*, a closely related species which is also endangered in China (Dong *et al.*, 2005), as well as the endangered aquatic fern *Isoetes sinensis* Palmer (Wen *et al.*, 2003). Considering that pH may influence the absorption of ions and metabolic activities of aquatic plants including *C. pteridoides*, it could be an important factor leading to extirpation of this endangered species from its habitats. This finding suggests that change in pH may be associated with the disappearance of *C. pteridoides* populations from the type B sites.

The different tests of water chemical parameters of these sample sites indicated that type B sites had significantly lower dissolved oxygen ($P < 0.05$) than type A sites (Table 2). Several studies, including Yang *et al.* (2001) and Shi *et al.* (1989), have demonstrated that dissolved oxygen is a principal factor that affects the distribution of aquatic plants. The absorption of minerals by plant roots is closely related to respiration in plants. Variation in oxygen availability influences absorbability of minerals by the roots. Generally

speaking, the higher the oxygen concentration, the more efficient the absorption of minerals by roots. *Ceratopteris pteridoides* is primarily a floating plant in lakes and ponds and absorbs minerals chiefly through its roots. It is probable that the lower concentrations of dissolved oxygen at these sites of extinct populations affects the intake of oxygen and consequently respiration in *C. pteridoides* plants, thus interfering with the physiological activities of the plants. This could be detrimental to the health of the populations and may have contributed to the extirpation of the species from these sites. Therefore, the significant differences in pH and dissolved oxygen between type A and type B sites indicated that distribution and occurrence of *C. pteridoides* are closely correlated with water chemical characteristics.

Biodiversity has declined in freshwater lakes in China in recent times (Jian *et al.*, 2001). The factors leading to biodiversity reduction mainly include water pollution and excessive aquaculture, with the latter having been identified as the single most important factor (Jian *et al.*, 2001). At the sites of the eight extinct populations at Taibai, Haikou, Honghu and Changhu Lakes, observations and interviews with the locals revealed a long history of intensive aquaculture. It is likely that this is a major reason for the decline of *Ceratopteris pteridoides* at these sites and elsewhere in China. Field surveys also showed that fishing activities by local fishermen destroyed *C. pteridoides* in the Yangxin (HB-4) and Jiayu (HB-5) populations in Hubei province, which compromised the self-maintenance and self-renewal abilities of the populations, leading to gradual decline of the populations.

Five populations among the eight extant populations of *Ceratopteris pteridoides* were relatively small with fewer than 1000 individuations (Table 1). Most of the eight extant *C. pteridoides* populations grew in ponds, ditches, shallow lakes, and kaleyards, while others (ZJ-1, HB-3, and HB-5) were located among city regions that have undergone rapid expansion. Such populations can be highly susceptible to the effects of environmental changes and disasters, which heighten the possibility of species extirpation.

We have shown that the distribution and occurrence of *Ceratopteris pteridoides* are correlated with water chemistry, with pH value and dissolved oxygen being the most important factors. Evidence from the present study supports the idea that the observed decline in *C. pteridoides* populations is associated with the destruction and the loss of their primary habitats, especially the reduction in wetland areas and the increase in water pollution. Human activities such as farming, tourism, real estate development, fisheries, and run-off water pollution are the most important reasons. It is of critical importance that measures are taken to establish appropriate conservation strategies to stem and even reverse the decline in populations of *C. pteridoides* observed in mainland China.

Both *in situ* and *ex situ* conservation approaches are important conservation strategies for rare and endangered species. However, the most appropriate conservation strategy is to protect the habitats of the species (Primack 1993). Because habitat and population characteristics of the remaining populations are different, a uniform approach may not apply for all sites, and different sites should adopt different conservation methods as appropriate. Some small

populations such as Jx-1, FJ-1, HB-2, ZJ-1, and HB-5 could be better conserved by establishing small, protected areas. Other relatively large populations, such as HB-1, HB-3, HB-4 should be conserved by establishing nature reserves. At the same time, there should be a government policy implementing complete cessation of farming activities in selected areas to allow forest to regenerate.

In recent years, in order to protect natural species and renew lake habitat, some government policies have been implemented at Xilianghu Lake (HB-2), Liangzihu Lake (HB-3) in Hubei province, including a complete cessation of farming activities, purse seine cultures, and fill-up of urban lakes. The HB-3 population, located at Wuhan, Hubei province, could be better conserved by actualizing local projects such as the expansion of lake areas and the connection of the surrounding lakes. These policies and measures could be a good approach towards improving the lacustrine ecosystem and protecting threatened aquatic life forms including *Ceratopteris pteridoides*. At the same time, in order to better protect threatened aquatic species, we advise establishing a list of lakes for conservation in China, and taking effective and long-term population and community characteristic measurements of these lakes, as well as monitoring the changes in water chemical parameters.

Botanical gardens have played an increasingly important role in the *ex situ* conservation of rare and endangered plants (Maunder, 1994). Wuhan Botanical Garden (WBG), is ranked as one of three core research botanical gardens in China. It houses the largest aquatic plant garden in East Asia, and has theme gardens such as Central China relicts, and a rare and endangered plant garden. WBG currently plays an important role in the conservation of aquatic species. The botanical garden has shown preliminary success in the conservation of *Ceratopteris* including *C. pteridoides* and *C. thalictroides*. *Ceratopteris pteridoides* was mainly protected at a pond in a zone of aquatic plants. The species in WBG mainly grows together with *Nelumbo nucifera* Gaertn., *Hydrocleys nymphoides* (Willd.) Buchenau., *Potamogeton lucens* Linn., *Triarrhena sacchariflora* (Maxin.) Nakai., *Pistia stratiotes* L., and *Vallisneria spiralis* (L.) Hara.

A key aim of conservation, in addition to habitat preservation, is to maintain a species' existing level of genetic variation in order to maximize its chances for persistence in the face of changing environments (Keiper and McConchie, 2003). Dong *et al.* (2007, 2010) used ISSR and RAPD data that revealed low levels of genetic diversity at the species level and low levels of genetic variation among populations of *Ceratopteris pteridoides* in China. The studies have also demonstrated a high level of interpopulation gene flow in the extant populations of *C. pteridoides* in China. In light of the genetic information for *C. pteridoides*, we recommend establishing as many *in situ* conservation spots as possible and cross-transplanting plants between populations in order to increase gene flow and preserve to the greatest extent possible the genetic resources of the species.

ACKNOWLEDGMENTS

The authors are particularly indebted to Dr. Jin-Ming Chen, Dr. Can Dai, Xing-Wei Wang, and Yu-Dei Huang for their assistance. The study was supported by grants from the Natural Science

Foundation of China (No. 31170341), the Natural Science Foundation of Hubei province (No. 2011CDB001), Science and Technologic Project of Wuhan City (201150699189-02), and State Key Laboratory of Lake Science and Environment (2010SKL011), and the "100 Talents Program" of CAS granted to WQF (KSCX2-YW-Z-0805).

LITERATURE CITED

- CARQUIST, S. and E. L. SCHNEIDER. 2000. SEM studies of vessels in ferns 14. *Ceratopteris*. *Aquat. Bot* 66:1–8.
- CARRIER, J. 1991. The Colorado: a river drained dry. *National Geographic Magazine* 179:4–35.
- DIAO, Z. S. 1990. *Aquatic weeds in China*. Chongqing Press, Chongqing.
- DONG, Y. H., W. G. ROBERT., J. M. CHEN and Q. F. WANG. 2005. Effect of habitat modification on the distribution of the endangered aquatic fern *Ceratopteris thalictroides* (Parkeriaceae) in China. *J. Freshwater Ecol* 20:689–693.
- DONG, Y. H., J. M. CHEN., W. G. ROBERT and Q. F. WANG. 2007. Gene flow in populations of the endangered aquatic fern *Ceratopteris pteridoides* in China as revealed by ISSR markers. *Aquat. Bot* 87:69–74.
- DONG, Y. H., W. G. ROBERT, J. M. CHEN and Q. F. WANG. 2010. Genetic variation and gene flow in the endangered aquatic fern *Ceratopteris pteridoides* in China, and conservation implications. *Ann. Bot. Fennici* 47:34–44.
- DOWNEY, D. M., C. R. FRENCH and M. ODOM. 1994. Low cost limestone treatment of acid sensitive trout streams in the Appalachian mountains of Virginia. *Water Air Soil Pollut* 77:49–77.
- FAN, Y. W. and S. J. DAI. 1999. Observation on spore morphology of the pteridophyta by SEM. *Nat. Sci. J. Harbin Normal Univ* 15:73–76.
- HICKOK, L. G., T. R. WARNE and R. S. FRIBOURG. 1995. The biology of the fern *Ceratopteris* and its use as a model system. *Int. J. Plant Sci* 156:332–345.
- HICKOK, L. G., T. R. WARNE and M. K. SLOCUM. 1987. *Ceratopteris richardii*: applications for experimental plant biology. *Am. J. Bot* 74:1030–1316.
- IVAHNENKO, T. I., J. J. RENTON and H. W. RAUCH. 1988. Effects of liming on water quality of two streams in West Virginia. *Water Air Soil Pollut* 41:331–357.
- JACKSON, L. J., J. KALFF and J. B. RASMUSSEN. 1993. Sediment pH and redox potential affect the bioavailability of Al, Cu, Fe, Mn, and Zn to rooted aquatic macrophytes. *Can. J. Fish. Aquat. Sci* 50:143–148.
- JIAN, Y. X., J. B. WANG, G. Q. HE., J. LI and J. K. CHEN. 2001. A comparative study of aquatic plant diversity of Haikou, Taibai and Wushan lakes in Hubei province of China. *Acta Ecol. Sin* 21:1815–1824.
- KEIPER, F. J. and R. MCCONCHIE. 2000. An analysis of genetic variation in natural populations of *Sticherus flabellatus* [R.Br.(St John)] using amplified fragment length polymorphism (AFLP) markers. *Mol. Ecol.* 9:571–581.
- KÖRNER, S., S. K. DAS., S. VEENTRA and J. E. VERMAAT. 2001. The effect of pH variation at the ammonium/ammonia equilibrium in wastewater and its toxicity to *Lemna gibba*. *Aquat. Bot* 71:71–78.
- LI, S. G. 2002. *Applied biology statistics*. Beijing University Press, Beijing.
- LIU, J. K. 1984. Lakes of the middle and lower basins of the Chang Jiang (China). Pp. 331–355, *In*: Tabu F. B. *Lakes and Reservoirs*. Elsevier, Amsterdam.
- LIU, X., X. A. PANG and Q. F. WANG. 2003. Character and variation of chemical properties of the water in the natural habitats of three species of *Isoetes* in China. *Acta Phytocologica Sin* 27:510–515.
- LLOYD, R. M. 1974. Systematics of the genus *Ceratopteris* Brongn. (Parkeriaceae). II. Taxonomy. *Brittonia* 26:139–160.
- LU, S. and J. H. JIANG. 2003. The wetland resources of Honghu Lake and countermeasures of its protection. *J. Lake Sci* 15:281–284.
- MAUNDER, M. 1994. Botanical gardens: future challenges and responsibilities. *Biodivers Conserv* 3:97–103.

- MOYLE, P. B. and R. A. LEIDY. 1992. Loss of biodiversity in aquatic ecosystems: Evidence from fish faunas. Pp. 127–169, In: Fiedler P. L. and S. K. Jain. *Conservation Biology: the Theory and Practice of Nature Conservation, Preservation and Management*. Chapman and Hall, New York.
- PRIMACK, R. B. 1993. *Essentials of conservation biology*. Sinauer Associates, Inc, Sunderland M.A.
- SHI, C. X., X. C. WANG., H. S. DOU, L. ZHANG and H. D. WANG. 1989. *A general outline of Chinese lakes*. Science Press, Beijing.
- TANG, T., Q. H. CAI, R. Q. LIU, D. F. LI and Z. C. XIE. 2002. Distribution of epilithic algae in the Xangxi River system and their relationships with environmental factors. *J. Freshwater Ecol* 17:345–352.
- TAO, L., L. Y. YIN and W. LI. 2008. Effects of herbicide bensulfuron-methyl on gametophyte development and sex organ differentiation in *Ceratopteris pteridoides*. *J. Plant Ecol (Chinese Version)*. 32:408–412.
- WAN, C. Q., Y. H. WU and J. T. LIU. 2004. Quality status urban lake water in Wuhan city with integrated approaches on restoration. *Resources and Environment in the Yangtze Basin*. 13:500–502.
- WANG, X. L., J. N. WU., H. L. WANG, C. J. KE and R. X. ZHU. 2009. Problems in lake protection and management of Hubei Province and countermeasures. *Yangtze River*. 20:10–11.
- WEN, M. Z., X. A. PANG and Q. F. WANG. 2003. Relationship between water chemistry and the distribution of the endangered aquatic quillwort *Isoetes sinensis* Palmer in China. *J. Freshwater Ecol* 18:361–367.
- YANG, H. L. and J. X. YE. 2001. Primary study on interrelation between aquatic vascular plant communities and environmental factors in Jiangxi province. *Chinese Journal of Ecology*. 20:45–47.
- YU, Y. F. 1999. A milestone of wild plant conservation in China. *Plants*. 5:3–11.

Negative Gravitropism in Dark-Grown Gametophytes of the Fern *Ceratopteris richardii*

HIROYUKI KAMACHI* and MUNENORI NOGUCHI

Graduate School of Science and Engineering (Science), University of Toyama, 3190 Gofuku, Toyama 930-8555, Japan

ABSTRACT.—This study examined whether gravity influences the growth direction of dark-grown gametophytes of the fern *Ceratopteris richardii*. Analyses of directional growth of gametophytes in response to gravitropic stimulation demonstrated that gametophytes showed negative gravitropism. Dark-grown gametophytes of *dkg1 her1* mutants, which germinate in complete darkness, displayed a more distinct negative gravitropism. Unlike *her1* spores, *dkg1 her1* spores do not require light irradiation to induce spore germination. Therefore, light irradiation on *her1* spores was possibly inhibiting the negative gravitropism of *her1* gametophytes. In the present study, prolonged white-light irradiation on *her1* spores inhibited negative gravitropism in the gametophytes. Light irradiation on spores therefore affects the later negative gravitropism of dark-grown gametophytes.

KEY WORDS.—*Ceratopteris richardii*, gametophyte physiology, gravitropism

Spore germination is the first event in the life cycle of ferns. Germinated spores progress autotrophically through many developmental stages to form a mature gametophyte with rhizoids and gametangia (Momose, 1967; Raghavan, 1989). During this time many environmental factors influence development and morphogenesis of the fern gametophyte. In vascular plants gravity is an important factor controlling plant morphogenesis and directional growth (Morita and Tasaka, 2004; Hoson *et al.*, 2010); similar responses in fern gametophytes have not yet been described in detail.

The fern *Ceratopteris richardii* Brogn. is often used as a plant model system (Hickok *et al.*, 1995; Banks, 1999; Salmi *et al.*, 2005). In the germinating spores of *C. richardii*, Edwards and Roux (1994, 1998) found that the primary rhizoid emerged in a downward direction with respect to gravity, suggesting that germinating spores could sense the direction of gravity. After germination the rhizoid failed to respond to changes in gravity, indicating that the rhizoid itself was not gravitropic (Edwards and Roux, 1994). Gravitropism in *Ceratopteris richardii* gametophytes other than in the rhizoids has not yet been examined. Investigation of gravity sensing by gametophytes is of interest because it is a poorly understood environmental response in gametophyte development. If *C. richardii* gametophytes can sense the direction of gravity and then show gravitropism, the gametophytes will be useful for investigating mechanisms of

* Corresponding Author: E-mail: kamachi@sci.u-toyama.ac.jp

gravitropic responses in non-vascular plants. In the present study, *C. richardii* gametophytes were examined for directional gravitropic responses.

MATERIALS AND METHODS

Morphogenesis between male and hermaphroditic gametophytes varies greatly in *Ceratopteris richardii* (Kamachi *et al.*, 2004). Male gametophytes, which are induced by the pheromone antheridiogen (Kamachi *et al.*, 2007), were insensitive to light for induction of asymmetric cell division followed by rhizoid development (Murata and Sugai, 2000), suggesting that male gametophytes might be less sensitive to environmental changes. Therefore *her1* mutants, which are antheridiogen-insensitive mutants and do not develop into males (Banks, 1994), were used for this work. In this study, *dkg1 her1* double mutants were also used. The *dkg1* (*dark-germinator 1*) mutant allele enables the spore to germinate in complete darkness (Scott and Hickok, 1991; Kamachi *et al.*, 2004).

Ceratopteris richardii spores of *her1* and *dkg1 her1* mutants were collected from fertile fronds in a greenhouse at Toyama University. The spores were sterilized for 3 min in commercial 5% NaOCl bleach, 0.02% (w/v) Triton X-100, rinsed with distilled water, and incubated in the dark for 7 days to synchronize germination. Spores were then irradiated for 24 h at 26°C under white light ($5.0 \text{ J m}^{-2} \text{ s}^{-1}$), and germinated in the dark to obtain strap-shaped gametophytes (Fig. 1B). Spores of the *dkg1 her1* mutants were germinated in the dark immediately after the sterilization because these mutants can germinate in the dark. A 1:4 dilution of Murashige and Skoog (MS) salt mixture (Wako Pure Chemical Industries, Osaka, Japan) solidified with 0.3% (w/v) Bacto Agar (Difco) was used as the germination medium.

Gravitropism of *Ceratopteris richardii* gametophytes was evaluated in 9-day old gametophytes grown on agar medium in the dark. Observations were made using a stereoscopic zoom microscope (Nikon, SMZ-1000). In each experiment 50–100 gametophytes were classified into three types: gametophytes that grew

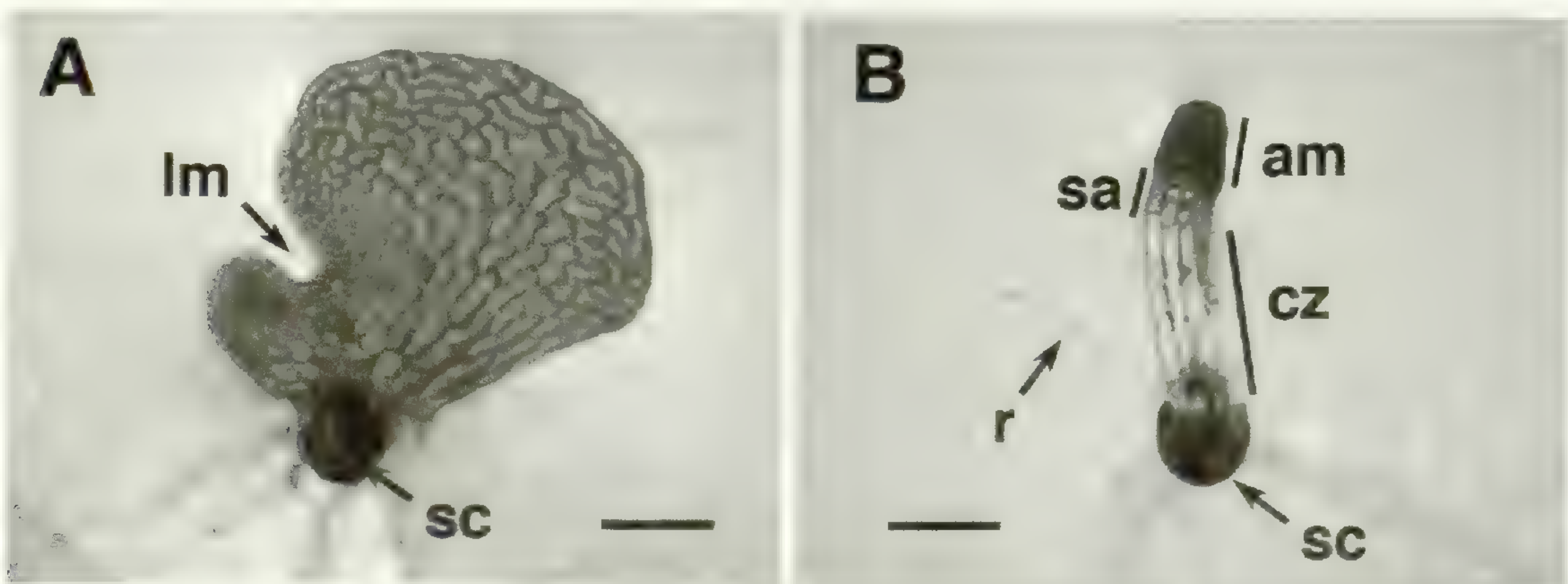


FIG. 1. Morphological profiles of 7-d-old *Ceratopteris richardii* gametophytes grown in the light (A) and in the dark (B). *am* Apical meristem, *cz* basal growth cessation zone, *lm* lateral meristem, *r* rhizoid, *sa* subapical elongation zone, *sc* spore coat. Scale Bar = 0.2 mm.

upward, downward, and horizontally with respect to the surface of the agar medium. Results were expressed as mean values of percentages obtained from three separate experiments.

RESULTS

Figure 1 shows typical morphological profiles of 7-day old *Ceratopteris richardii* gametophytes grown in the light (A) and dark (B). The dark-grown gametophytes have a strap-shape with 3–6 rows of cells in a single plane, an apical meristem, a subapical elongation zone, and a basal growth cessation zone where the cells are extremely elongated. A similar growth habit was also described in a study by Murata *et al.* (1997).

Dark-grown gametophytes were first examined for a display of gravitropism. Sixty one percent of the 8-day old, dark-grown gametophytes grew upward with respect to gravity, and 10% grew downward (Fig. 2), which suggests that *Ceratopteris richardii* gametophytes display a tendency toward negative gravitropism. For a clearer demonstration of negative gravitropism, gametophytes were turned upside down one and two days before observation. These gametophytes changed their direction of growth from “upward” to “downward” following this rotation (Fig. 2), which further demonstrates that *C. richardii* gametophytes display negative gravitropism.

Figure 2 shows results from gametophytes with the *dark germinator 1 (dkg1)* mutant allele, which enables spores to germinate in complete darkness (Scott and Hickok, 1991; Kamachi *et al.*, 2004). Interestingly, 89% of these gametophytes grew upward, and only 1% grew downward. Thus, these mutants showed an enhancement of negative gravitropism compared with the

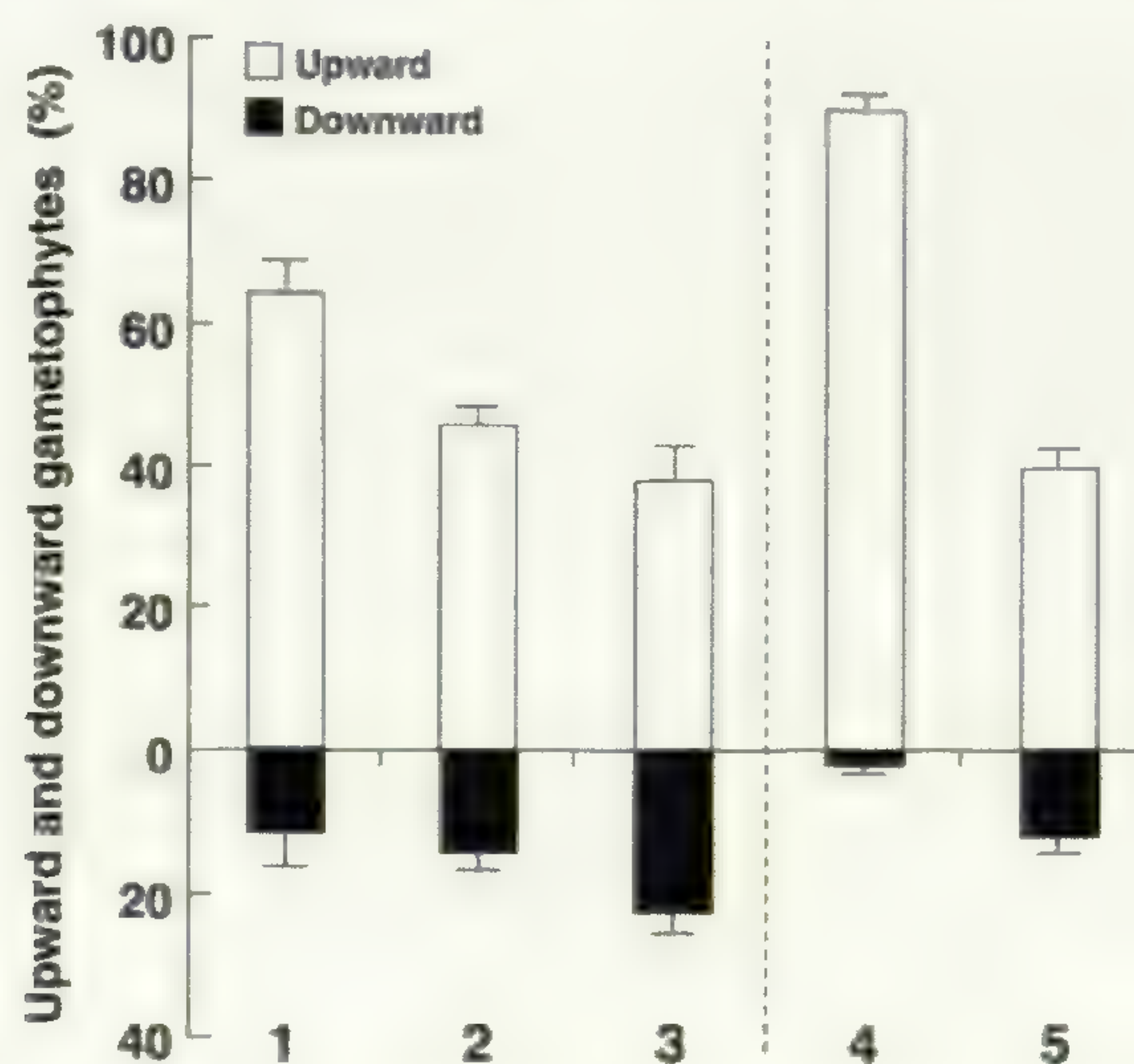


FIG. 2. Percentages of dark-grown *Ceratopteris richardii* gametophytes that grew upward and downward directions in *her1* mutants (1, 2, 3) and *dkg1 her1* mutants (4, 5). Gametophytes grown on agar medium placed horizontally (1 and 4); gametophytes turned upside down one day before the observation (2); gametophytes turned upside down two days before the observation (3 and 5). Values represent the means evaluated from three separate experiments. In each experiment 50–100 gametophytes were observed. Bars are standard errors.

her1 mutants. These results imply that white light, which is required to induce spore germination, may inhibit the subsequent gravitropism of developing gametophytes.

To confirm this hypothesis, white-light irradiation effects on gravitropism were examined using *her1* gametophytes (Fig. 3). When the length of irradiation time was changed from 18 h to 48 h, the negative gravitropic response weakened. Seventy-four percent of gametophytes grew upward when the irradiation time was 18 h, as opposed to 48% when irradiation time was extended to 48 h. On the other hand, the percentages of gametophytes that grew downward and horizontally increased to 14 and 35% from 4 and 22%, respectively, when the length of irradiation time was changed from 18 h to 48 h. Thus, the white light irradiation during the initial developmental steps in spore germination inhibited negative gravitropism in *Ceratopteris richardii* gametophytes.

DISCUSSION

This research was conducted to determine whether gravity affects the directional growth of dark-grown gametophytes of *Ceratopteris richardii*. Gametophytes showed negative gravitropism similar to that as seen in most seedlings of vascular plants, caulonema of the moss *Physcomitrella patens*

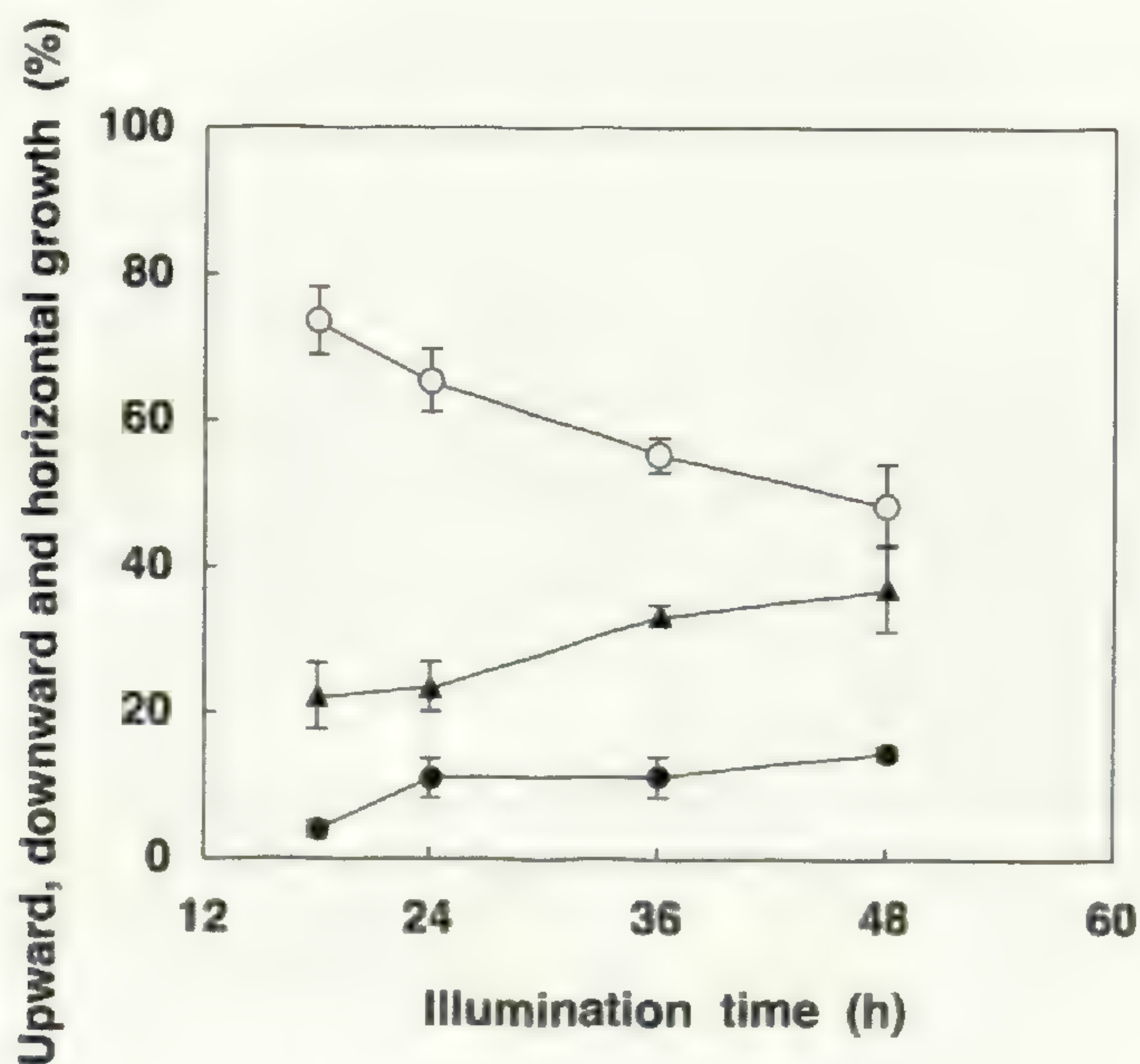


FIG. 3. White-light irradiation effects on negative gravitropism in *Ceratopteris richardii* gametophytes. The *her1* spores were irradiated by white light for the designated times to induce spore germination, then germinated and grown in the dark. Percentages of gametophytes that grew upward (open circles), downward (closed circles) and horizontally (closed triangle) were determined from the 9-d-old gametophytes. Values represent the means evaluated from five separate experiments. In each experiment 50–100 gametophytes were observed. Bars are standard errors.

(Martin *et al.*, 2009), and protonemata of characean algae (Braun and Limbach, 2006).

Gravitropism in plants occurs in three temporal stages: gravity perception, signal transduction, and organ response (Kumar *et al.*, 2008). The detailed mechanisms of gravity perception have been unveiled mostly in vascular plants. In *Arabidopsis thaliana* (L.) Heynh. amyloplast movement along the gravity vector within gravity-sensing cells is the most likely trigger of a subsequent gravitropic response (reviewed in Morita and Tasaka, 2004). In contrast, no data are available to explain how *Ceratopteris richardii* gametophytes might sense the direction of gravity. Amyloplasts would not seem to be involved in gravity perception in *C. richardii* gametophytes because no starch-accumulating amyloplasts were found in dark-grown gametophyte cells following I₂-KI staining (data not shown). This suggests the involvement of some other statolith in triggering the gravitropic response in *C. richardii* gametophytes.

Edwards and Roux (1994) found that germinating spores of *Ceratopteris richardii* could sense the direction of gravity because gravity directed the nuclear migration in the germinating spores, as well as the initial direction of growth of the primary rhizoid. They detected a calcium flux in the germinating spores as the earliest gravity-directed event (Chatterjee *et al.* 2000), suggesting that calcium channels and pumps may be involved in the primary gravity perception mechanism in *C. richardii* spores. Recently, Salmi *et al.* (2011) proposed that the gravity-directed calcium current is regulated primarily by the activation of mechanosensitive calcium channels at the bottom of the spore, based on data obtained from a silicon microfabricated sensor array. Thus, the nuclear migration and the following calcium flux might be important in the gravity perception mechanism in *C. richardii* gametophytes.

As shown in Figure 3, the white-light irradiation that is required to induce spore germination weakened the negative gravitropism in *her1* gametophytes, indicating that light irradiation on spores influences the later negative gravitropism of dark-grown gametophytes. In fact, the dark-grown gametophytes with the *dkg1* mutant allele showed distinct negative gravitropism as compared with the gametophytes without the *dkg1* allele (Fig. 2). The *dkg1* mutants were shown to be constitutively active in several photomorphogenic responses mediated by phytochrome (a red and far-red light photoreceptor) through the gametophytic phase (Kamachi *et al.*, 2004), in addition to the dark-germinating property. Considering these characteristics of the *dkg1* mutants, phytochrome may not be responsible for the inhibitory effect of white light on subsequent negative gravitropism. In preliminary experiments, blue- and green-light, but not red light, affected the inhibition of negative gravitropism (Adachi and Kamachi, unpublished data).

The gravitropic growth-orientation of the seedlings of flowering plants is also inhibited by light (Correll and Kiss, 2002). In contrast to *C. richardii* gametophytes, however, phytochrome is responsible for the inhibition of gravitropism in *Arabidopsis thaliana* (Poppe *et al.* 1996; Lariguet and Fankhauser, 2004), where phytochrome is found to promote the conversion of amyloplasts to other forms of plastids in the endodermis, causing cessation

of hypocotyl gravitropism (Kim *et al.*, 2011). Thus, the mechanisms involved in the light-induced inhibition of negative gravitropism in *C. richardii* gametophytes are likely to be different than those operating in *A. thaliana* seedlings. Further analyses are required to determine how the negative gravitropism of the gametophytes is inhibited by light and to identify the gravity-sensing mechanisms of *C. richardii* gametophytes.

ACKNOWLEDGMENTS

A part of this research was supported by Grants-in-Aid for Scientific Research, MEXT (No. 21657011).

LITERATURE CITED

- BANKS, J. A. 1994. Sex-determining genes in the homosporous fern *Ceratopteris*. *Development* 120:1949–1958.
- BANKS, J. A. 1999. Gametophyte development in ferns. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50:163–186.
- BRAUN, M. and C. LIMBACH. 2006. Rhizoids and protonemata of characean algae: model cells for research on polarized growth and plant gravity sensing. *Protoplasma* 229:133–142.
- CHATTERJEE, A., D. M. PORTERFIELD, P. S. SMITH and S. J. ROUX. 2000. Gravity-directed calcium current in germinating spores of *Ceratopteris richardii*. *Planta* 210:607–610.
- COOKE, T. J., L. G. HICKOK, W. J. VAN DER WOUDE, J. A. BANKS and R. J. SCOTT. 1993. Photobiological characterization of a spore germination mutant *dkg1* with reversed photoregulation in the fern *Ceratopteris richardii*. *Photochem. Photobiol.* 57:1032–1041.
- CORRELL, M. J. and J. Z. KISS. 2002. Interactions between gravitropism and phototropism in plants. *J. Plant Growth Regul.* 21:89–101.
- EDWARDS, E. S. and S. J. ROUX. 1994. Limited period of graviresponsiveness in germinating spores of *Ceratopteris richardii*. *Planta*. 195:150–152.
- EDWARDS, E. S. and S. J. ROUX. 1998. Influence of gravity and light on the developmental polarity of *Ceratopteris richardii* fern spores. *Planta* 205:553–560.
- HICKOK, L. G., T. R. WARNE and R. S. FRIBOURG. 1995. The biology of the fern *Ceratopteris* and its use as a model system. *Int. J. Plant Sci.* 156:332–345.
- HOSON, T., S. MATSUMOTO, K. SOGA and K. WAKABAYASHI. 2010. Cortical microtubules are responsible for gravity resistance in plants. *Plant Signal. and Behav.* 5:752–754.
- KAMACHI, H., E. MATSUNAGA, M. NOGUCHI and H. INOUE. 2004. Novel mutant phenotypes of a dark-germinating mutant *dkg1* in the fern *Ceratopteris richardii*. *J. Plant Res.* 117:163–170.
- KAMACHI, H., O. IWASAWA, L. G. HICKOK, M. NAKAYAMA, M. NOGUCHI and H. INOUE. 2007. The effects of light on sex determination in gametophytes of the fern *Ceratopteris richardii*. *J. Plant Res.* 120:629–634.
- KIM, K., J. SHIN, S.-H. LEE, H.-S. KWEON, J. N. MALOOF and G. CHOI. 2011. Phytochromes inhibit hypocotyl negative gravitropism by regulating the development of endodermal amyloplasts through phytochrome-interacting factors. *Proc Natl. Acad. Sci. USA* 108:1729–1734.
- KUMAR, N. S., M. H. H. STEVENS and J. Z. KISS. 2008. Plastid movement in statocytes of the *arg1* (altered response to gravity) mutant. *Am. J. Bot.* 95:177–184.
- LARIGUET, P. and C. FANKHAUSER. 2004. Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. *Plant J.* 40:826–834.
- MARTIN, A., L. DANIEL, S. T. HANKE, S. J. X. MUELLER, E. SARNIGHAUSEN, M. VERVLIIET-SCHEEBAUM and R. RESKI. 2009. Targeted gene knockouts reveal overlapping functions of the five *Physcomitrella patens* FtsZ isoforms in chloroplast division, chloroplast shaping, cell patterning, plant development, and gravity sensing. *Mol. Plant* 2:1359–1372.

- MORITA, M. T. and M. TASAKA. 2004. Gravity sensing and signaling. *Curr. Opin. Plant Biol.* 7:712–718.
- MOMOSE, S. 1967. *Prothallia of the Japanese ferns (Filicales)*. University of Tokyo Press, Tokyo.
- MURATA, T., A. KADOTA and M. WADA. 1997. Effects of blue light on cell elongation and microtubule orientation in dark-grown gametophytes of *Ceratopteris richardii*. *Plant Cell Physiol.* 38:201–209.
- MURATA, T. and M. SUGAI. 2000. Photoregulation of asymmetric cell division followed by rhizoid development in the fern *Ceratopteris prothalli*. *Plant Cell Physiol.* 41:1313–1320.
- POPPE, C., R. P. HANGARTER, R. A. SHARROCK, F. NAGY and E. SCHAFER. 1996. The light-induced reduction of the gravitropic growth-orientation of seedlings of *Arabidopsis thaliana* (L.) Heynh. is a photomorphogenic response mediated synergistically by the far-red-absorbing forms of phytochromes A and B. *Planta* 199:511–514.
- RAGHAVAN, V. 1989. *Developmental biology of fern gametophytes*. Cambridge University Press, Cambridge.
- SALMI, M. L., T. J. BUSHUART, S. C. STOUT and S. J. ROUX. 2005. Profile and analysis of gene expression changes during early development in germinating spores of *Ceratopteris richardii*. *Plant Physiol.* 138:1734–1745.
- SALMI, M. L., A. HAQUE, T. J. BUSHART, S. C. STOUT, S. J. ROUX and D. M. PORTERFIELD. 2011. Changes in gravity rapidly alter the magnitude and direction of a cellular calcium current. *Planta* 233:911–920.
- SCOTT, R. J. and L. G. HICKOK. 1991. Inheritance and characterization of a dark-germinating, light-inhibited mutant in the fern *Ceratopteris richardii*. *Can. J. Bot.* 69:2616–2619.

Antimicrobial and Modulatory Activity of Ethanol Extract of the Leaves from *Lygodium venustum* SW.

MARIA F. B. MORAIS-BRAGA*, TEÓGENES M. SOUZA, KARLA K. A. SANTOS, JACQUELINE C. ANDRADE, GLÁUCIA M. M. GUEDES, SAULO R. TINTINO, CELESTINA E. SOBRAL-SOUZA, JOSÉ G. M. COSTA, IRWIN R. A. MENEZES, ANTONIO A. F. SARAIVA, and HENRIQUE D. M. COUTINHO

Universidade Regional do Cariri – URCA, Crato-CE, Brasil. Rua Cel. Antonio Luis 1161, Pimenta, 63105-000

ABSTRACT.—The evolution of microorganism defense systems has led to intensive searches for new drugs extracted from various natural products to fight microbial infections. This study evaluated the antibacterial and antifungal activity of *Lygodium venustum*, a climbing fern. A phytochemical screening was performed using ethanol extract from leaves of *L. venustum* (EELV), detecting the presence of phenols, tannins, flavonoids and alkaloids. The test of Minimal Inhibitory Concentration (MIC) against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. krusei* and *C. tropicalis* was evaluated using the microdilution method, resulting in inhibitory concentrations $\geq 1024 \mu\text{g/mL}$. Using a subinhibitory concentration of $128 \mu\text{g/mL}$ of EELV, the modulatory potential of the extract was tested against multidrug-resistant clinical isolates, resulting in synergism when combined with Gentamicin and actually altering the phenotype of *S. aureus* from sensitive to resistant. The extract also increased the effect of the kanamycin against *S. aureus*. This was the first report of modulatory antibiotic activity by a member of *Lygodium*.

KEY WORDS.—*Lygodium venustum*, microdilution, antimicrobial, modulator

Microbial infectious diseases have prompted the development of studies to understand their drug resistance mechanisms and the creation of drugs to avoid these defenses. Infection by *Staphylococcus aureus* is among the most common problems in hospitals due to its resistance against several antibiotics. *Pseudomonas aeruginosa* is the cause of nosocomial infections, particularly in people with cystic fibrosis. *Escherichia coli* is commonly found in the intestinal tract, but certain strains have been closely linked to serious urinary tract infections and diarrhea (Tortora *et al.*, 2008). *Klebsiella pneumoniae*, although confined to the normal flora, has emerged as an important hospital pathogen capable of causing severe morbidity and mortality in pediatric patients (Pfaller *et al.*, 1998). Strains of *Candida* have concerned the medical community due to their role in high-morbidity and mortality infections,

* Corresponding author: Laboratório de Microbiologia e Biologia Molecular, Departamento de Química Biológica, Universidade Regional do Cariri – URCA, Crato-CE, Brasil. Rua Cel. Antonio Luis 1161, Pimenta, 63105-000. Phone: +55(88)31021212; Fax +55(88) 31021291. E-mail: flavianamoraisb@yahoo.com.br

particularly in immunocompromised patients (Richardson and Lass-Flörl, 2008; Coutinho, 2009).

Through natural selection plants have developed several mechanisms against parasitism and herbivory. The production of defensive chemical compounds, such as secondary metabolites, indicates evolutionary adaptive responses from the pressure caused by these ecological relationships (Rhodes, 1994). Products derived from plants that feature antimicrobial properties or the ability to improve the antimicrobial potential of existing drugs play an important role in battling infectious diseases (Coutinho *et al.*, 2009). They can serve as alternative therapeutic agents with the ability to directly counter natural microbial resistance to drugs.

Lygodium venustum is a fern with a pantropical distribution used as a bioindicator of degraded environments (Mehltreter, 2006). This fern is used as a medicinal plant in Latin America due to its antifungal, trichomonocidal, antidiarrheal, anti-inflammatory and analgesic activity (Duke and Ottesen, 2009; Argueta *et al.*, 1994). It is used in the Peruvian Amazon as an adaptogen and as an ingredient of the sacred beverage “ayahuasca” (Rivier and Lidgren, 1972). Few studies have reported on the bioactivity of *L. venustum* in preclinical studies (Alanis *et al.*, 2005; Calzada *et al.*, 2007; Calzada *et al.*, 2010), as is true in others ferns (Xavier, 2007).

In this work, a phytochemical screening was performed on the ethanol extract from leaves of *L. venustum*; its antimicrobial activity was assayed against bacterial and fungal strains, as well the modulatory potential against aminoglycosides and antifungal drugs.

MATERIAL AND METHODS

Plant Material

Leaves of *L. venustum* were collected in the city of Crato, Ceará, Brazil. The plant was identified by Dr. Antonio Álamo Feitosa Saraiva and voucher specimens were deposited at the Herbarium Caririense Dárdano de Andrade-Lima of the Regional University of Cariri – URCA, under number 5569 HCDAL.

Preparation of Ethanol Extract from Leaves of *L. venustum* (EELV)

The leaves were partially milled and 211.18 g of powdered material was extracted by maceration using 1 L of 95% ethanol as solvent at room temperature. The mixture was allowed to stand for 72 h at room temperature. The extract was then filtered and concentrated under vacuum in a rotary evaporator at 60°C and 760 mm/Hg, yielding 103.9 g (Brasileiro *et al.*, 2006).

Phytochemical characteristics

The phytochemical assays were used for the qualitative analysis of the presence of secondary metabolites. The detection tests to evaluate the presence

TABLE 1. Bacterial source and antibiotic resistance profile.

Bacteria	Source	Antibiotic resistance
<i>Escherichia coli</i> 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
<i>Staphylococcus aureus</i> 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
<i>Pseudomonas Aeruginosa</i> 03	Urine culture	Cpm, Ctz, Imi, Cip, Ptz, Lev, Mer, Ami

Ast-Aztreonam; Ax-Amoxicillin; Amp-Ampicillin; Ami-Amicilina; Amox-Amoxillin, Ca-Cefadroxil; Cfc-cefaclor; Cf-Cephalothin; Caz-Ceftazidime; Cip-Ciprofloxacin; Clo-Chloramphenicol; Imi-Imipenem; Can-Canamycin; Szt-Sulfamethoxazole; Tet-Tetracycline; Tob-Tobramycin; Oxa-Oxacillin; Gen-Gentamicin; Neo- Neomycin; Para- Paramomycin; But-Butirosin; Sis-Sisomicin; Net-Netilmicin; Com-Cefepime; Ctz-Ceftazidime; Ptz-Piperacillin-tazobactam; Lev-Levofloxacin; Mer-Meropenem.

of heterosides, saponins, phenols, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids were performed according to the method described by Matos (2009). The tests are based on the visual observation of color modifications and formation of precipitate after the addition of specific reagents.

Microbial strains

The bacteria used in the Minimal Inhibitory Concentration (MIC) test were the standard strains of *E. coli* ATCC25923, *S. aureus* ATCC10536, *P. aeruginosa* ATCC15442 and *K. pneumonia* ATCC4362. The antifungal assays used standard strains of *Candida albicans* ATCC40006, *C. krusei* ATCC2538 and *C. tropicalis* ATCC40042. To evaluate the modulatory activity of the extract, the following multi-resistant bacterial strains were used, isolated from clinical environments: *P. aeruginosa* 03, *E. coli* 27 and *S. aureus* 358, with the resistance profile demonstrated in Table 1 and the same fungal strains used in the MIC test. All strains were obtained from the Laboratory of Clinical Mycology – UFPB.

Drugs

The drugs used in the tests were the aminoglycosides kanamycin, amikacin, neomycin and gentamicin, and antifungals mebendazole, amphotericin B, nystatin and benzoyl metronidazole (Sigma Co., St. Louis, USA). All drugs were diluted in sterile water.

Minimal Inhibitory Concentration

Broth microdilution was the method used. The EELV solution was dissolved using DMSO and diluted to 1024 µg/mL using sterile distilled water. The bacterial inoculum was diluted using BHI to a final concentration of 10⁵ CFU/mL. A total of 100 µL of each inoculum was distributed in each well of a microtiter plate with 96 wells, and then submitted to a twofold serial dilution

TABLE 2. Phytochemical characterization of ethanol extracts of *L. venustum*.

Metabolites														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
+	-	+	-	-	+	+	+	+	-	+	-	-	+	+

1 - Phenols; 2 - Tannin pyrogallates; 3 - Tannin Phlobaphenes; 4 - Anthocyanidins; 5 - Anthocyanins; 6 - Flavones; 7 - flavonols; 8 - Xanthonnes; 9 - Chalcones; 10 - Aurones; 11 - Flavonols; 12 - Leucoanthocyanidins; 13 - Catechins; 14 - Flavonones; 15 - Alkaloids; (+) presence; (-) absence.

using 100 μ L of the extract, with concentrations ranging between 8 and 512 μ g/mL. The plates were incubated for 24 hours at 35 °C (Javadpour *et al.*, 1996). Bacterial MIC was determined using resazurin, while the MIC of fungi was determined by turbidity. The MIC was defined as the lowest concentration where no growth can be observed, according to NCCLS (2008).

Drug Modulation Test

To observe whether the extract would alter the action of antimicrobial drugs against the tested strain, the method proposed by Coutinho *et al.* (2008) was used. The EELV was tested at a sub-inhibitory concentration (MIC/8 = 128 μ g/mL). A 100 μ L sample of a solution containing BHI, the microbial inoculums and extract were placed in each well. After this, 100 μ L of the antimicrobial drug was mixed with the first well, following the twofold dilution. Concentrations of aminoglycosides and antifungals ranged between 2.44 and 2500 μ g/mL and 2 to 512 μ g/mL, respectively.

RESULTS

The phytochemical characterization showed the presence of phenols, tannins, flavonoids and alkaloids, as shown in Table 2.

The antibacterial and antifungal assays of EELV did not demonstrate clinically relevant results, with MICs \geq 1024 μ g/mL. However, when the modifying activity of EELV against aminoglycosides was evaluated, the Gram-negative *E. coli* 27 and Gram-positive *S. aureus* 358 strains showed synergistic activity when combined with gentamicin and kanamycin (Table 3). The combination of the extract with antifungals did not show any modulatory activity against strains of *Candida*.

DISCUSSION

Several plants used in the religious beverage "ayahuasca", such as *L. venustum*, contain alkaloids (Rivier and Lidgren, 1972). This fact is corroborated by the results of our phytochemical screening. Other species from the genus *Lygodium* have been the subjects of more detailed chemical studies, including the isolation of some compounds (Zhang *et al.*, 2005;

TABLE 3. Antibacterial activity modulating the ethanol extract from leaves of *L. venustum* ($\mu\text{g/mL}$).

Extract/ Antibiotic	Modulation of antibiotic for EELV			
	<i>E. coli</i> 27		<i>S. aureus</i> 358	
	MIC combined	MIC alone	MIC combined	MIC alone
EELV/ Kanamycin	156.25	156.25	39.06	156.25
EELV/ Amikacin	312.5	312.5	78.125	78.125
EELV/ Neomycin	156.25	156.25	39.06	39.06
EELV/ Gentamicin	39.06	1250	2.44	19.53

MIC: Minimal Inhibitory Concentration; EELV: Ethanol Extract of *L. venustum*.

Kurumatani *et al.*, 2001; Achari *et al.*, 1986). However, this is the first work to focus on the chemical composition of *L. venustum*.

The lack of the antibiotic activity of *L. venustum* against strains of *E. coli* was verified in another report (Alanis *et al.*, 2005). The results demonstrate that the extracts were not efficient inhibitors of bacterial growth, as their inhibition percentages were lower than 50%. A relevant note regarding this research is the value of the extract concentration used in the test, 8 mg, which is considered high for clinical applications (Houghton *et al.*, 2007), as demonstrated in our work. Additionally, it is important to note that the microdilution method used in present study is currently the preferred technique to evaluate antimicrobial activity, compared to other methodologies using agar diffusion (Greger and Hadacek, 2000).

The methanol extracts of other plants from the genus *Lygodium* such as *Lygodium japonicum* (Thumb.) SW. was tested against strains of *P. aeruginosa*, *S. aureus*, *E. coli* and *C. albicans* using the disk diffusion method, impregnated with 40 μg of dried plant material/disk, but no bioactivity was demonstrated (Taylor *et al.*, 1995). Our results corroborate those obtained in this work by Taylor *et al.* (1995).

Compared with the isolated action of drugs, EELV increased the antibiotic activity of amikacin against *S. aureus*. When associated with gentamicin, it demonstrated a very promising modulatory activity against *E. coli* and *S. aureus*, causing a reversal of the resistant phenotype of this strain to sensitive according to the classification of NCCLS (2005). The observed bioactivity of the extract in combination with the antibiotics may indicate that secondary metabolites such as tannins, flavonoids and alkaloids –all secondary metabolites with well-known antimicrobial activity and found in the EELV– could be acting in association with the assayed drugs, enhancing the activity of these drugs at lower concentrations (Scalbert 1991; Bylka *et al.*, 2004; Zongo *et al.*, 2009).

This is the first report on the modulatory activity against aminoglycosides by a fern. This activity indicates the possibility of development of new drugs derived from the association between natural products isolated from *L. venustum* with antibiotics, to be used in antibiotic therapy against multi-drug resistant bacteria, as well as prevent the emergence of drug resistant bacteria.

LITERATURE CITED

- ACHARI, B., K. BASU, C. R. SAHA and S. C. PAKRASHI. 1986. A New Triterpene Ester, an Anthraquinone and Other Constituents of the Fern *Lygodium flexuosum*. *Plant Med* 52:329–30.
- ALANIS, A. D., F. CALZADA, J. A. CERVANTES and G. M. CEBALLOS. 2005. Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *Journal of Ethnopharmacology* 100:153–157.
- ARGUETA, A., L. CANO and M. RODARTE. 1994. Atlas de las Plantas de la Medicina Tradicional Mexicana, vol. I–III. Instituto Nacional Indigenista, Mexico City.
- BRASILEIRO, B. G., V. R. PIZZILOLO, D. S. RASLAN, C. M. JAMAL and D. SILVEIRA. 2006. Antimicrobial and cytotoxic activities screening of some Brazilian medicinal plants used in Governador Valadares district. *Braz. J. Pharm. Sci.* 42:195–202.
- BYLKA, W., I. MATLAWSKA and N. A. PILEWSKI. 2004. Natural flavonoids as antimicrobial agents. *JANA* 7:24–31.
- CALZADA, F., Y. M. LILIAN and A. T. CONTRERAS. 2007. Effect of Mexican medicinal plant used to treat trichomoniasis on *Trichomonas vaginalis* trophozoites. *Journal of Ethnopharmacology* 113:248–251.
- CALZADA, F., R. ARISTA and H. PÉREZ. 2010. Effect of plants used in Mexico to treat gastrointestinal disorders on charcoal - gum acacia - induced hyperperistalsis in rats. *Journal of Ethnopharmacology* 128:49–51.
- COUTINHO, H. D. M. 2009. Factors Influencing the Virulence of *Candida Spp.* *West Indian Med J* 58(2):160.
- COUTINHO, H. D. M., J. G. M. COSTA, E. O. LIMA, V. S. FALCÃO-SILVA and J. P. SIQUEIRA-JÚNIOR. 2008. Enhancement of the Antibiotic Activity against a Multiresistant *Escherichia coli* by *Mentha arvensis* L. and Chlorpromazine. *Chemotherapy* 54:328–330.
- COUTINHO, H. D. M., J. G. M. COSTA, E. O. LIMA, V. S. FALCÃO-SILVA and J. P. SIQUEIRA-JÚNIOR. 2009. Increasing of the Aminoglycoside Antibiotic Activity Against a Multidrug-Resistant *E. coli* by *Turnera ulmifolia* L. and Chlorpromazine. *Biol Res Nurs* 11:332–335.
- DUKE, J. A. 2009. *Duke's Handbook of Medicinal Plants of Latin America*. 2008. New York: CRC Press Taylor & Francis group. 832 p.
- GREGER, H. and F. HADACEK. 2000. Testing of antifungal natural products: Methodologies, comparability of results and assay choice. *Phytochemical Analysis* 11:137–147.
- HOUGHTON, P. J., M. J. HOWES, C. C. LEE and G. STEVENTON. 2007. Uses and abuses of in vitro tests in ethnopharmacology: Visualising an elephant. *J Ethnopharmacol* 110:391–400.
- JAVADPOUR, M. M., M. M. JUBAN, W. C. LO, S. M. BISHOP, J. B. ALBERTY, S. M. COWELL, C. L. BECKER and M. L. McLAUGHLIN. 1996. De novo antimicrobial peptides with low mammalian cell toxicity. *J Med Chem* 39:107–3113.
- KURUMATANI, M., K. YAGI, T. MURATA, M. TEZUKA, L. N. MANDER, M. NISHIYAMA and H. YAMA. 2001. Isolation and identification of antheridiogens in the ferns, *Lygodium microphyllum* and *Lygodium reticulatum*. *Biosci Biotechnol Biochem* 65(10):2311–4.
- MATOS, F. J. A. 2009. *Introdução à fitoquímica experimental*. 3 ed. Fortaleza. Editora: UFC.
- MEHLTRETER, K. 2006. Leaf Phenology of the Climbing Fern *Lygodium venustum* in a Semideciduous Lowland Forest on the Gulf of Mexico. *American Fern Journal* 96(1):21–30.
- NCCLS. 2005. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard, 6th ed.* NCCLS document M7-A6. Wayne: National Committee for Clinical Laboratory Standards.
- PFALLER, A. M., R. N. JONES, G. V. DOERN and K. KUGLER. 1998. Bacterial pathogens isolated from patients with bloodstream infection: frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). *Antimicrob Agent Chemother* 42:1762–70.
- RHODES, M. J. C. 1994. Physiological roles for secondary metabolites in plants: from progress, many outstanding problems. *Plant Mol. Biol* 24:1–20.
- RICHARDSON, M. and C. LASS-FLORL. 2008. Changing epidemiology of systemic fungal infections. *Clinical Microbiology and Infection* 14:5–24.

- RIVIER, L. and J. E. LIDGREN. 1972. "Ayahuasca: the South American hallucinogenic drink. An ethnobotanical and chemical investigation". *Economic Botany* 26(2):101–129.
- SCALBERT, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30:3875–83.
- TAYLOR, R. S., N. P. MANANDHAR and G. H. N. TOWERS. 1995. Screening of selected medicinal plants of Nepal for antimicrobial activities. *Journal of Ethnopharmacology* 46:153–159.
- TORTORA, G. J., B. R. FUNKE and C. L. CASE. 2008. *Microbiologia*, 8^a ed. Porto Alegre, Artmed.
- XAVIER, S. R. S. 2007. Pteridófitas da Caatinga: Lista anotada, Análise da Composição Florística e Padrões de Distribuição Geográfica. Tese (Doutorado em Botânica) - Universidade Federal Rural de Pernambuco, Recife. 147 p.
- ZHANG, L. H., Z. Q. YIN, W. C. YE, S. X. ZHAO, L. WANG and F. HU. 2002. Studies on the chemical constituents in herb of *Lygodium japonicum*. *Zhongguo Zhong Yao Za Zhi* 30(19):1522–4.
- ZONGO, C., E. F. O. AKOMO, A. SAVADOGO, L. C. OBAME, J. KOUDOU and A. S. TRAORE. 2009. *In vitro* antibacterial properties of total alkaloids extract from *Mitragyna inermis* (Willd.) O. Kuntze, a West African traditional medicinal plant. *Asian J. Plant Sci.* 8:172–177.

A New Species and a New Hybrid in the Grammitid Fern Genus *Stenogrammitis* (Polypodiaceae)

PAULO H. LABIAK

Universidade Federal do Paraná, Departamento de Botânica, Caixa Postal 19031, 81531-980, Curitiba-PR, Brazil, e-mail: plabiak@ufpr.br

ABSTRACT.—A new species and a new hybrid in the genus *Stenogrammitis* are here described, and descriptions, illustrations, and comments on the most similar species are provided. *Stenogrammitis brevipubens* is characterized by having hemidimorphic laminae, with the fertile portion narrower and less dissected than the sterile portion, and by its indument, which is composed of simple, hyaline, and 2-celled hairs that are appressed upon the petiole, rachis, and laminar tissue. The hybrid, *Stenogrammitis* × *guatemalensis*, has hemidimorphic laminae and reddish, 1-furcate and 3-celled hairs that are spreading on the petiole and lamina. Based on the hybrid morphology, the putative parents are *S. prionodes* and *S. limula*.

KEY WORDS.—diversity, Guatemala, hybridization, Panama

Stenogrammitis Labiak is a pantropical genus recently segregated from *Lellingeria*. It comprises about 25 species, of which 12 are currently known from the Neotropics, six species from the continental Africa, four from Madagascar, and three that occur in some Islands of the Atlantic and Pacific Oceans (Labiak, 2011). The genus is characterized by its small size and narrow laminae (fronds generally less than 10 cm long and 0.5 cm wide), clathrate and iridescent rhizome scales that are glabrous throughout or with only one apical cilium, laminae with only one sorus per segment, and fertile veins that present the sclerenchyma visible beneath the sporangia.

Phylogenetic studies (Labiak *et al.*, 2010; Ranker *et al.*, 2010) showed that *Stenogrammitis* is most closely related to *Lellingeria* A. R. Sm. & R. C. Moran and *Melpomene* A. R. Sm. & R. C. Moran. *Lellingeria* differs by having broader laminae (usually more than 1 cm wide), rhizome scales that are usually ciliate, multiple sori per segment (except for *L. militaris* that has one), and fertile veins not visible beneath the sporangia (Labiak, 2011). *Melpomene* differs by presenting reddish setae on the fronds and also by rhizome scales with papillate apex (Lehnert, 2008; Labiak, 2011).

While studying the Neotropical species of *Stenogrammitis* I found a new species and a new hybrid that deserve recognition, which I describe as follow.

***Stenogrammitis brevipubens* Labiak, *sp. nov.* TYPE.**—PANAMA. Prov. de Panamá: Cerro Jefe, cabecera del Río San Cristobal, 900 m, 27 Dec 1986, *I. Valdespino et al.* 268 (holotype: US; isotype PMA). **Fig. 1. A–E**

Species *Stenogrammitidi myosuroidi* similaris, sed pilis brevibus, indivisis (vs. furcatis), hyalinis (vs. subrubris) differt.

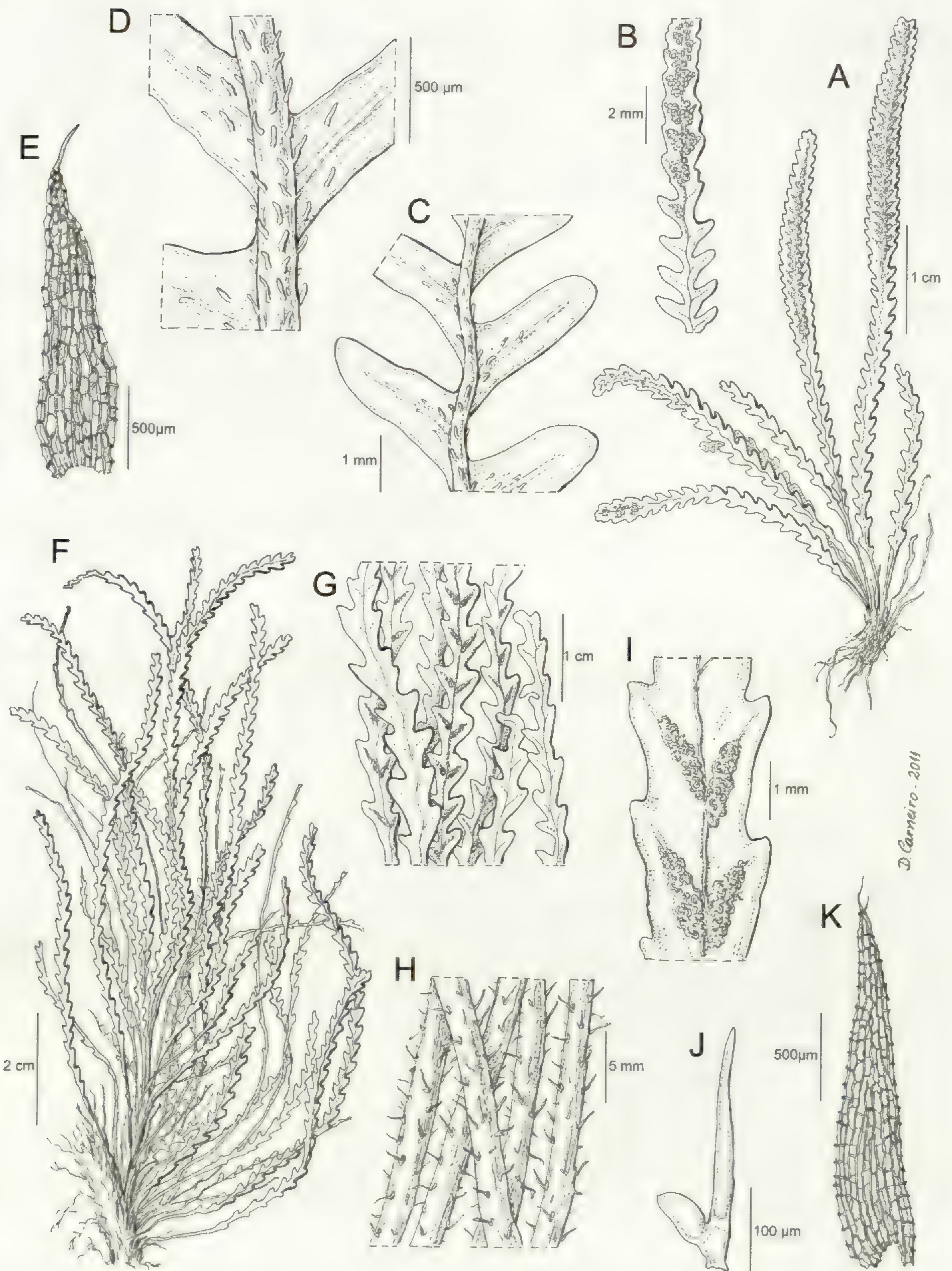


FIG. 1. A–E. *Stenogrammitis brevipubens* (all from the holotype). A. Habit. B. Detail showing the hemidimorphic lamina. C. Detail of the segments from the sterile portion. D. Detail showing the hairs on the abaxial surface. E. Rhizome scale. F–K. *Stenogrammitis* × *guatemalensis* (all from the holotype). F. Habit. G. Detail of the segments. H. Petioles showing the hairs. I. Detail of the fertile portion of the lamina. J. Furcate hair from the laminar tissue. K. Rhizome scale.

Plants epipetric; rhizome short creeping, radially symmetrical, scaly, the scales lanceolate, castaneous, clathrate, 1.5–2 mm long, glabrous or with a single apical cell, the cells in the medial portion isodiametric to elongate (two or three times longer than wide); petiole 0.5–1 cm × 0.3 mm, dark brown, slightly pubescent, the hairs hyaline, soft, straight, 2-celled, simple, appressed; lamina 2–5 cm long, erect or slightly arcuate, linear, chartaceous, hemidimorphic; sterile portion deeply pinnatisect, abruptly reduced at the base, the basal segment decurrent, but not ending in a long and narrow wing to the petiole base, the segments at the medial portion 1.8–2 × 0.5–0.7 mm, linear, set at 70–80° to the rachis, symmetrical or slightly asymmetrical at the base, the apex acute to obtuse, laminar tissue and veins slightly pubescent on both sides, the hairs hyaline, 1 or 2-celled, simple, appressed, margin glabrous; fertile portion slightly crenulate, cut ca. 1/5 the way to the rachises, equal to or shorter than the sterile portion; rachis with dark sclerenchyma exposed abaxially, covered by the laminar tissue adaxially, flexuous, pubescent, the hairs hyaline, soft, 0.1 mm long, 2-celled, simple, appressed; sinuses as broad as the width of the segments; veins simple, not visible in the sterile portion, the dark sclerenchyma slightly exposed in the fertile portion beneath the sporangia, adaxially with linear, well-marked hydathodes; sori inframedial, rounded to oblong, indistinct, confluent when mature, extending beyond the bases of the sinuses and the rachis, slightly sunken, leaving an impression on the laminar upper surface; sporangia glabrous; spores green, trilete.

ETYMOLOGY.—The specific epithet “*brevipubens*” refers to the short hairs that are present on the petiole, rachis, and laminar tissue, which are the main character that helps to distinguish this species from its congeners.

DISTRIBUTION.—*Stenogrammitis brevipubens* is known by a single collection from Panama.

Stenogrammitis brevipubens is characterized by having a hemidimorphic lamina, flexuous rachis, and hyaline, simple, soft, straight, 1 or 2-celled, and appressed hairs present on the petiole, rachis and laminar tissue. Among the species with hemidimorphic lamina *S. myosuroides* (Sw.) Labiak and *S. jamesonii* (Hook.) Labiak are the most similar species, which can be distinguished by having straight (or only slightly flexuous) rachis and reddish, furcate, 3–4-celled, spreading hairs. Furthermore, *S. brevipubens* is an epipetric species, whereas *S. myosuroides* and *S. jamesonii* are usually epiphytes.

Another Neotropical species that also present simple and hyaline hairs on the petiole and rachis is *S. pumila* (Labiak) Labiak, from southeastern Brazil. It differs from *S. brevipubens* by its monomorphic lamina.

CONSERVATION STATUS.—This species is currently known by a single collection, but with several individuals present in the type collection. This suggests that it might have a very narrow distribution but may form a dense population on the rocks of Cerro Jefe. Therefore, according to IUCN Red List Criteria (IUCN, 2001), it is assessed here as Data Deficient (DD).

Stenogrammitis × *guatemalensis* Labiak, **hybrid nov.** TYPE.—GUATEMALA. Depto. Alta Verapaz: Mun. Cobán Chicusha'b, 8 km al SW de Cobán, 15°26'N, 90°27'W, 22 Jul. 1988, P. Tenorio et al. 14729 (holotype: MO).

Fig. 1. F–K

Hybrida inculta e *Stenogrammitide prionode* et *S. limula* genita. Plantae epiphyticae; rhizoma erectum, paleis brunneis, iridescentibus. Frondes fasciculatae; petioli setis brevibus, furcatis vestiti; laminae pro partibus sterilibus pinnatisectae; segmenta deltata vel deltato-lineararia, nervis indivisis; sori lineares, impressi.

Plants epiphytic; rhizome erect, radial, scaly, the scales lanceolate, dark brown, iridescent, clathrate, 1–1.4 mm long, glabrous or with a single and furcate cilium at the apex, the cells in the medial portion isodiametric; petiole 0.5–1.5 cm × 0.3 mm, dark brown, slightly pubescent, the hairs spreading, reddish, rigid, straight, 3-celled, 1-furcate; lamina 10–15 cm long, arcuate, linear, chartaceous, slightly hemidimorphic; sterile portion deeply pinnatifid to pinnatisect, gradually reduced at the base, basal segment long-decurrent, ending in a long and narrow wing to the petiole base, the segments at the medial portion 1.5–2 × 1–1.5 mm, deltate to linear-deltate, set at 70–80° to the rachis, symmetrical to slightly asymmetrical at the base, the apex obtuse, laminar tissue, veins, and margins glabrous; fertile portion pinnatifid, cut ca. 1/3 the way to the rachises, about as long as the sterile portion, the segments deltate, obtuse; rachis on both sides with dark sclerenchyma exposed, pubescent, the hairs spreading, reddish, rigid and straight, 1-furcate, ca. 0.2 mm long; sinuses equal to or broader than the width of the segments; veins simple, not visible in the sterile portion, but with the dark sclerenchyma exposed in the fertile portion beneath the sporangia, adaxially with linear, well-marked hydathodes; sori inframedial, linear, distinct, not extending beyond the bases of the sinuses and the rachis, sunken, leaving an impression on the laminar upper surface; sporangia not well formed; spores not seen.

ETYMOLOGY.—The epithet “*guatemalensis*” refers to the type locality where the hybrid was found: Guatemala.

DISTRIBUTION.—*Stenogrammitis* × *guatemalensis* is known only from the type collection from Guatemala.

Stenogrammitis × *guatemalensis* is known by a single collection, which suggests a sporadic event of hybridization. Based on its intermediate morphology, the most probable parents are *Stenogrammitis limula* (H. Christ) Labiak and *S. prionodes* (Mickel & Beitel) Labiak, two of the commonest species of *Stenogrammitis* in Guatemala.

With *S. limula* it shares reddish, rigid, straight and 1-furcate hairs on the petiole and rachis, rhizome scales with isodiametric cells, and also linear and sunken sori that leave an impression on the adaxial surface of the lamina. It differs from *S. limula* by its slightly hemidimorphic lamina (vs. monomorphic), and longer rhizome scales (1–1.4 vs. 0.8–1.2), characters that are shared with *S. prionodes*.

TABLE 1. Comparison of the main characters that distinguish the species of *Stenogrammitis* known to Guatemala, and the hybrid.

	Leaf dimorphism	Rhizome scales length	Cells of the rhizome scales	Sori
<i>S. limula</i>	monomorphic	0.8–1.2 mm	isodiametric	linear and sunken, distinct
<i>S. prionodes</i>	hemidimorphic	2–3 mm	elongate	roundish and superficial, distinct
<i>S. jamesonii</i>	hemidimorphic	1.5–2 mm	isodiametric to elongate	confluent at maturity, indistinct
<i>S.</i> × <i>guatemalensis</i>	slightly hemidimorphic	1–1.4 mm	isodiametric to elongate	linear and sunken, distinct

Other than *Stenogrammitis limula* and *S. prionodes*, the only other species of *Stenogrammitis* known to Guatemala is *S. jamesonii* (Hook.) Labiak. Like *S. prionodes*, it also has a hemidimorphic lamina, and reddish and furcate hairs on the petiole and rachis. However, its fertile portion is shallowly crenulate, and the sori are conspicuously confluent when mature—characteristics that are not present in the hybrid. A summary of characters useful for distinguishing the species and hybrid of *Stenogrammitis* known from Guatemala is presented in Table 1.

Although hybridization is a common event among some groups of terrestrial ferns (e.g., *Anemia*, *Blechnum*, and *Dryopteris*), it seems to be very rare and occasional among epiphytic plants (Gómez, 1985). Records of hybrids among the Grammitid ferns are scarce in the literature and, as far as I know, only four cases have been reported so far (Parris, 1977; Parris, 1984; Labiak and Matos 2007; Christenhusz, 2009). Therefore, because it is an uncommon event among the Grammitid ferns, I consider it worthwhile to recognize this hybrid, even though it is represented by only a few individuals.

ACKNOWLEDGMENTS

This study was funded by a grant from the CNPq—Brazil (CNPq Proc. n° 301997/2010-1). I thank The New York Botanical Garden for providing herbarium and laboratory facilities, as well as the Curators of the following herbaria for loans: AAU, B, BM, BR, F, FR, GH, K, MAPR, MICH, MO, NY, P, PMA, S, SP, U, UC, US, and WU. I also thank Robbin Moran, John Mickel, Alejandra Vasco, and Michael Sundue for their help during my visit to NY. Diana Carneiro prepared the line drawings.

LITERATURE CITED

- CHRISTENHUSZ, M. 2009. Index Pteridophytorum Guadalupensium or a revised checklist to the ferns and club mosses of Guadeloupe (French West Indies). *Botanical Journal of the Linnean Society* 161:213–277.
- GÓMEZ, L. D. 1985. Ecology of some neotropical hybrid pteridophytes. *Proceedings of the Royal Society of Edinburgh* 86B:347–351.
- IUCN. 2001. Red List Categories and Criteria, Version 3.1. Prepared by the IUCN Species Survival Commission, IUCN, Gland, Switzerland, and Cambridge, United Kingdom.

- LABIAK, P. H. 2011. *Stenogrammitis*, a new genus of Grammitid Ferns segregated from *Lellingeria* (Polypodiaceae). *Brittonia* 63:139–149.
- LABIAK, P. H. and F. B. MATOS. 2007. A new hybrid and two new combinations in neotropical grammitid ferns. *Brittonia* 59:182–185.
- LABIAK, P. H., M. SUNDUE and G. ROUHAN. 2010. Molecular phylogeny, character evolution and biogeography of the grammitid fern genus *Lellingeria* (Polypodiaceae). *American Journal of Botany* 97:1354–1364.
- LEHNERT, M. 2008. Eleven new species in the Grammitid fern genus *Melpomene* (Polypodiaceae). *American Fern Journal* 98:214–250.
- PARRIS, B. S. 1977. A naturally occurring intergeneric hybrid in Grammitidaceae (Filicales): *Ctenopteris heterophylla* × *Grammitis billardierei*. *New Zealand Journal of Botany* 15:597–599.
- PARRIS, B. S. 1984. Another intergeneric hybrid in Grammitidaceae: *Ctenopteris longiceps* × *Grammitis sumatrana*. *Fern Gazette* 12:337–340.
- RANKER, T. A., M. SUNDUE, P. H. LABIAK, B. S. PARRIS and G. ROUHAN. 2010. New insights into the phylogeny and historical biogeography of the *Lellingeria myosuroides* clade (Polypodiaceae). [Internet]. Version 48. PLoS Currents: Tree of Life. 2010 Nov 18 [revised 2010 Nov 22]:PMC2989831.

Diplazium fimbriatum (Athyriaceae), a New Species from Brazil

CLAUDINE M. MYNSSEN

Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rua Pacheco Leão 915, Rio de Janeiro, RJ 22460-030, Brazil. e-mail: cmynssen@jbrj.gov.br

FERNANDO B. MATOS

The New York Botanical Garden, 2900 Southern Blvd., Bronx, NY 10458, U.S.A.
e-mail: fmatos@nybg.org

ABSTRACT.—A new species, *Diplazium fimbriatum* (Athyriaceae), is described and illustrated. It is endemic to the humid montane forests of eastern Brazil, a region known for its high level of endemism and species richness. A comparative table to distinguish it from similar species of *Diplazium* occurring in Brazil is provided.

KEY WORDS.—Athyrioid ferns, Atlantic Forest, Bahia, Espinhaço Mountains, Minas Gerais, taxonomy

The Athyriaceae comprises five genera and ca. 600 species (Christenhusz *et al.*, 2011; Rothfels *et al.*, 2012), the majority of which were placed in Woodsiaceae by Smith *et al.* (2006, 2008). *Diplazium* comprises approximately 400 species, with well over 100 in tropical America, where the highest diversity is concentrated in the Andean and Guyanan regional centers (Tryon, 1972). The present work is part of a revision of the genus *Diplazium* in Brazil, which has indicated the occurrence of 22 species in the country, most of these being widely distributed in the coastal Atlantic Forest (Mynssen *et al.*, 2009), including eight endemics (Mynssen, 2010). During this revision more than 30 localities were visited, including the states of Bahia, Espírito Santo, Minas Gerais, Paraná, and Rio de Janeiro. Furthermore, specimens from 45 herbaria were examined, including B, BM, BHCN, CEPEC, CESJ, HB, HBR, INPA, IPA, K, MBM, MBML, MG, NY, OUP, P, R, RB, S, SP, SPF, UPCB, and US (Thiers, 2009).

The species of *Diplazium* are predominantly terrestrial, rarely epipetric, and can be distinguished by the following characters: stems usually ascending to erect, rarely long-creeping, bearing scales at the apex; scales usually non-clathrate, brown to blackish-brown, margin entire or toothed; leaves monomorphic, rarely dimorphic; petioles with two crescent-shaped vascular bundles at the base (in cross section), these uniting distally; lamina simple to 1–4-pinnate-pinnatifid, glabrous or pubescent; veins generally free (simple or furcated), or anastomosing without included veinlets; sori elongate, elliptic to linear, borne on both sides of the vein (diplazioid) or on only one side; indusia generally present, rarely absent (among Brazilian species, indusia are absent, or nearly so, only in *D. lindbergii*), paraphyses absent; spores ellipsoidal, monolete, with wing-like folds, the surface smooth, reticulate or papillate to echinate between the folds.

Diplazium fimbriatum Mynssen & F. B. Matos, *sp. nov.* TYPE.—BRAZIL. Bahia: Camacan, Serra Bonita, Fazenda Uiraçu, trilha da Bapeba, 15°23'30"S, 39°33'55"W, 890 m, 22 Oct 2009, C. M. Mynssen & F. B. Matos 1167 (holotype: RB; isotypes: CEPEC, MO, NY, UC, UPCB). **Figs. 1, 2.**

Diplazium fimbriatum *D. mutilo* Kunze multis notis simile a quo margine longo-fimbriato indusii et praesentia gemmarum in rachidi recedit. *Diplazium mutilum* a *D. fimbriato* etiam margine integro vel dentato indusii sui et gemmis in rachidi absentibus differt.

ETYMOLOGY.—From Latin “fimbriatus,” fringed; referring to the fimbriate margins of the indusia.

Plants terrestrial; *stem* 20–40 × 1–1.5 cm, ascending to erect, bearing scales at the apex; *scales* 1.2–2 × 0.1–0.3 cm, concolorous, brown, lanceolate, basifixed, the apices acuminate, sinuate, margins entire to dentate; *leaves* to 150–180 cm long, erect to arched, fasciculate; *petioles* 55–80 × 0.8–1 cm, brown, tomentose, with septate hairs 0.1–0.3 mm, and scales like those of the stems; *lamina* 94–149 × 40–65 cm, chartaceous, 1-pinnate-pinnatifid, lanceolate, gradually tapering to a pinnatifid apex, with proliferous buds in the axils of distal pinnae; *rachises* with septate hairs 0.2–0.4 mm long in adaxial grooves, linear scales 1.5–2.5 × 0.1–0.2 mm and lanceolate scales 1.5–4 × 0.5–0.8 mm abaxially and adaxially; *pinnae* 28–37 × 3–5.5 cm, stalked to 0.4–1.2 cm, 6–14 pairs per lamina, oblong-ovate to deltate, incised 2/3 or less to the costae, bases truncate or round, apices acuminate, margins crenate to slightly serrate, without differentiated marginal cells; *costae* grooved adaxially, with vertical laminar wings 0.2–0.5 mm wide; *veins* free, pinnate, simple or furcate; *indument* adaxially restricted to costal grooves, the hairs similar to those on the rachis, abaxial side of the veins with linear scales, 0.8–1 × ca. 0.1 mm, laminar tissue between veins glabrous on both sides of the lamina; *sori* 5–8 × 0.3 mm, oblong, diplazioid or not; *indusia* commonly persistent, membranaceous, brownish, margins fimbriate, the fimbriae 0.4–0.6 mm long; *spores* brown, ellipsoidal, monolete.

DISTRIBUTION AND ECOLOGY.—Restricted to the humid montane forests of southern Bahia and Minas Gerais, Brazil, where it grows in deep shade and along stream banks, at 250–1200 m. *Diplazium fimbriatum* occasionally shares its habitat with *D. celtidifolium* Kunze, *D. lindbergii* (Mett.) Christ, and *D. mutilum* Kunze, which could favor hybridization within the genus.

CONSERVATION STATUS.—Vulnerable (VU), under criteria D2 (IUCN, 2010). Based on the available collections and recent expeditions carried out by the authors throughout the Brazilian Atlantic Forest, *Diplazium fimbriatum* has a very restricted distribution, being known from only six localities in eastern Brazil (Fig. 2). Although most of these populations are within legally protected areas (i.e., national parks and biological reserves), human activities such as logging and replacement of forests by cattle ranches and plantations (cocoa, coffee and sugarcane) continue to be serious threats. Particularly alarming is the rapid deforestation of Serra do Corcovado, in the municipality of Almadina, a

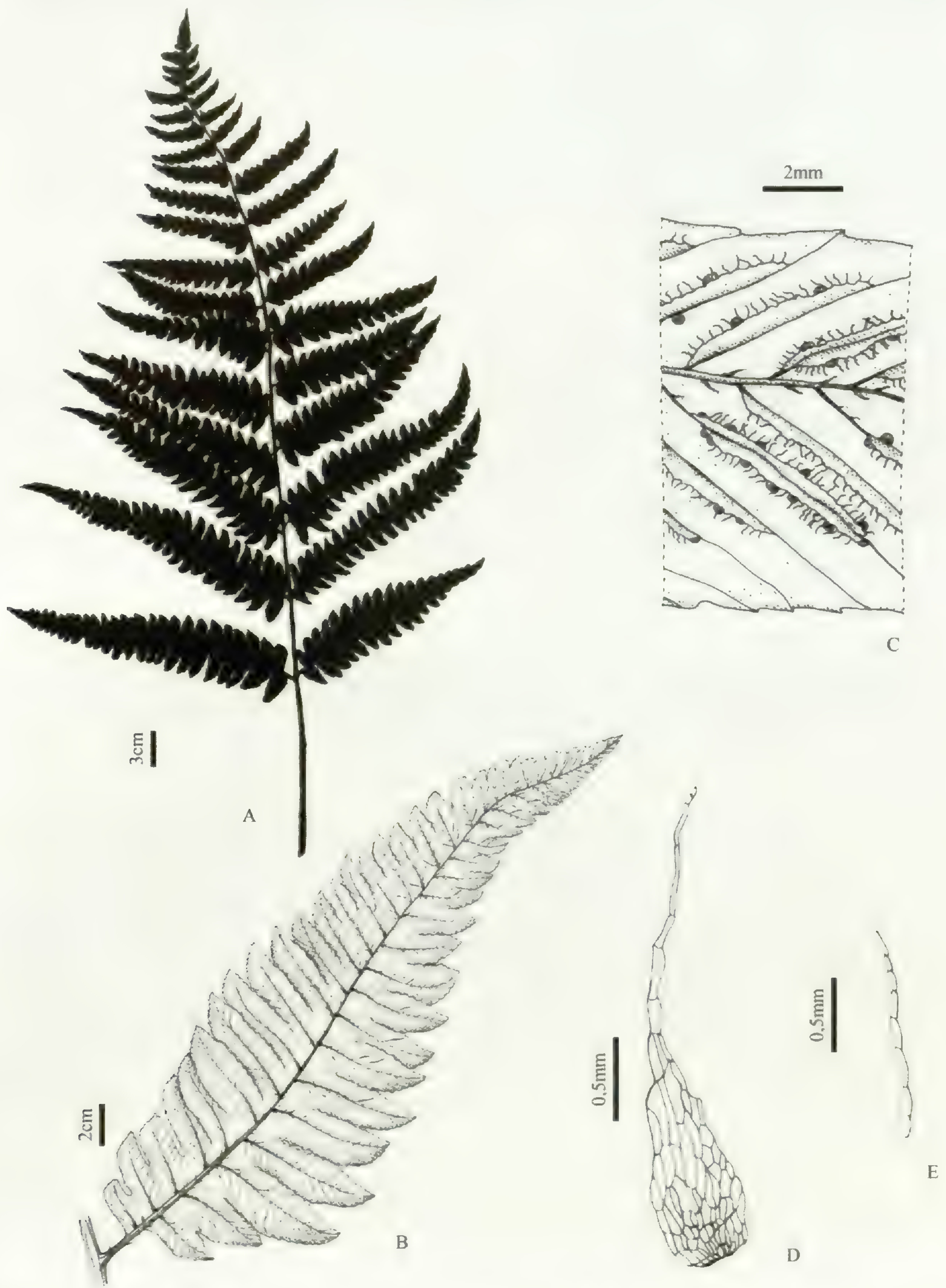


FIG. 1. *Diplazium fimbriatum* A. Lamina (C. M. Mynssen & F. B. Matos 1167). B. Proximal pinnae. C. Abaxial side of median segment, showing sori and indusial margin. D. Lanceolate scale of the lamina. E. Septate hair of the lamina (F. B. Matos et al. 236).



FIG. 2. Distribution map of *Diplazium fimbriatum*, with protected areas indicated.

locality that is becoming well known for increasing records of rare species of ferns (e.g., *Adiantum diphyllum* (Fée) Maxon, *Asplenium truncorum* F. B. Matos, Labiak & L. Sylvestre, and *Megalastrum indusiatum* R. C. Moran, J. Prado & Labiak) and angiosperms (A.M. Amorim, pers. comm.).

PARATYPES.—BRAZIL. **Bahia:** Almandina, Serra do Corcovado, 9.8 km ao SW de Coarací pela estrada para Almandina, seguindo N até a Fazenda São José, 14°42'21"S, 39°36'12"W, 650–750 m, 29 Jan 2005, *F. B. Matos et al.* 236 (CEPEC, MBM, NY, RBR, UPCB); Idem, 19 Apr 2007, *F. B. Matos et al.* 1395 (CEPEC, NY, UPCB); Arataca, Serra do Peito de Moça, estrada entre Arataca e Una, ramal ca. 22.4 km de Arataca, entrada pelo assentamento Santo Antônio, Reserva Particular do Patrimônio Natural Caminho das Pedras, 15°10'25"S, 39°20'30"W, 1000 m, 16 Feb 2006, *F. B. Matos et al.* 994 (CEPEC, UPCB); Camacan, Serra Bonita, Fazenda Paris, trilha às margens de córrego, 15°30'52"S, 39°40'27"W, 250m, 21 Oct 2009, *C. M. Mynssen & F. B. Matos* 1154, 1155 (CEPEC, RB); Wenceslau Guimarães, Estação Ecológica Estadual Nova Esperança, início da trilha para o Pico do Urubu, 13°35'34"S, 39°42'58"W, 450–650 m, 4 May 2007, *J. Jardim et al.* 5024 (CEPEC, UPCB). **Minas Gerais:** Conceição do Mato Dentro, Parque Natural do Ribeirão do Campo, 1 Oct 2002, *R. C. Mota et al.* 1540 (BHCB); Idem, mata de galeria do córrego da mina, 19°06'19"S, 43°34'04"W, 1175m, 30 May 2005, *A. Salino et al.* 8748 (BHCB).

Diplazium costale (Sw.) C. Presl var. *robustum* (Sodirol) Stolze, endemic to Ecuador (Stolze *et al.* 1994), resembles *D. fimbriatum* in blade dissection, leaf length, and presence of proliferous buds on the axils of distal pinnae. Nevertheless, it differs from *D. fimbriatum* by abundant costal scales and the robust falcate sori closer to the costae with entire indusia (vs. delicate oblong sori with fimbriate indusia). Fée (1869) applied the name *D. costale* to two specimens collected in Brazil; however, a careful examination of these specimens shows that *Blanchet* 535 (NY) refers to *D. celtidifolium* Kunze, whereas *D. rostratum* Fée would be the correct name for *Miers* 164 (K).

Also similar is *Diplazium macrophyllum* Desv. (Venezuela, Colombia, Ecuador, Peru, and Bolivia), which differs by the broader pinnae (9–22 vs. 3–5.5 cm), and the shape of the apices of ultimate segments, which are attenuate or acuminate (vs. obtuse to acute in *D. fimbriatum*). Furthermore, the veins of *D. macrophyllum* are sparsely provided abaxially with lanceolate to deltate scales (1–1.5 × 0.3–0.5 mm), whereas in *D. fimbriatum* these scales are filiform to linear.

Several other species that occur in Brazil are also probably related to the new species (Table 1). *Diplazium celtidifolium* Kunze has similar linear and lanceolate scales on the rachises, and also has buds on the axils of distal pinnae, but differs from *D. fimbriatum* by the shape of the pinnae (oblong to lanceolate vs. oblong-ovate to deltate), and indusia margins (entire to dentate vs. fimbriate). Moreover, the pinnae of *D. celtidifolium* are usually less incised, often entire. From *D. mutilum* Kunze, a Brazilian endemic, *D. fimbriatum* differs by the presence of proliferous buds and fimbriate indusia (vs. entire to

TABLE 1. Comparisons between *Diplazium fimbriatum* and other similar species of *Diplazium* occurring in Brazil.

Species	Proliferous buds	Proximal pinnae	Pinnae indument	Indusia
<i>D. fimbriatum</i>	Present (axils of distal pinnae)	oblong to ovate	septate hairs plus linear and lanceolate scales	long-fimbriate catenate
<i>D. mutilum</i>	Absent	oblong	septate hairs and filiform scales	entire to dentate
<i>D. celtidifolium</i>	Present or not (axils of distal pinnae)	oblong to lanceolate	septate hairs and linear scales	entire to dentate
<i>D. lindbergii</i>	Absent	oblong	septate hairs and filiform scales	absent or vestigial

dentate in *D. mutilum*). Another species in Brazil, *D. lindbergii* (Mett.) Christ, has similar habit, blade dissection and leaf length, but can be easily distinguished by indusia absent or vestigial and lack of proliferous buds on the lamina.

ACKNOWLEDGMENTS

We thank Dr. André M. Amorim (CEPEC, UESC), Dr. Vitor O. Becker (RPPN Serra Bonita), and staff of the CEPEC herbarium for supporting field work in southern Bahia, Brazil. Thanks also to Dr. William A. Rodrigues (UFPR) for the Latin diagnosis, Tainan Messina and Nina P. Monteiro (CNCFlora) for drafting the map, and Maria Alice Rezende for preparing the illustrations. Special thanks to Dr. Lana S. Sylvestre for supervising and suggestions, Dr. Robbin C. Moran and Dr. James L. Luteyn for helpful comments on the manuscript. This research was largely funded by CAPES and the Kew Latin America Research Fellowship Programme - Prance Fellowship in Neotropical Botany (2007).

LITERATURE CITED

- IUCN STANDARDS AND PETITIONS SUBCOMMITTEE. 2010. Guidelines for Using the IUCN Red List Categories and Criteria. Version 8.1. Prepared by the Standards and Petitions Subcommittee in March 2010. Downloadable from <http://intranet.iucn.org/webfiles/doc/SSC/RedList/RedListGuidelines.pdf>.
- CHRISTENHUSZ, M. J. M., X.-C. ZHANG and H. SCHNEIDER. 2011. A linear sequence of extant families and genera of lycophytes and ferns. *Phytotaxa* 19:7–54.
- FÉE, A. L. A. 1869. *Cryptogames vasculaires du Brésil*. v. I. Veuve Berger-Levrault & Fils Libraires, Paris.
- MYNSSEN, C. M. 2010. Woodsiaceae. Pp. 566–567. In: R. C. Forzza, J. F. Baumgratz, C. E. M. Bicudo, A. Carvalho Jr, A. Costa, D. P. Costa, M. J. G. Hopkins, P. Leitman, L. G. Lohmann, L. C. Maia, G. Martinelli, M. Menezes, M. P. Morim, M. A. Nadruz, A. L. Peixoto, J. R. Pirani, J. Prado, L. P. Queiroz, V. C. Souza, J. R. Stehmann, L. S. Sylvestre, B. M. T. Walter and D. Zappi (Orgs.). *Catálogo de plantas e fungos do Brasil 1*. Instituto Jardim Botânico do Rio de Janeiro.
- MYNSSEN, C. M., A. SALINO and T. E. ALMEIDA. 2009. Woodsiaceae. In: J. R. Stehmann, R. C. Forzza, A. Salino, M. Sobral, D. P. Costa and L. H. Y. Kamino. *Plantas da Floresta Atlântica*. Instituto Jardim Botânico do Rio de Janeiro 1:111.
- PROCTOR, G. R. 1985. *Ferns of Jamaica*. British Museum (Natural History), London.
- ROTHFELS, C. J., M. A. SUNDUE, L. -Y. KUO, A. LARSEN, M. KATO, E. SCHUETTELZ and K. M. PRYER. 2012. A revised family-level classification for eupolypod II ferns (Polypodiidae: Polypodiales). *Taxon*, (in press).

- SMITH, A. R., K. M. PRYER, E. SCHUETTPPELZ, 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. SCHUETTPPELZ, P. KORALL, H. SCHNEIDER and P. G. WOLF. 2008. Fern classification. Pp. 417–467. *In*: T. A. Ranker and C. H. Hafler, eds. *Biology and evolution of ferns and lycophytes*. Cambridge University Press, United Kingdom.
- STOLZE, R. G., L. PACHECO and B. ØLLGAARD. 1994. Polypodiaceae–Dryopteridaceae–Physematiaceae. *In*: G. Harling and L. Andersson, eds. *Flora of Ecuador* 49:1–108.
- THIERS, B. 2009 [continuously updated]. Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. <http://sweetgum.nybg.org/ih/>
- THOMAS, W. W., A. M. CARVALHO, A. M. AMORIM, J. GARRISON and A. L. ARBELÁEZ. 1998. Plant endemism in two forests in southern Bahia, Brazil. *Biodiversity and Conservation* 7:311–322.
- TRYON, R. M. 1972. Endemic areas and geographic speciation in tropical American ferns. *Biotropica* 4:121–131.

Isoetes mourabaptistae, a New Species from Southern Brazil

JOVANI B. PEREIRA

Universidade Federal do Paraná, Depto. de Botânica, C.P. 19031, 81531-980, Curitiba, PR, Brazil,
e-mail: giovanibio@gmail.com

PAULO G. WINDISCH

Universidade Federal do Rio Grande do Sul, Depto. de Botânica, C.P. 43433, 91501-970, Porto Alegre, RS, Brazil, e-mail: pteridos@gmail.com

MARIA L. LORSCHETTER

Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Depto. de Botânica, C.P. 9500, 91540-000, Porto Alegre, RS, Brazil, e-mail: mlorsch@uol.com.br

PAULO H. LABIAK*

Universidade Federal do Paraná, Depto. de Botânica, C.P. 19031, 81531-980, Curitiba, PR, Brazil,
e-mail: plabiak@ufpr.br

ABSTRACT.—*Isoetes mourabaptistae*, a new species from southern Brazil, is described, illustrated, and compared to the most similar species. This new species is apparently restricted to southern Brazil, and is characterized by cristate to irregularly reticulate megaspores and microechinate microspores. It is an aquatic plant, occurring among submersed rocks along rivers, at about 900–1100 m in elevation.

KEY WORDS.—Isoetaceae, taxonomy, lycophytes, diversity, aquatic plants

The genus *Isoetes* L. comprises approximately 350 species (Hickey *et al.*, 2003), most of them occurring as aquatic plants in lakes and streams, but also as terrestrials on permanent or seasonally wet soils. The genus is widely distributed around the world, but many of its species tend to have very narrow distributions (Hickey, 1986; Hickey *et al.*, 2003; Small and Hickey, 2001; Luebke and Budke, 2003; Choi *et al.*, 2008).

Hickey (1990) considered South America to be a center of both morphological and taxonomic diversity for *Isoetes*. However, this is still one of the less known regions, and published estimates of the diversity of *Isoetes* in South America vary considerably. Two of the most important contributions to South American *Isoetes*, provided by Fucks-Eckert (1982) and Hickey (1985), suggest, respectively, between 75 and 47 species for this region. Undoubtedly much work is still needed to assess the actual diversity in South America. In Brazil particularly, *Isoetes* is one of the less known groups of lycophytes, and most of the species are represented by a very limited number of collections. Prado and Sylvestre (2010) recorded 14 species for Brazil (12 endemics), most of them

* Corresponding author

distributed along the coastal mountains of the southeastern and southern regions.

A study on the spore morphology of ferns and lycophytes of Rio Grande do Sul (Lorscheitter *et al.*, 2009), and additional fieldwork conducted by the authors, has identified the presence of a new species of *Isoetes* that we describe herein.

Isoetes mourabaptistae J. B. S. Pereira, P. G. Windisch, M. L. Lorscheitter, and Labiak, *sp. nov.* TYPE.—BRAZIL. Rio Grande do Sul, São Francisco de Paula, Rio Tainhas, 1 Oct. 2005, P. G. Windisch 10056 (Holotype: PACA 107431). **Figs. 1 A–B; 2 A–D; 3 A–D.**

Plantae parvae, minus quam 15 cm altae. Cormus erectus, bilobatus. Radices synchronae, dichotome divisae, conspicue succulentae. Folia chartacea, 10–35, ascendunt, 6–11 cm longa. Alae castaneae, apice vulgo truncatae, 20–25% per foliae longitudinem ascendentes. Subula olivacea, ascendens, apice attenuata. Squamae absentes. Labium absens. Ligula deltato-lanceolata. Velum incompletum. Sporangium basale ellipticum, hyalinum. Megasporae albae, triletae, 558–736 μm diametro, hemisphaero distali reticulato vel cristato, hemisphaero proximali reticulato. Microsporae monoletae, microechinatae, 24–31 μm longae, 13–22 μm latae.

Plants submerged, growing among rocks in streams, less than 15 cm tall. Corm erect, bilobed, 3–10 mm wide. Roots synchronous, dichotomous. Microphylls 10–35, ascending, linear, chartaceous, 6–11 cm long, 4–6 mm wide at the base and 1–2.5 mm wide in the medial portion, base of the microphylls alate, the alae 1.4–2.3 cm long, 0.5–0.8 mm wide (above the sporangium), extending 20–25% the length of the microphyll, castaneous, apices often truncate. Subula olive green, ascending, semiterete, the apex attenuate. Scales absent. Labium absent. Ligule deltate-lanceolate, membranaceous, 2.2–2.5 mm long and 1.4–1.8 mm wide. Velum incomplete, covering 50–75% of the sporangium width, and 25–50% of the sporangium length. Sporangium basal, elliptic, hyaline, 3.1–3.4 mm long, 1.9–2.4 mm wide. Megaspores whitish, trilete, globose in equatorial view, globose to subtriangular in proximal view, 558–736 (\bar{x} = 669) μm wide, irregularly reticulate to densely cristate distally, irregularly reticulate proximally. Microspores gray, monolete, elliptic, 30–38 (\bar{x} = 34) μm long, 23–28 (\bar{x} = 25) μm wide, perispore microechinate.

ADDITIONAL SPECIMENS EXAMINED.—BRAZIL. Rio Grande do Sul: São José dos Ausentes, Silveira, 09 Dez. 1994, R. Bueno 4471 (ICN).

ETYMOLOGY.—The specific epithet honors Dr. Luis Rios de Moura Baptista, botanist from the Federal University in Porto Alegre, Rio Grande do Sul.

DISTRIBUTION AND ECOLOGY.—*Isoetes mourabaptistae* is known from only two collections: one from São Francisco de Paula, and the other from São José dos Ausentes, both in the northeastern part of Rio Grande do Sul. It was found

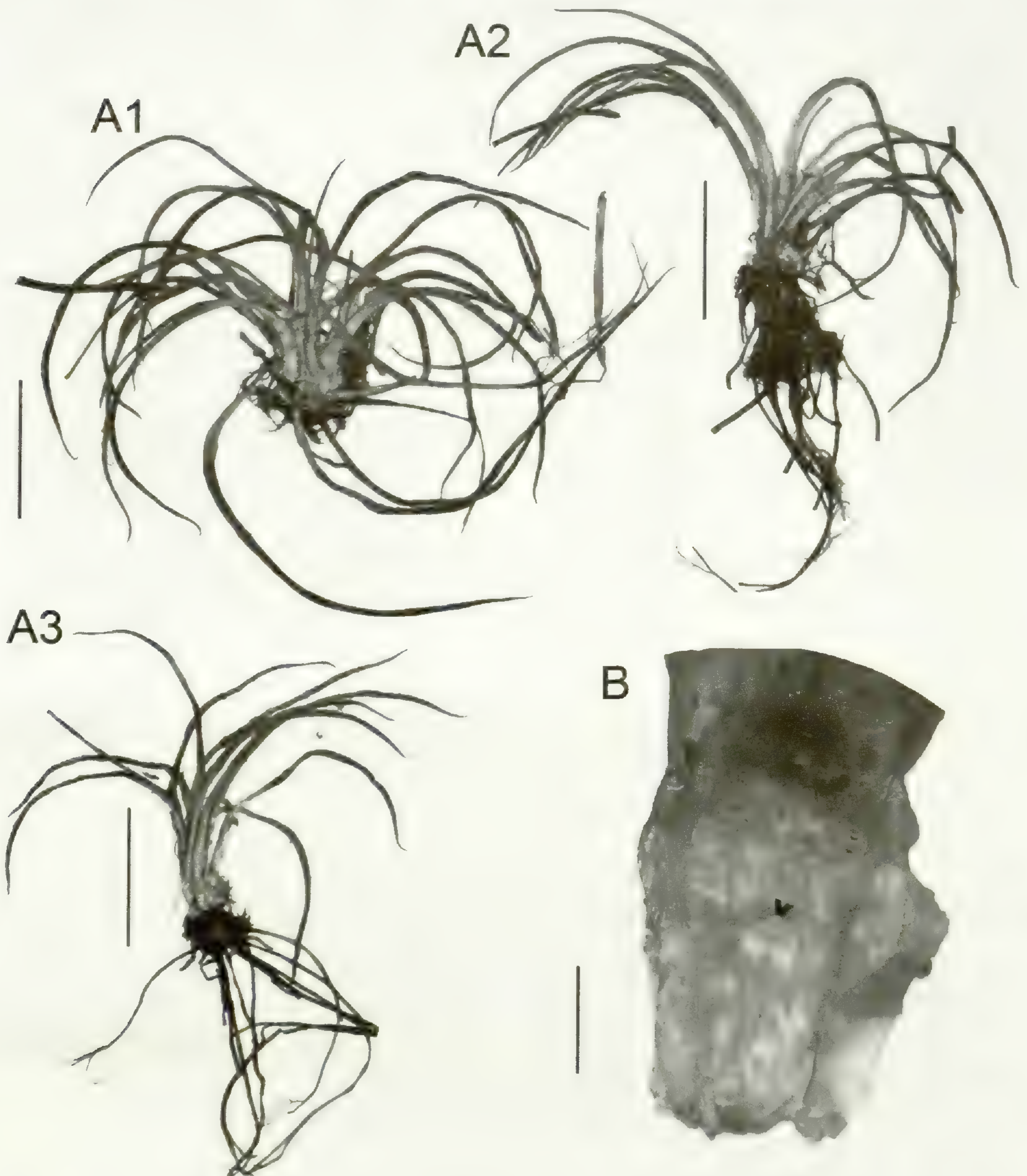


FIG. 1. A–B. A. Habit of *Isoetes mourabaptistae*. B. Velum (the arrow indicates the edge of the velum with the fenestra below). Scale bars: A = 2 cm and B = 1 mm. (All from the Holotype, P. G. Windisch 10056, PACA).

growing submerged, on rocks in shallow, clear waters, at altitudes of about 900–1100 m.

TAXONOMIC NOTES.—According to Hickey *et al.* (2009), in the southern part of South America there is a group of species with reticulate megaspores that is very complex taxonomically. This group includes species such as *I. brasiliensis* H. P. Fuchs, *I. ekmanii* U. Weber, *I. fuscomarginata* H. P. Fuchs,

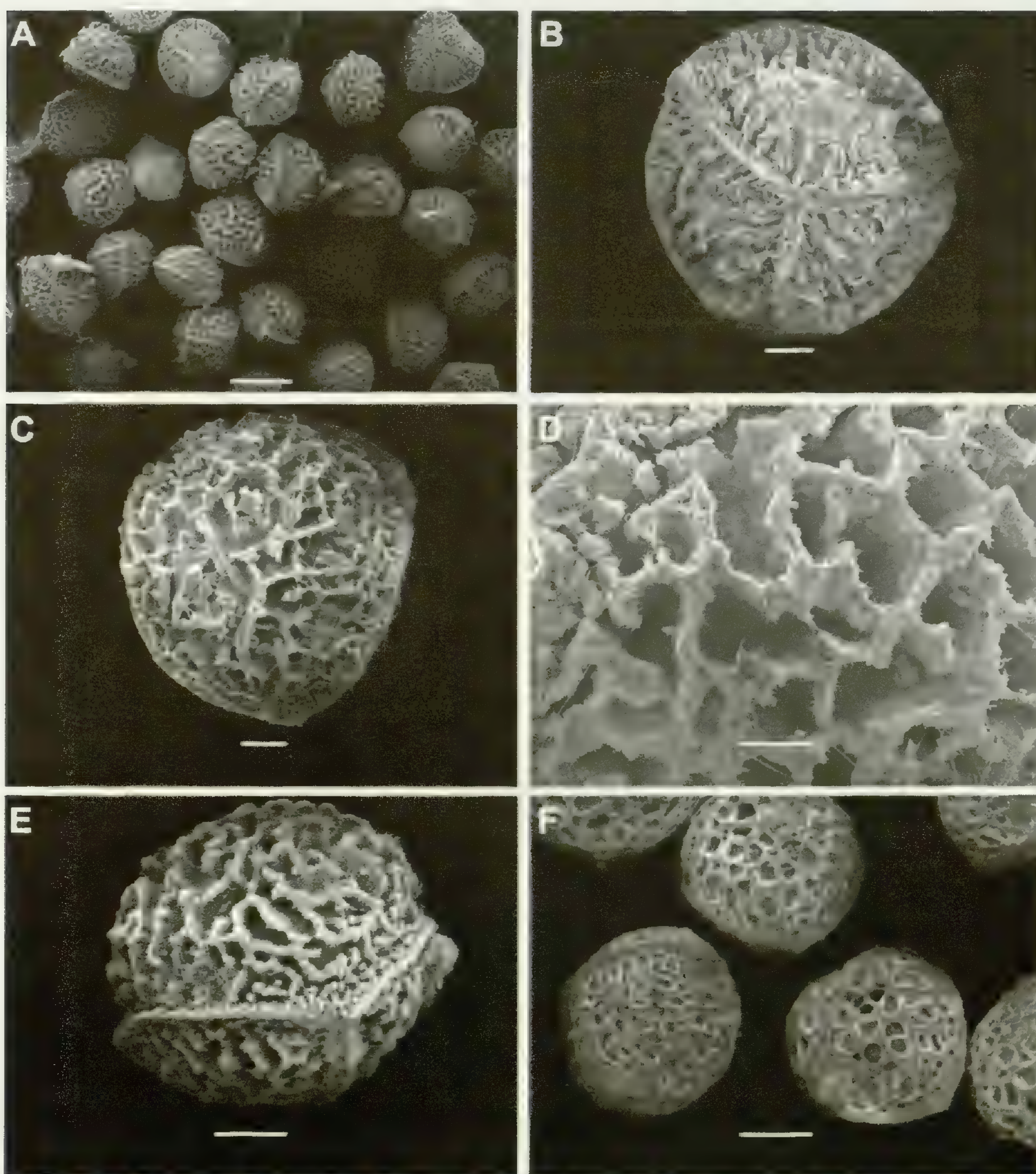


FIG. 2. A–F. SEM images of the megaspores of *Isoetes mourabaptistae*, *I. sehnemii*, and *I. spannagelii*. A–D. *Isoetes mourabaptistae* (Windisch 10056, PACA). A. Overview of the megaspores. B. Proximal view. C. Distal view. D. Detail of the megaspores in distal view. E. *I. sehnemii* (Sehnem 17094, PACA), megaspore in equatorial view. F. *I. spannagelii* (Pereira et al. 626, UPCB), megaspores in different positions. Scale bars: A = 500 μm ; B, C, and E = 100 μm ; D = 50 μm and F = 200 μm .

I. martii A. Br., *I. organensis* U. Weber, *I. ramboi* Herter, *I. smithii* H. P. Fuchs, *I. sehnemii* H. P. Fuchs, and *I. spannagelii* H. P. Fuchs. Because *Isoetes mourabaptistae* has megaspores that are irregularly reticulate, we believe that it may belong to this group. It can be distinguished from most of those species, however, by its larger megaspores (558–736 μm in diameter), and by its

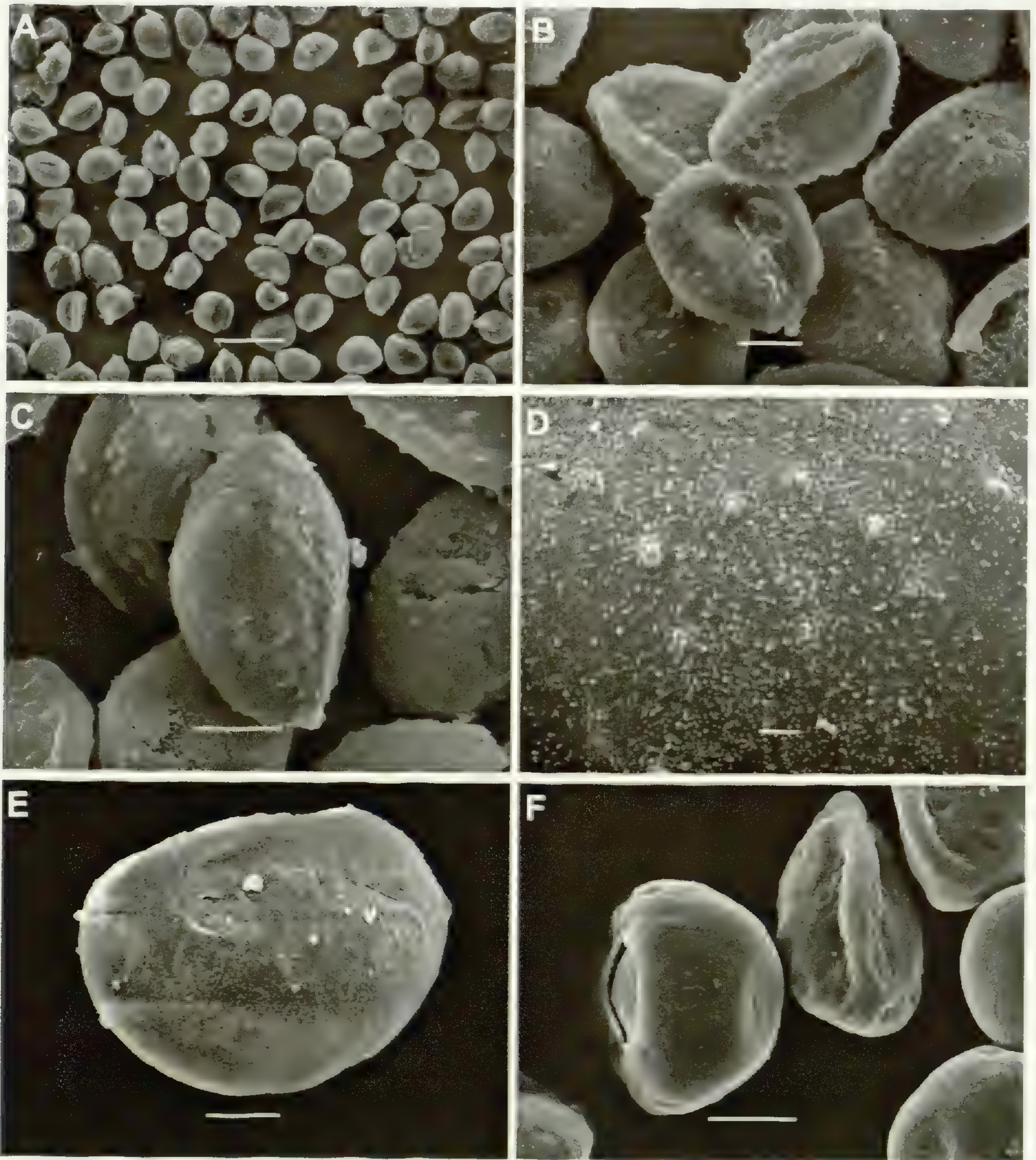


FIG. 3. A–F. SEM of the microspores of *Isoetes mourabaptistae*, *I. sehnemii* and *I. spannagelii*. A–D. *Isoetes mourabaptistae* (Windisch 10056, PACA). A–B. Overview of the microspores. C. Distal view of a microspore. D. Detail of the outer coating showing the microechinate surface. E. *I. sehnemii* (Sehnem 17094, PACA), equatorial view. F. *I. spannagelii* (Pereira et al. 626, UPCB), overview of the microspores. Scale bars: A = 50 μm ; B, C and F = 10 μm ; D = 1 μm and E = 5 μm .

microechinate microspores. In contrast, the megaspores of most of the species from southern Brazil are usually less than 400 μm in diameter, and the microspores always have smooth outer coatings.

In the size of the microphylls (up to 11 cm long) and habit (Fig. 1 A), *Isoetes mourabaptistae* resembles *I. sehnemii* H. P. Fuchs and *I. spannagelii* H. P. Fuchs. However, the megaspores of *I. sehnemii* are much smaller (332–480

(–554) μm diam.) and the microspores are smooth (Figs. 2E and 3E). In *I. spannagelii* the megaspores are 406–562(–603) μm in diameter and distinctly and consistently reticulate. The microspores are also smooth (Figs. 2F and 3F).

Hans Peter Fucks-Eckert labeled a collection made by Luis Rios Moura-Baptista as '*Isoetes batistae*' (*Moura-Baptista 7719*, PACA), and also cited this name on pages 237 and 256 of his work published in 1982 (Fucks-Eckert, 1982). However, Fucks-Eckert never validly published this new name (therefore a *nomen nudum*). The specimen at the PACA Herbarium is very depauperate, with only fragments of the microphylls. Even though it was collected in the same river, and likely represents the species described here, we cannot be sure about its identity given the absence of the characters that distinguish this species from its congeners.

CONSERVATION STATUS.—*Isoetes mourabaptistae* is currently known from only two collections, from two different rivers in southern Brazil. This suggests that it is locally rare and has a narrow distribution, therefore deserving special attention relative to its conservation status. However, based on our current knowledge of its population size and geographic distribution, and according to IUCN Red List Criteria (IUCN, 2001), it is assessed here as Data Deficient (DD).

ACKNOWLEDGEMENTS

We thank the “Centro de Microscopia Eletrônica da UFPR” and Priscila Izabel Tremarin for their help with the SEM images. We also thank Dr. Thelma Veiga Ludwig and Dr. William Rodrigues for their valuable help in this work. This study is part of Jovani Souza's Master Dissertation, which was made possible through a fellowship from CAPES. Paulo Labiak, Paulo Windisch, and M. Luisa Lorscheitter are Research Grant recipients from the Brazilian Research Council (CNPq).

LITERATURE CITED

- CHOI, H.-K., J. JUNG and C. KIM. 2008. Two new species of *Isoetes* (Isoetaceae) from Jeju Island, South Korea. *J. Pl. Biol.* 51:354–358.
- FUCHS-ECKERT, H. P. 1982. Zur heutigen Kenntnis von Vorkommen und Verbreitung der südamerikanischen *Isoetes*-Arten. *Proc. Koninkl. Neder. Akad. Wetensch. Ser. C.* 85:205–260.
- FUCHS-ECKERT, H. P. 1986. Isoetáceas. Pp. 3–43, in R. Reitz, ed. *Flora Ilustrada Catarinense*. Itajaí, Santa Catarina, Brasil.
- HICKEY, R. J. 1985. Revisionary studies of Neotropical *Isoetes* Ph.D. Dissertation, The University of Connecticut, Storrs, CT.
- HICKEY, R. J. 1986. The early evolutionary and morphological diversity of *Isoetes*, with description of two new Neotropical species. *Syst. Bot.* 11:309–321.
- HICKEY, R. J. 1990. Studies of Neotropical *Isoetes* L. I. *Euphyllum*, a new Subgenus. *Ann. Missouri Bot. Gard.* 77:239–245.
- HICKEY, R. J., W. C. TAYLOR and N. T. LUEBKE, N. T. 1989. The species concept in Pteridophyta with special reference to *Isoetes*. *Amer. Fern. J.* 79:78–89.
- HICKEY, R. J., C. C. MACLUF and W. C. TAYLOR. 2003. A re-evaluation of *Isoetes savatieri* Franchet in Argentina and Chile. *Amer. Fern. J.* 93:126–136.
- HICKEY, R. J., C. C. MACLUF and M. LINK-PERÉZ. *Isoetes maxima*, a new species from Brazil. *Amer. Fern. J.*, 99:194–199.
- LORSCHETTER, M. L., A. R. ASHRAF, P. G. WINDISCH and V. MOSBRUGGER. 2009. Pteridophyte spores of Rio Grande do Sul flora, Brazil, Part VI. *Palaeontographica* 281:1–96.

- LUEBKE, N. T. and J. M. BUDKE. 2003. *Isoetes tennesseeensis* (Isoetaceae), an octoploid quillwort from Tennessee. *Amer. Fern. J.* 93:184–190.
- PRADO, J. and L. S. SYLVESTRE. 2010. Isoetaceae in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. (<http://floradobrasil.jbrj.gov.br/2010/fb091270>).
- SMALL, R. L. and R. J. HICKEY. 2001. Systematics of the Northern Andean *Isoetes karstenii* Complex. *Amer. Fern. J.* 91:41–69.
- TAYLOR, W. C. and R. J. HICKEY. 1992. Habitat, evolution, and speciation in *Isoetes*. *Ann. Missouri Bot. Gard.* 79:613–622.

SHORTER NOTES

Agravitropic Growth of the Early Leaves of Apogamous Sporophytes of *Dryopteris tyrrhena*.—*Dryopteris tyrrhena* Fraser-Jenk. & Reichst., a Western Mediterranean endemic, is a threatened species represented by a few populations in Spain, France, and Italy (Magrini and Scoppola, Inform. Bot. Ital. 42(2):595–597. 2010). The relict type of its distribution (rare and scattered with big gaps between different localities, mostly in crevices and caves) suggests that *D. tyrrhena* is an old species of the Tertiary flora of the Mediterranean mountains (Fraser-Jenkins *et al.*, Fern Gaz. 11(2–3):177–198. 1975; Bernardello and Martini, *Felci e piante affini in Liguria e in Italia*. Le Mani-Microart's Edizioni, Recco-Genova. 2004). It is an allotetraploid species ($2n=164$) originated by interspecific hybridization between the diploid species *D. oreades* Fomin ($2n=82$) and *D. pallida* (Bory) Maire & Petitm. ($2n=82$) with subsequent chromosomes doubling (Fraser-Jenkins *et al.*, 1975).

This study on the *in vitro* development of apogamous sporophytes of *Dryopteris tyrrhena* (Magrini, *Plant Biosystems* 145(3):635–637. 2011; Magrini *et al.*, *Studi Trent. Sci. Nat.* 90:165–169. 2012) was undertaken in summer 2008 at the Tuscia Germplasm Bank of the Botanic Gardens of Viterbo (Italy) in order to learn about its reproductive biology, and for conservation purposes, to obtain information on the biological factors that may have contributed to the strong fragmentation of its distribution. Fresh spores were collected in September 2007 from a wild population of *D. tyrrhena* growing within the Cinque Terre National Park (Riomaggiore, La Spezia, Italy). Studies of gametophyte and sporophyte development were carried out according to the protocol of Menendez *et al.* (*Plant Cell Rep.* 25:85–91. 2006; Quintanilla and Escudero, *Ann. Bot.* 98:609–618. 2006; Magrini *et al.*, 2012). All the spores were separated from sporangia using sieves with a mesh size of 71 μm , and then were soaked in Eppendorf tubes with 1.5 ml of distilled water for 24 h. After, they were surface sterilized for 3 min. in a 0.5% NaOCl solution, supplemented with a drop of Tween 20 to improve the efficiency of the sterilization. They were then rinsed three times with sterile distilled water and centrifuged at 6,000 rpm for 3 min. between rinses. The spores were sown with three replicates in sterile plastic Petri dishes (6 cm diameter) containing 15 ml of MS medium (Murashige and Skoog, *Physiol. Plant.* 15:473–497. 1962) (PhytoTechnology Laboratories® Shawnee Mission, KS, USA), supplemented with 0.7% agar (Plant tissue culture grade, AppliChem, Darmstadt, Germany) and a Nystatin solution (100 Uml^{-1}), which was added as a fungicide (AppliChem, Darmstadt, Germany). The cultures were maintained under cool-white fluorescent illumination (Osram Dulux L 36W/840 Lumilux, 2900 lm), a 12-h photoperiod, and a temperature of $20 \pm 1 \text{ }^\circ\text{C}$ (Sheffield *et al.*, Amer. Fern J. 91(4):179–186. 2001; Magrini *et al.*, 2012). The dishes were examined daily for spore germination (defined as the first emergence of the rhizoid) and weekly for gametophyte growth and sporophyte development.

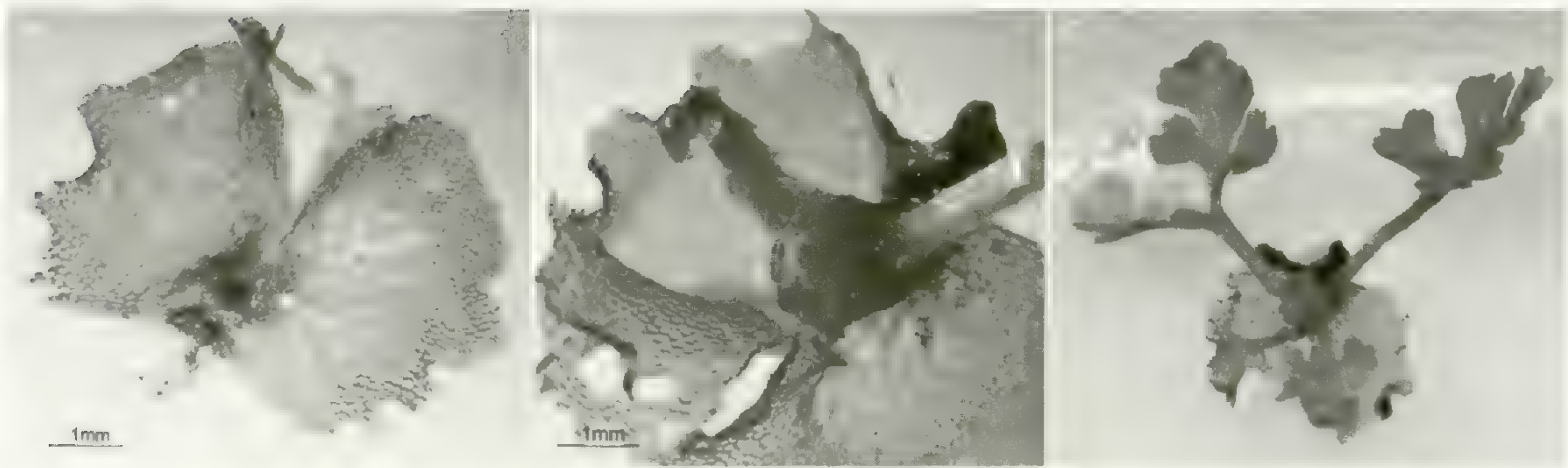


FIG. 1. a) Cordate gametophytes of *Dryopteris tyrrhena*, with rhizoids in the ventral side and an apogamous bud in the dorsal side, in the cushion area behind the apical notch. b) First fronds arose from the apogamous bud, showing circinate vernation. c) Apogamous sporophyte with young pinnate fronds grown upward, each diverging at an angle of about 100° from the previous one.

A low percentage (<5%) of the spores germinated after 36 days after sowing. All gametophytes were filamentous at the beginning and they followed a normal trend of development in the transition from protonema to the laminar-cordate phase: the first oblique division of the terminal cell of the germ filament started after the fourth longitudinal division (Raghavan, *Developmental biology of fern gametophytes*. Cambridge University Press. 1989), resulting in laminar heart-shaped gametophytes 15 days after germination. All the cordate gametophytes produced rhizoids in the ventral side and developed apogamous buds in the dorsal side, in the cushion area behind the apical notch (Fig. 1a), about 60–70 days after germination (Magrini *et al.*, 2012). This same pattern has been previously recorded for *D. affinis* (Lowe) Fraser-Jenk. subsp. *affinis* (Menéndez *et al.*, 2006). Sporophytes arose from these buds in a few days, with young pinnate fronds, which started to quickly grow, showing circinate vernation (Fig. 1b).

During the apogamous sporophyte development, an interesting phenomenon was noticed. In the beginning, the leaves grew upward, each diverging at an angle of about 100° from the previous one (Fig. 1c), as has been observed by



FIG. 2. Agravitropic response: some leaves developed downward and they grew into the agar of the culture medium.

Duncan (Bot. Gaz., 105:202–211, 1943) for certain apogamous forms of *D. affinis* (Lowe) Fraser-Jenk. subsp. *borreri* (Newman) Fraser-Jenk. An anomalous growth was observed a few days later as the leaves showed an agravitropic response. They developed both upward and downward, growing also into the agar of the culture medium (Fig. 2). Other studies are testing the already known induction factors of apogamy, like the presence of sucrose in the culture medium, high light intensity, and the vertical growth of the gametophytes (Magrini *et al.*, 2012). Their preliminary results led us to hypothesize that apogamy may have been induced by ethylene production during *in vitro* culture (Elmore and Whittier, Can. J. Bot. 53:375–381, 1975). In fact, the dishes were sealed with Parafilm, in order to reduce contamination, to prevent excessive water loss, and to reduce air exchange. Menéndez *et al.* (2006) showed how auxins play a stimulatory role during the induction and differentiation of apogamous embryo development in *D. affinis*. Van der Laan (1934) was the first to consider the possibility that ethylene acts on auxin in plants. Given its potential role in apogamy, it is possible that ethylene could also be influencing the normal gravitropic response. However, more experimental work is needed to study the influence of ethylene on tropisms and to clarify the effective cause of this agravitropic response.—SARA MAGRINI, Tuscia Germplasm Bank, Botanic Gardens of Viterbo, Tuscia University, largo dell'Università, Blocco C, 01100, Viterbo, Italy, e-mail: magrini@unitus.it, and ANNA SCOPPOLA, Department of Agriculture, Forestry, Nature, and Energy, Tuscia University, via S. Camillo de Lellis, 01100, Viterbo, Italy, e-mail: scoppola@unitus.it.

SHORTER NOTES

***Cheilanthes feei* T. Moore (Pteridaceae) and *Dryopteris erythrosora* (D.C. Eaton) Kunze (Dryopteridaceae) New for the Flora of North Carolina.**—Recent field and herbarium work has added these two species to the spontaneous flora of the state of North Carolina, USA. *Cheilanthes feei* is native to the center of the continent; its core range extends from eastern Minnesota and Texas, south to northern Coahuila and Chihuahua, and west to southern Nevada and extreme southeastern British Columbia (Windham and Rabe, *Cheilanthes*. In: Flora of North America. Oxford University Press, New York. 1993; Mickel and Smith, *The Pteridophytes of Mexico*. The New York Botanical Garden Press, New York. 2004). From this core area, there are two reported disjunctions. The first, in central northern Kentucky (Reed. Amer. Fern J. 42:53–56. 1952.) is approximately 200 km east of the main range, and the second, along the New River in western Virginia (Wieboldt and Bentley, Amer. Fern J. 72:76–78. 1982.) is another 450 km to the east. In both cases, the species was found on limestone or dolomitic cliffs, its typical habitat.

Recent curatorial activities at the Duke University herbarium (DUKE) resulted in the discovery of this species as new for North Carolina. A single specimen was collected in 1930 by Hugo Blomquist in Durham County (some 200 km SE of the nearest station, in Virginia), but was misidentified as the more common *Cheilanthes tomentosa* Link. The Blomquist specimen is undoubtedly *C. feei* and not *C. tomentosa*, based on the absence of scales on the rachises and costae, and the glabrescent adaxial surfaces of the ultimate segments. Unfortunately, the label data describe the collection location merely as “Eno River, Durham, NC.” Given that Blomquist was based in Durham, and that his databased collections from June 1930 are all from North Carolina (including one from Durham County: www.herbarium.unc.edu/seflora; www.tropicos.org), it seems unlikely that this record is due to a labeling error. Subsequent searches by the authors and associates along the Eno River failed to discover any extant populations, but such may well still exist.

This record continues a noteworthy pattern of disjunction for western cheilanthoid ferns: in most cases where a predominantly western species has populations east of the Appalachians, the species involved is an apomictic triploid. Species fitting this pattern include *Cheilanthes feei*, *C. eatonii* Baker (*C. castanea* Maxon), *C. alabamensis* (Buckley) Kunze, and *C. tomentosa*. This pattern is also seen in the related genus *Astrolepis*, where two apomictic triploids (*A. sinuata* (Lagasca ex Swartz) D.M. Benham & Windham ssp. *sinuata* and *A. integerrima* (Hooker) D.M. Benham & Windham) have eastern disjunct populations far from their core western ranges (disjunct to Georgia and Alabama, respectively; Weakley, *Flora of the Southern and Mid-Atlantic States*. The University of North Carolina Herbarium, Chapel Hill, NC. 2011). This tendency for apomictic taxa to have long-distance geographic disjunctions is consistent with their breeding system. As asexuals, they need only a



FIG. 1. *Dryopteris erythrosora* along the Ellerbe Creek, Durham, North Carolina. A) Habit and habitat. B) Close-up of a leaf. Photos: Sarah Zylinski.

single spore to land in a suitable habitat in order to potentially establish a new population. In contrast, many sexual cheilanthoid ferns appear unable to undergo intragametophytic selfing, and thus require two spores to establish and produce gametophytes in close enough proximity to permit cross-fertilization before a population can be established.

Cheilanthes feei T. Moore. Eno River, Durham, North Carolina. Exposed rocks. H.L. Blomquist 1649 (DUKE 10973). June, 1930. Det'd M.D. Windham, 2006.

Like *C. feei*, *Dryopteris erythrosora* is an apomictic triploid. It is native to woodlands of eastern Asia but is common in the North American horticultural trade, where it is sold under the name Autumn Fern (Hoshizaki and Wilson, *Amer. Fern J.* 89:1–98. 1999; Hoshizaki and Moran, *Fern Grower's Manual*. Timber Press, Portland, OR. 2001). In North America, it has been reported from Georgia (Weakley, 2011) and Arkansas (Simpson *et al.*, *Amer. Fern J.* 98:111–112. 2008). We found it growing in a typical habitat—a disturbed suburban woodlot—in Durham County, North Carolina. It was uncommon, in the company of other native and nonnative taxa, without any indication of it (or anything else) having been planted on the site or in the vicinity (Fig. 1).

While *Dryopteris erythrosora* does not appear to be spreading aggressively in Durham, this report extends a potentially worrisome trend of non-native temperate ferns naturalized from presumably horticultural sources (e.g., *Macrothelypteris*—Gorman *et al.*, *Journal of the Botanical Research Institute of Texas* 5:343–344. 2011; *Athyrium nipponicum* and *Deparia petersenii*—Weakley, 2011; *Hypolepis tenuifolia* (G. Forster) Bernh.—Peck, *Phytoneuron* 30:1–33. 2011; *Arachniodes*—Gordon, *Amer. Fern J.* 71:65–68. 1981).

Dryopteris erythrosora (D.C. Eaton) Kunze. Durham County, North Carolina. Durham, Old West Durham neighborhood. Tributary of Ellerbe Creek, just

north of Sunset Ave, between Carolina Ave and Albany St. N36.02361 W78.92577 \pm 8m. Elevation: 153 m. Two plants seen (but there were more earlier in the year; they presumably got washed away). Just above scour zone of small suburban creek, on disturbed, steep sandy banks, with *Impatiens*, *Toxicodendron*, *Polystichum*, *Viola*, *Boehmeria*, *Vinca*, other invasive shrubs, etc. Under *Morus*, *Acer negundo*, *Fraxinus*, etc., in weedy ridge through floodplain forest. 2010-August-08. C.J. Rothfels 3959, with S. Zylinski (DUKE 401869). Det'd C.J. Rothfels & E. Sigel, August 2010. ! C. Fraser-Jenkins (from photos), August 2010.

We thank Christopher Fraser-Jenkins for confirming the identification of the *Dryopteris*, Dylan Burge, Amanda Grusz, Beth Guy, Layne Huiet, Fay-Wei Li, and Lisa Pokory for their efforts in our as-yet unsuccessful attempts to locate extant North Carolina populations of *Cheilanthes feei*, and Jennifer Geiger (Editor) and two anonymous reviewers for improvements to the manuscript.— C. J. ROTHFELS, E. M. SIGEL, and M. D. WINDHAM, Department of Biology, Duke University, Durham, North Carolina.

REVIEW

Flora de la Región del Parque Nacional Amboró, Bolivia, Vol. 1 Licofitas y Helechos, Gimnospermas, the flora edited by Michael H. Nee, the volume authored by Michael Sundue and Michael H. Nee. 2011. Published by Editorial FAN, Santa Cruz de la Sierra, Bolivia. Hardcover Price \$20. ISBN 978-99905-66-54-3, 464 pages, 61 color and 115 black-and-white photographs. In Spanish.

The tropical Andean fern flora is – along with that of Southeast Asia and New Guinea – the richest worldwide. Despite (or perhaps because of) this, floristic treatments of Andean ferns are rare. Peru and Venezuela are the only countries with fully published fern floras, although these are now becoming outdated as fern taxonomy evolves and new species are recorded. For Ecuador and Colombia, only selected families have been treated so far in the series of national floras. In addition, there are a number local or regional floras and checklists. Bolivia, which harbors about 1200 known fern species, has so far had no floristic fern treatment. Now, Michel H. Nee and Michael Sundue have produced the first flora for part of the country, namely Amboró National Park and surrounding areas.

Amboró National Park is located on the “Andean elbow” where the Andes turn abruptly southwards and where the humid tropical Andes that extend from western Venezuela to central Bolivia meet the arid subtropical Andes that reach from here to northwestern Argentina. As a result of this position at a major biogeographical meeting point and because of its high diversity of habitats, Amboró is one of most diverse protected areas worldwide. Covering 442,500 hectares plus 195,000 hectares in a surrounding “Natural Area of Integrated Management”, Amboró ranges from 235 m to 3100 m elevation and includes vast tracts of evergreen rainforest as well as smaller areas of drier and seasonally deciduous forest types. So far, over 3000 species of vascular plants have been recorded from the park. Dr. Michael H. Nee from the New York Botanical Garden has been working intensively of the flora of this region for decades and is the driving force behind the preparation of the flora.

The first volume of the flora of Amboró, published in Spanish in 2011 by the Bolivian conservation NGO Fundación Amigos de la Naturaleza (FAN), covers all 429 species of ferns and lycophytes as well as 11 species of gymnosperms so far known from the park and adjacent areas. Because large parts of the national park, especially in the wet montane rainforests at 1000–2000 m which harbour the highest fern diversity are largely inaccessible and have not yet been explored botanically, there is no doubt that many more fern species actually occur in the park, probably in the order of 600–800 species. However, this is true for much of the Bolivian Andes where collecting activity is very localized and where new records are commonplace.

The fern treatment was prepared over several years by Dr. Michael Sundue, with significant input from numerous taxonomic specialists for a variety of

taxa. The treatment covers 331 pages and follows the standard format of floras, including dichotomous keys to families, genera, and species, full descriptions of families, genera and species, taxonomic or morphological notes, specimen citations, and some ecological information for each species (elevation and vegetation types in which the species has been found); nomenclatural remarks are kept to a minimum. In addition, the flora includes 61 color photographs (49 of ferns) and 115 black-and-white photographs of herbarium specimens (107 of ferns). There are 20 pages of general introduction giving a brief outline of the vegetation types of the flora region and of the history of its botanical exploration plus no less than 15 pages of glossary explaining botanical terms.

Overall, the taxonomic treatment is of very high quality. Dr. Sundue has invested an inordinate amount of work into clarifying even the most obscure taxonomic problems and into making sure that all the latest developments of fern classification/taxonomy, both at the family and generic level as well as at species level, are taken into account. Unavoidably, some of the very latest developments, such as the segregation of Neotropical species of *Micropolypodium* into the new genus *Moranopteris*, have not been considered. I have used some of the keys and have found them to work very well even for difficult groups such as *Elaphoglossum* or *Campyloneurum*. This is certainly one of the best fern floras currently available for any part of the tropical Andes.

As a result, this is a highly important work for anybody interested in tropical Andean ferns. Because of its location in central Bolivia, the flora – which covers about one third of the Bolivian fern flora – nicely fills the gap between the fern floras of Peru and Jujuy in northern Argentina, and includes a number of endemic Bolivian species not covered by either of the other floristic works. Until the publication of a forthcoming fern flora for the entire country (Kessler and Smith, in prep.), this will be the major reference for Bolivian ferns, and will also be useful for northern Argentina and to a lesser degree southern Peru. That the flora is written in Spanish makes it readily accessible to the Bolivian researchers and public (the main audience) while perhaps limiting its use to non-Spanish speakers, although the technical terminology is so similar that most of the text can be used by anybody with a good foundation in English botanical terminology. If you are interested in Andean ferns, then there is much to learn from and enjoy in this book. The authors and FAN are to be highly commended for producing this important piece of literature for what is probably the botanically least well known South American country.

Unfortunately, I must end this review on a critical note. While preparing this review, I also inquired about the availability of the book outside of Bolivia and to my great disappointment had to learn that the book can only be ordered directly from the publisher (www.fan-bo.org) and that this is very complicated. At the time of writing, the book was not even listed on their website so that you had to know that it exists and specifically ask for it via e-mail, and payment was only possible by money order. I have also learnt that Missouri Botanical Garden considered obtaining a number of these books to be able to make them available to a wider public, but that this project was cancelled due to

communication problems with the publisher. It is sad to realize that unless a solution is found for these problems, this important publication will likely receive much less distribution outside of Bolivia than it would deserve.—

MICHAEL KESSLER, Systematic Botany and Botanical Garden, University of Zurich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland.

ERRATUM

AFJ volume 101, issue 4, pp. 282–294

In describing the new species *Bolbitis lanceolata* J.Y. Xiang & S.K. Wu (Amer. Fern J. 101(4): 287–290. 2011), we inadvertently omitted the explicit designation of a holotype from among the duplicates of the type gathering. The corrected type citation appears below. This validates the species description by direct association with the original protologue and delays the effective date of publication for the name to that of the present issue.

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* (holotype: KUN; isotypes: MO, TMRC). **Fig. 1: A–C, Fig. 2.**

INFORMATION FOR AUTHORS

Authors are encouraged to submit manuscripts pertinent to pteridology for publication in the *American Fern Journal*. Manuscripts should be sent to the managing editor at hauk@denison.edu. Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication.

Authors should adhere to the following guidelines; manuscripts not so prepared may be returned for revision prior to review. **Electronic submission is strongly encouraged**; however if it is necessary to submit hard copy, please submit one copy of the manuscript and include a review copy of illustrations and originals of illustrations. After acceptance, please submit final versions of manuscripts via FTP (contact the managing editor for instructions), email, or on diskette or CD ROM (see below for figure formatting). If submitting hard copies, use standard 8.5 by 11 inch paper of good quality, not "erasable" paper. **Double-space manuscripts throughout**, including title, author's names and full addresses, a short, informative abstract, key words, text (including heads and keys), literature cited, tables (separate from text), and figure captions (grouped as consecutive paragraphs separate from figures). Arrange parts of manuscript in order just given. Include author's name and page number in the upper right corner of every sheet, and provide an abbreviated running title. Provide margins of at least one inch (25 mm) all around on typed pages. Do not submit right-justified text, avoid footnotes, and do not break words at end of lines. Make table headings and figure captions self-explanatory. Use S.I. (metric) units for all measures (e.g., distance, elevation, weight) unless quoted or cited from another source (e.g., specimen citations). For nomenclatural matters (i.e., synonymy and typification), use one paragraph per basionym (see *Regnum Veg.* 58:39–40, 1968). Abbreviate titles according to *Botanico-Periodicum-Huntianum* (Lawrence *et al.*, 1968, Hunt Botanical Library, Pittsburgh) and its supplement (1991). References cited only as part of nomenclatural matter are not included in literature cited. For shorter notes and reviews, omit the abstract and put all references parenthetically in text. Use *Index Herbariorum* (*Regnum Veg.* 120:1–693, 1990; or <http://websun.nybg.org/bsci/ih/>) for designations of herbaria. For more detailed instructions on manuscript preparation, see <http://amerfernsoc.org/>.

Illustrations should be proportioned to fit page width (5 inches or 12.5 cm) with caption ultimately to be included on the same page. Halftone and color images should be scanned at a minimum of 300 pixels per inch (ppi). Line art should be scanned at 1200 ppi whenever possible. Please note that nearly all images that are downloaded from the Internet or that are in JPEG or GIF format will be 72 dpi and not acceptable for the printing process. Indicate the file format of the graphics. Please submit image files in TIFF (preferable) or EPS format. Provide margins of at least 25 mm on all illustrations. For continuous-tone illustrations, design originals for reproduction without reduction or by uniform amount. In composite blocks, abut edges of adjacent photographs. Avoid combining continuous-tone and line-copy in single illustrations or blocks. Coordinate sequence and numbering of figures (and tables), with order of citation in text. Explain scales and symbols in figures themselves, not in captions. Include a scale and reference to latitude and longitude in each map.

Instructions for downloading electronic proofs and reprint order forms are sent to authors by the printer. Authors should send proof corrections of corrected proofs to the editor and reprint orders to the printer. Authors will be assessed charges for extensive alterations made after type has been set.

For other matters of form or style, consult recent issues of the *American Fern Journal* and *The Chicago Manual of Style*, 14th ed. (1993, Univ. of Chicago Press, Chicago). Occasionally, departure from these guidelines may be justified. Authors are encouraged to consult the editor for assistance with any aspect of manuscript preparation.

Papers of longer than 32 printed pages may be sent to the Editor of *Pteridologia* (memoir Editor, see journal cover page 2).

PTERIDOLOGIA ISSUES IN PRINT

The following issues of *Pteridologia*, the memoir series of the American Fern Society, are available for purchase:

1. Wagner, David H. 1979. Systematics of *Polystichum* in Western North America North of Mexico. 64 pp. \$10.00 plus postage and handling.

2A. Lellinger, David B. 1989. The Ferns and Fern-allies of Costa Rica, Panama, and the Chocó (Part 1: Psilotaceae through Dicksoniaceae). 364 pp. \$32.00 plus postage and handling.

3. Lellinger, David B. 2002. A Modern Multilingual Glossary for Taxonomic Pteridology. 263 pp. \$28.00 plus postage and handling.

For orders and more information, please contact our authorized agent for sales at: Missouri Botanical Garden Press, P.O. Box 299, St. Louis, MO 63166-0299, tel. 314-577-9534 or 877-271-1930 (toll free). For online orders, visit: <http://www.mbgpress.org>.

AMERICAN FERN JOURNAL ON MICROFICHE

Volumes 1–61 of the *American Fern Journal* are available as archival quality, silver positive microfiches. Single volumes or the entire run may be purchased. The fiches are easily read with 10x or greater magnification (using a dissecting microscope and transmitted illumination or a fiche reader). Silver negative microfiches of vols. 1–50 are also available. The price is \$4.00 per volume or \$244.00 per set of 61 volumes, postpaid.

Send your inquiry or order with a check or money order to: American Fern Society, Inc., % Ecology III, 804 Salem Blvd., Berwick, PA, 18603-9801.

FIDDLEHEAD FORUM

The editor of the Bulletin of the American Fern Society welcomes contributions from members and non-members, including miscellaneous notes, offers to exchange or purchase materials, personalia, horticultural notes, and reviews of non-technical books on ferns.

SPORE EXCHANGE

Ms. Denia Mandt, 12616 Ibbetson Ave., Downey, CA 90242-5050, is Director. Spores exchanged and lists of available spores sent on request. <http://amerfernsoc.org/sporexy.html>

GIFTS AND BEQUESTS

Gifts and bequests to the Society enable it to expand its services to members and to others interested in ferns. Back issues of the Journal and cash or other gifts are always welcomed and are tax-deductible. Inquiries should be addressed to the Membership Secretary.

VISIT THE AMERICAN FERN SOCIETY'S WORLD WIDE WEB HOMEPAGE:

<http://amerfernsoc.org/>

AMERICAN FERN JOURNAL

Volume 102

Number 3

July–September 2012

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

- Farina Production by Gametophytes of *Argyrosma nivea* (Poir.) Windham (Pteridaceae) and its Implications for Cheilanthoid Phylogeny**
Jose María Gabriel y Galán and Carmen Prada 191
- Cytotoxic and Tripanocide Activities of *Pityrogramma calomelanos* (L.) Link.**
Teogenes M. de Souza, Maria F. B. M. Braga, Rogério A. Saraiva, Pablo A. Nogara, Diones C. Bueno, Aline A. Boligon, Margareth L. A. Fone, João B. T. da Rocha, Miriam Rolon, Celeste Vega, Antonieta Rojas de Arias, Jose G. M. Costa, Irwin R. Alencar de Menezes, Henrique D. M. Coutinho and Antônio A. F. Saraiva 198
- Effects of *Ageratina adenophora* on Spore Germination and Gametophyte Development of *Neochheiropteris palmatopedata***
K. M. Zhang, J. H. Liu, X. Cheng, G. F. Zhang, Y. M. Fang, and H. J. Zhang 208
- Occurrence of Dark Septate Endophytes in the Sporophytes of *Christella dentata***
Rebeca Ghanta, Sikha Dutta, and Radhanath Mukhopadhyay 216
- Azolla cristata* in the Kashmir Himalaya**
Burhan Ahad, Zafar A. Reshi, Aijaz H. Ganaie, and A. R. Yousuf 224
- SHORTER NOTES
- New Country and Regional Records from the Brazilian Side of Neblina Massif**
Fernanda Antunes Carvalho, Alexandre Salino, and Charles Eugene Zartman 228
- New Records Of Ferns From Chiapas, Mexico**
Miguel A. Pérez-Farrera, Ma. Evangelina López-Molina, Nayely Martínez-Meléndez, and Héctor Gómez-Domínguez 233
- Obituary: The Rev. Father Dr. V. S. Manickam S. J. (1944–2012)**
Dr. A. Benniamin and Chris Fraser-Jenkins 236

The American Fern Society

Council for 2012

KATHLEEN PRYER, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President</i>
JAMES E. WATKINS, JR., Dept. of Biology, Colgate University, Hamilton, NY 13346.	<i>President-Elect</i>
MARY STENSVOLD, USDA Forest Service, 204 Siginaka Way, Sitka, AK 99835.	<i>Secretary</i>
JAMES D. CAPONETTI, Div. of Biology, University of Tennessee, Knoxville, TN 37996-0830.	<i>Treasurer</i>
GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.	<i>Membership Secretary</i>
JAMES D. MONTGOMERY, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.	<i>Curator of Publications</i>
JENNIFER M. O. GEIGER, Dept. of Natural Sciences, Carroll College, Helena, MT 59625.	<i>Journal Editor</i>
R. JAMES HICKEY, Dept. of Botany, Miami University, Oxford, OH 45056.	<i>Memoir Editor</i>
JOAN N. E. HUDSON, Dept. of Biological Science, Sam Houston State University, Huntsville, TX 77341-2116. DAVID SCHWARTZ, 9715 Christey Way, Bakersfield, CA 93312-5617.	<i>Bulletin Editors</i>

American Fern Journal

EDITOR

JENNIFER M. O. GEIGER Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-4461, e-mail: jgeiger@carroll.edu

MANAGING EDITOR

JILL ANNE DILL Dept. of Natural Sciences, Carroll College, Helena, MT 59625,
ph. (406) 447-5176, e-mail: jdill@carroll.edu

ASSOCIATE EDITORS

NAN CRYSTAL ARENS Dept. of Geoscience, Hobart & William Smith Colleges, Geneva, NY 14456
JOSE MARÍA GABRIEL Y GALÁN Dept. de Biología Vegetal I, Universidad Complutense de Madrid,
Madrid, Spain 28040
GERALD J. GASTONY Dept. of Biology, Indiana University, Bloomington, IN 47405-6801
GARY K. GREER Biology Dept., Grand Valley State University, Allendale, MI 49401
CHRISTOPHER H. HAUFLER Dept. of Ecology and Evolutionary Biology, University of Kansas,
Lawrence, KS 66045-2106
R. JAMES HICKEY Dept. of Botany, Miami University, Oxford, OH 45056
PAULO LABIAK Universidade Federal do Paraná, Dept. de Botânica, 81531-980, Curitiba-PR, Brasil
ROBBIN C. MORAN New York Botanical Garden, Bronx, NY 10458-5126
NATHALIE NAGALINGUM Royal Botanical Garden Sydney, Sydney, NSW, Australia
CARL ROTHFELS Botany Dept., Duke University, Durham, NC 27708
EMILY SESSA Botany Dept., University of Wisconsin-Madison, Madison, WI 53706
MICHAEL SUNDUE Pringle Herbarium, The University of Vermont, Burlington, VT 05405
JAMES E. WATKINS, JR. Dept. of Biology, Colgate University, Hamilton, NY 13346

The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, c/o Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

Claims for missing issues, made 6 months (domestic) to 12 months (foreign) after the date of issue, and orders for back issues should be addressed to Dr. James D. Montgomery, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.

Changes of address, dues, and applications for membership should be sent to the Membership Secretary.

General inquiries concerning ferns should be addressed to the Secretary.

Back volumes are available for most years as printed issues or on microfiche. Please contact the Back Issues Curator for prices and availability.

Subscriptions:

Society Membership – USA, Canada, Mexico (includes Journal and Fiddlehead Forum)	\$30
Society Membership – All other countries (includes Journal and Fiddlehead Forum)	\$40
Society Life Membership – \$360 (add \$120 mailing surcharge for outside USA, Canada, Mexico)	
Regular Membership – USA, Canada, Mexico (includes Fiddlehead Forum)	\$15
Regular Membership – All other countries (includes Fiddlehead Forum)	\$22
Institutional Membership – \$40 to USA, Canada, Mexico; \$50 elsewhere (-\$3 agency fee)	

POSTMASTER: Send address changes to American Fern Journal, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

Farina Production by Gametophytes of *Argyrochosma nivea* (Poir.) Windham (Pteridaceae) and its Implications for Cheilanthoid Phylogeny

JOSE MARÍA GABRIEL Y GALÁN and CARMEN PRADA

Departamento de Biología Vegetal I, Universidad Complutense de Madrid, Avda. Jose Antonio Nováis, 2, 28040 Madrid (Spain)

ABSTRACT.—Modern molecular phylogenetic studies of the Pteridaceae have recognized a well supported cheilanthoid clade that includes four major subclades: myriopteroids, pellaeoids, hemionitidoids and notholaenoids. Many of the morphological characters used in delimitation of the cheilanthoid lineages and genera appear to be the result of convergent evolution, a result of adaptation to xeric environments. Faced with the apparent lack of sporophytic synapomorphies for the cheilanthoid subclades, farina production by gametophytes has been proposed as a character of possible phylogenetic utility. All the notholaenoid species observed to date produce farina in their gametophytes, but species of the other cheilanthoid clades (pellaeoids, hemionitidoids, and myriopteroids) do not. In this work we provide the first account of farina production in the gametophyte of a non-notholaenoid: two accessions of *Argyrochosma nivea* from different geographical localities were found to have farina on their gametophytes, suggesting that this gametophytic character is not a synapomorphy for the notholaenoids, and may have had several independent evolutionary origins.

KEY WORDS.—Pteridaceae, cheilanthoids, *Argyrochosma*, farina, gametophyte, *Notholaena*

Pteridaceae is a large family with ca. 50 genera and about 1000 species. In its modern conception it is monophyletic and includes, among others, the Adiantaceae and Vittariaceae (Smith *et al.*, 2008). However, internal relationships within the family and circumscription of many genera are still under review. Based on recent molecular analyses, five monophyletic groups can be recognized: cryptogrammoids, ceratopteridoids, pteridoids, cheilanthoids and adiantoids (Schuettpelz *et al.*, 2007; Schuettpelz and Pryer, 2008; Smith *et al.*, 2008). Cheilanthoid ferns comprise around 400 species, and form a very well supported clade (Gastony and Rollo, 1995; Schuettpelz and Pryer, 2007). Within this group, molecular studies suggest the existence of four major clades (Fig. 1): myriopteroids, pellaeoids, hemionitidoids, and notholaenoids (Rothfels *et al.*, 2008; Windham *et al.*, 2009). Cheilanthoid taxonomy is particularly difficult, due to their great morphological diversity (for example, in frond architecture and presence of pseudoindusium) and to convergent evolution resulting from their adaptation to arid environments (Rothfels *et al.*, 2008). In this last sense, one of the most confusing characters is the presence of farina in the sporophytes. Farina is a white or yellow powder, produced by glandular hairs and of variable composition, but often containing flavonoids. Farina is produced by sporophytes of a number of species within each of these cheilanthoid subclades, and is thus a homoplastic character.

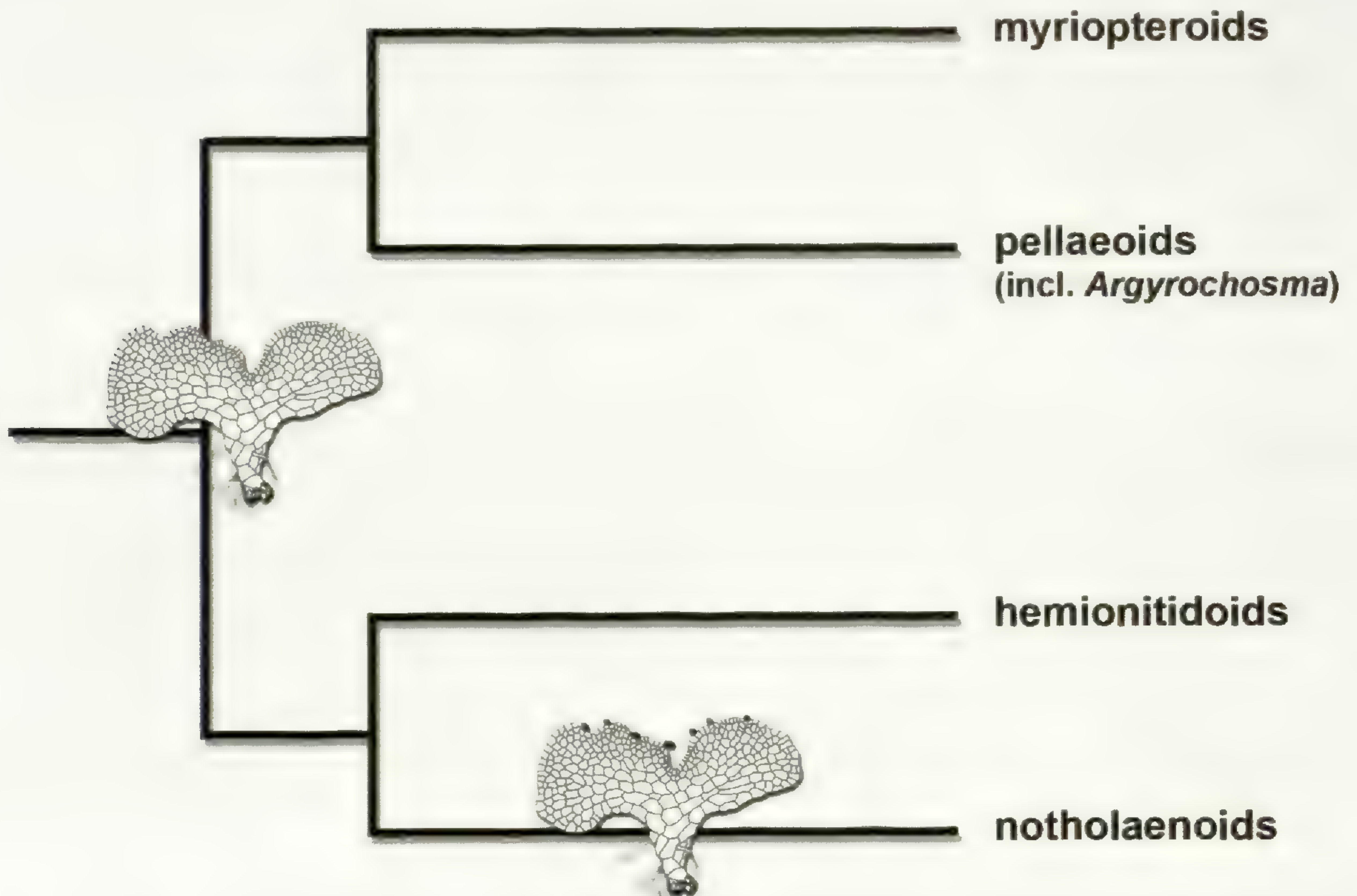


FIG. 1. Simplified to-date phylogenetic scheme of cheilanthoid clade (Pteridaceae), showing the position of *Argyrochosma* within the pellaoids. A hypothetical history of gametophyte evolution has been represented over this tree: non-farinose gametophyte is the ancestral condition, shared by all the lineages except the notholaenoids.

Some morphological characters, however, may function as synapomorphies for some of the cheilanthoid lineages. One of these potential synapomorphies is the production of farina by the gametophyte (Rothfels *et al.*, 2008). All notholaenoid species observed to date produce farina on their gametophytes, but those of the other cheilanthoid subclades (pellaoids, hemionitidoids, myriopteroids) do not (Fig. 1). Thus, the presence of farina in the gametophyte is potentially an important feature for defining the notholaenoid group. This is very interesting, given the traditional lack of attention paid by taxonomists to gametophytic characters, a fact that is likely attributable to their reduced levels of morphological variation.

Argyrochosma is a New World genus, segregated from *Notholaena* R. Brown some decades ago (Windham, 1987), and comprises around 16 species (Sigel *et al.*, 2011). *Argyrochosma nivea* (Poir.) Windham consists of a complex of three mainly apogamous taxa, considered as varieties: two of them, var. *nivea* and var. *flava* (Hook.) Ponce, produce farina on their sporophytes, and the other, var. *tenera* (Gilles ex Hook.) Ponce, has glabrous (non-farinose) sporophytes (Sigel *et al.*, 2011).

The haploid phase of *A. nivea* var. *nivea* is very well known, from studies on spore morphology (Morbelli *et al.*, 2001), spore germination and gametophyte development (Gabriel y Galán, 2011) and reproduction via apogamy (Woronin,

1907; Gabriel y Galán, 2011). These studies report the gametophyte of *A. nivea* as non-farinose, in agreement with the pellaoid gametophyte concept described above.

Nevertheless, as relatively few cheilanthoid gametophytes have been studied, more effort is required before accepting definitively the synapomorphic status of gametophytic farina for the notholaenoids. With this in mind, and within a broader framework of observing morphology, development, and reproduction of Pteridaceae gametophytes, the goal of the current study was to determine if other *A. nivea* varieties besides var. *nivea* also produce non-farinose gametophytes.

MATERIAL AND METHODS

Samples of *Argyroschisma* used in this study were collected from the following locations: *Argyroschisma nivea* var. *nivea*: Peru, Cuzco Department, Urubamba Province, Ollantaytambo, beyond Huilloq, 13°14'50.5"S 72°15'28.8"W, 2920 m, in rocks, JM Gabriel y Galán, 28-04-2008. Argentina, Catamarca, El Alto Department, Sierra de Ancasti, between El Portezuelo and El Alto, 28°27'52"S 65°35'10"W, 1855 m, granite rocks, Prada, 16-09-2010. *Argyroschisma nivea* var. *flava*: Argentina, Córdoba, San Alberto Department, Quebrada Los Pozos, dique La Viña, 31°52'24"S 65°01'54"W, 910 m, granite rocks, Prada, 17-09-2010. Material was identified following local floras (Tryon and Stolze, 1989; de la Sota *et al.*, 2009), and is deposited in the Herbarium of the Real Jardín Botánico de Madrid (MA).

Spore samples for cultures were taken from two different sporophytes at each location and kept dry at room temperature. Multispore cultures on mineral agar medium (Dyer, 1979) were established by manually removing spores from fertile pinnae on a weigh paper, and placing them in Petri dishes 6 cm in diameter. The sowing of each sample was replicated three times. Gametophytes were grown under fluorescent light on a 12-h light, 12-h dark cycle at $20 \pm 2^\circ\text{C}$, for c. 8 months. At different stages of maturity, random gametophyte samples of each culture were stained with chloral hydrate acetocarmine (Edwards and Miller, 1972). Fixed and *in vivo* materials were mounted in water and observed under a light microscope. In total, c. 150 gametophytes were assayed, c. 50 of each of the three samples (two of var. *nivea* and one of var. *flava*).

RESULTS

Both varieties of *Argyroschisma nivea* produced cordate gametophytes following the *Vittaria* type of development, although a notable number of prothalli acquired an irregular shape. Because description of the development and complete morphological features of the gametophytes exceeds the objectives of this work, we will focus specifically on farina production.

Gametophytes of *A. nivea* var. *flava* remained glabrous throughout the observational period. In contrast, some of the gametophytes of *A. nivea* var. *nivea*, about 20% of the total observed, produced short glandular hairs along their margin, which produced farina with time (Fig. 2). With very few

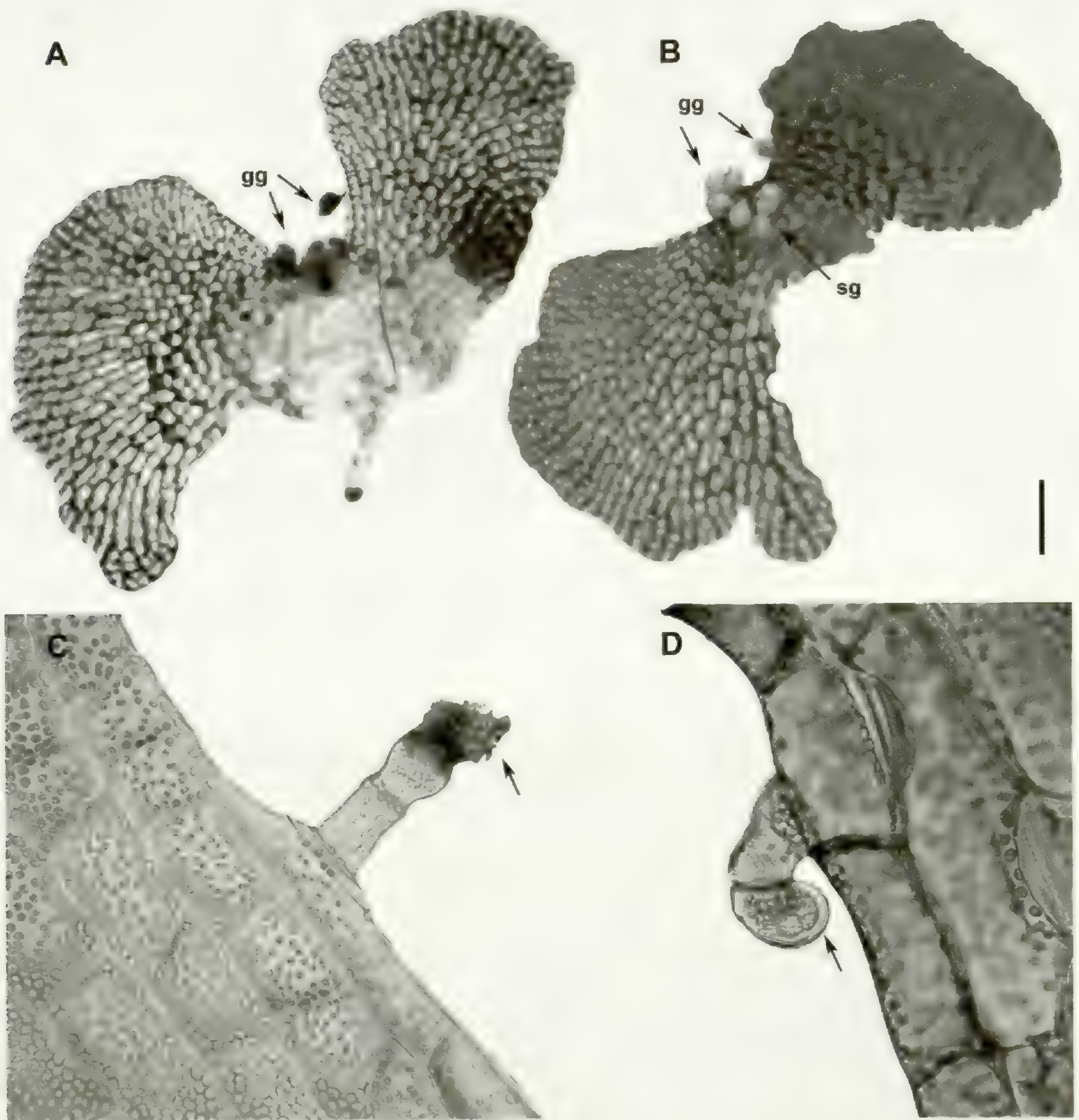


FIG. 2. Farina production in gametophytes of *Argyrochosma nivea* var *nivea*. A–B: gametophytes with glandular hairs and white farina, under transmitted light binocular microscope (A) and under incident light binocular microscope (B); gg=gametophyte glands; sg= apogamous sporophyte glands. C: detail of a glandular hair in the margin of gametophyte, with the farina secretion (arrow). D: detail of a glandular hair with the farina washed, showing the apical gland (arrow). Bar= 0.12 cm in A–B; 20 μ m in C; 14 μ m in D.

exceptions, these hairy gametophytes were those with cordate shape. The morphology of the hairs was constant in all the gametophytes: two cells, a more-or-less elongated basal one and a capitate secretory apical one. The hairs appeared more abundant near the apical notch, but they also spread towards the wings.

These marginal gametophytic trichomes could be detected towards maturity of the gametophytes, which occurred 30–40 days after sowing. Their

emergence coincided in time with the first visible indications of an apogamous sporophyte, which was the production under the notch of 2–5 very long hairs that finally reached up to 40 cells. These hairs associated with the apogamous sporophyte were very different to the ones of the gametophyte. All the gametophytes that produced sporophytes (about 75% of the total observed) also formed these long hairs associated with the sporophytes, but not all produced short marginal ones. Secretion of white farina by the marginal gametophytic hairs began within a few days following their formation. All of the observed marginal hairs produced farina. Sometimes, the long glandular hairs associated to the apogamous sporophyte produced farina also.

While the long glandular hairs elongated and the short hairs began to produce farina, proliferative areas appeared below the notch that gave rise to the new apogamous sporophyte. Some 3–5 days later, a dense cluster of cells emerged from these areas and began to produce 2–6 celled hairs that also secreted farina. Finally, as the new sporophyte elongated and acquired a leafy shape, glandular hairs appeared over its margins and lamina.

DISCUSSION

Observations to date of cheilanthoid gametophytes have shown that development of naked, hairless, non-glandular bodies predominates (Nayar and Kaur, 1971; Gabriel y Galán and Prada, 2009). Some gametophytes were known to produce hairs, however (Gabriel y Galán and Prada, 2010), and less frequently, even glandular secretions including farina (Tryon, 1947; Giauque, 1949; Nayar and Kaur, 1971; Atkinson, 1973).

Following the availability of molecular phylogenies, it became clear that all the glandular farinose gametophytes observed were restricted to members of the notholaenoid lineage (Rothfels *et al.*, 2008). This was then proposed as a synapomorphic character supporting the notholaenoid clade, although with caution, due to the small number of observed gametophytes, (Rothfels *et al.*, 2008; Sigel *et al.*, 2011).

In this work we document for the first time farina production in gametophytes of the pellaoid species *Argyrochosma nivea*. As far as is known, there are only three previous studies on gametophytes of *Argyrochosma*, one of *A. incana* (C. Presl) Windham (Nayar and Bajpai, 1964) and two of *A. nivea* (Woronin, 1907; Gabriel y Galán, 2011). All of these studies described naked, non-farinous gametophytes. The new observations made in this work report the presence of marginal glandular hairs in some of the gametophytes, which produce white farina at maturity. Timing in farina production is quite delayed in comparison with other known farinous gametophytes, as in some *Notholaena* (Nayar and Kaur, 1971): in *Argyrochosma* the phenomenon seems to occur towards gametophyte maturity. Several characters of the gametophytic farina indicate that it is independent of the sporophytic farina, including hair morphology (2-celled farinous gametophytic hairs vs. up to 40-celled first sporophytic farinous hairs), timing and amount of farina production (marginal gametophytic farina appears days before the proliferative clusters of sporophytes produce signals of farina,

and reduced farina production by the gametophyte compared to the sporophyte is similar to that in some notholaenoid species [Nayar and Kaur, 1971]), and location of farinose cells (marginal hairs develop near the notch but also in the wings). However, gametophytic farina appears identical to that produced by the sporophytes, in both color and texture, as seen under a microscope. Chemical analyses to confirm these observations should be undertaken.

Farina production in gametophytes of *A. nivea* var. *nivea* seems to be facultative, as only some individuals from each of the two different localities showed the character, while the majority remained glabrous. There is no obvious correlation between farina production and ecology by which to explain this facultative nature, as all of the sporophytes collected for this work lived under the same xeric conditions in exposed rock crevices, at more or less similar altitude. It may be that farina production in the gametophyte is triggered by the development of the apogamous sporophyte, because farina production by the gametophytes only occurs at gametophytic maturity. This deserves further investigation, but if this hypothesis is considered, it will be necessary to explain why some gametophytes produced sporophytes but not farina.

Whatever the trigger for farina production may be, its occurrence in gametophytes of *Argyrochosma* is no longer in doubt. The presence of farinous gametophytes in members of the pellaoid subclade of cheilanthoid ferns indicates that this character should no longer be considered a synapomorphy for the notholaenoids. It seems that farina production by gametophytes, like farina production by sporophytes, is a convergent character within cheilanthoids, appearing independently in at least the notholaenoid and pellaoid lineages (Rothfels *et al.*, 2008; Sigel *et al.*, 2011). In addition to observation of more gametophytes from all the Pteridaceae lineages, a more detailed ecological study is necessary in order to determine which variables may influence farina production in gametophytes of *A. nivea*.

ACKNOWLEDGMENTS

Field trips were made with financial support from the Spanish Red Universitaria de Investigación sobre Cooperación para el Desarrollo (project G07/10) and the Ministerio de Ciencia e Innovación (project CGL 2009-13622, Subprograma BOS).

LITERATURE CITED

- ATKINSON, L. R. 1973. The gametophyte and family relationships. *J. Linn. Soc. Lond. Bot.* 67:73–90.
- DE LA SOTA, E. R., M. L. LUNA, G. E. GIUDICE and J. P. RAMOS GIACOSA. 2009. Sinopsis de las pteridofitas de la provincia de San Luis (Argentina). *Bol. Soc. Argent. Bot.* 44:367–385.
- DYER, A. 1979. The culture of fern gametophytes for experimental investigation. Pp. 254–305, *In*: A. Dyer, ed. *The experimental biology of ferns*. Academic Press, London.
- EDWARDS, M. E. and J. H. MILLER. 1972. Growth regulation by ethylene in fern gametophytes .3. Inhibition of spore germination. *Am. J. Bot.* 59:458–465.
- GABRIEL Y GALÁN, J. M. 2011. Gametophyte development and reproduction of *Argyrochosma nivea* (Poir.) Windham (Pteridaceae). *Biologia* 65:50–54.

- GABRIEL Y GALÁN, J. M. and C. PRADA. 2009. Gametophytes of *Pleurosorus papaverifolius* (Kunze) Fee (Aspleniaceae) and *Cheilanthes glauca* (Cav.) Mett. (Pteridaceae), two South American ferns. *Acta Bot. Bras.* 23:805–811.
- GABRIEL Y GALÁN, J. M. and C. PRADA. 2010. Gametophyte of the Andean fern *Cheilanthes pilosa* Goldm. (Pteridaceae). *Amer. Fern J.* 100:32–38.
- GASTONY, G. J. and D. R. ROLLO. 1995. Phylogeny and generic circumscriptions of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) inferred from *rbcL* nucleotide sequences. *Amer. Fern J.* 85:341–360.
- GIAUQUE, M. F. A. 1949. Wax glands and prothallia. *Amer. Fern J.* 39:33–35.
- MORBELLI, M. A., M. M. PONCE, C. C. MACLUF and M. R. PINEIRO. 2001. Palynology of South American *Argyrochosma* and *Notholaena* (Pteridaceae) species. *Grana* 40:280–291.
- NAYAR, B. K. and N. BAJPAL. 1964. Morphology of the gametophytes of some species of *Pellaea* and *Notholaena*. *J. Linn. Soc. Lond. Bot.* 59:63–76.
- NAYAR, B. K. and S. KAUR. 1971. Gametophytes of homosporous ferns. *Bot. Rev.* 37:295–396.
- ROTHFELS, C. J., M. D. WINDHAM, A. L. GRUSZ, G. J. GASTONY and K. M. PRYER. 2008. Toward a monophyletic *Notholaena* (Pteridaceae): resolving patterns of evolutionary convergence in xeric-adapted ferns. *Taxon* 57:712–724.
- SCHUETTPELZ, E. and K. M. PRYER. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. *Taxon* 56:1037–1050.
- SCHUETTPELZ, E. and K. M. PRYER. 2008. Fern phylogeny. Pp. 395–416, *In*: T. A. Ranker and C. H. Haufler, eds. *Biology and evolution of ferns and lycophytes*. Cambridge University Press, Cambridge.
- 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. *Mol. Phylogenet. Evol.* 44:1172–1185.
- SIGEL, E. M., M. D. WINDHAM, L. HUIET, G. YATSKIEVYCH and K. M. PRYER. 2011. Species relationships and farina evolution in the Cheilanthoid fern genus *Argyrochosma* (Pteridaceae). *Syst. Bot.* 36:554–564.
- SMITH, A. R., K. M. PRYER, E. SCHUETTPELZ, P. KORALL, H. SCHNEIDER and P. G. WOLF. 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, A. F. 1947. Glandular prothalli of *Notholanea standleyi*. *Amer. Fern J.* 37:88–89.
- TRYON, R. M. and R. G. STOLZE. 1989. Pteridophyta of Peru. Part. II: Pteridaceae-Dennstaedtiaceae. *Fieldiana Bot., new series* 22:1–128.
- WINDHAM, M. D. 1987. *Argyrochosma*, a new genus of cheilanthoid ferns. *Amer. Fern J.* 77:37–41.
- WINDHAM, M. D., L. HUIET, E. SCHUETTPELZ, A. L. GRUSZ, C. J. ROTHFELS and J. BECK. 2009. Using Plastid and Nuclear DNA Sequences to Redraw Generic Boundaries and Demystify Species Complexes in Cheilanthoid Ferns. *Amer. Fern J.* 99:127–132.
- WORONIN, H. 1907. Apogamie und Aposporie bei einigen Farnen. *Ber. Deutsch. Bot. Ges.* 25:115–135.

Cytotoxic and Tripanocide Activities of *Pityrogramma calomelanos* (L.) Link.

TEOGENES M. DE SOUZA*, MARIA F. B. M. BRAGA, ROGÉRIO A. SARAIVA,
PABLO A. NOGARA, DIONES C. BUENO, ALINE A. BOLIGON, MARGARETH L. A. FONE,
and JOÃO B. T. DA ROCHA

Laboratório de Microbiologia e Biologia Molecular, Universidade Regional do Cariri, Crato (CE), Brasil

MIRIAM ROLON, CELESTE VEGA, and ANTONIETA ROJAS DE ARIAS

Centro para el Desarrollo de la Investigación Científica (CEDIC), Fundación Moisés Bertoni/
Laboratorios Díaz Gill, Asunción-Paraguay

JOSE G. M. COSTA

Laboratório de Pesquisa em Produtos Naturais, Universidade Regional do Cariri, Crato (CE), Brasil

IRWIN R. ALENCAR DE MENEZES

Laboratório de Farmacologia e Química Molecular, Universidade Regional do Cariri, Crato (CE), Brasil

HENRIQUE D. M. COUTINHO and ANTÔNIO A. F. SARAIVA

Laboratório de Microbiologia e Biologia Molecular, Universidade Regional do Cariri, Crato (CE), Brasil

ABSTRACT.—Chagas disease is caused by *Trypanosoma cruzi*, and is considered a public health problem. The current treatments for this disease are the synthetic drugs nifurtimox and benznidazol, which are highly toxic. *Pityrogramma calomelanos*, a plant used in traditional medicine as an astringent, analgesic, anti-hemorrhagic, pectoral depurative, emmenagogue, anti-hypertensive, anti-pyretic and an anti-tussive was tested for antiepipastigote activity in vitro. An ethanol extract and hexane fraction of *P. calomelanos* was prepared and tested against *T. cruzi* (CL-B5 clone). The effective concentration capable of killing 50% of parasites (EC₅₀) was 55.26 µg/mL and 73.57 µg/mL for the ethanol extract and hexane fraction, respectively. This is the first record of tripanocidal activity for *P. calomelanos*. Our results indicate that *P. calomelanos* could be a source of antiepipastigote natural products with only moderate toxicity toward healthy human cells.

KEY WORDS.—Antiepipastigote activity, Chagas disease, cytotoxicity, *Pityrogramma calomelanos*

Developing countries with abundant traditional knowledge and rich biodiversity, as in the case of Brazil, still grapple with a high incidence of so-called “neglected diseases,” such as tuberculosis, malaria and Chagas disease (Croft *et al.*, 2005); diseases that have the potential to be treated with natural products of plant origin from Brazil (Croft *et al.*, 2005). Brazil has the greatest biodiversity in the world, with more than 55,000 species of plants cataloged, and an estimated total of 550,000 species (Croft and Sundar, 2006), but only 8% have been studied in the search for bioactive compounds (Simões *et al.*, 2007).

Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi*, transmitted mainly by the insects from the genus *Triatoma*, representing a

* Corresponding author: Departamento de Química Biológica, Universidade Regional do Cariri – URCA, Crato-CE, Brasil. Rua Cel. Antonio Luis 1161, Pimenta, 63105-000. Fone: +55(88)31021212; Fax +55(88) 31021291. E-mail: teogenesms@yahoo.com.br

public health problem in South America with 20 million people infected and 90 million at risk in endemic areas (WHO, 2000). The parasite can be transmitted to humans by triatomine insects, foods contaminated with feces, blood, organ transplants, and by the transplacental route (Prata, 2001).

The treatment of this disease remains ineffective. Several compounds have been tested to evaluate their ability to eliminate infection by *T. cruzi* (Lana and Tafuri, 2005). Two drugs are currently used: nifurtimox and benznidazole. These drugs are active against the parasite blood and tissue forms. However, side effects of these drugs are the most significant argument against their general use: the use of nifurtimox is associated with weight loss, psychic alterations and gastric problems; the use of benznidazole is associated with cutaneous problems such as hypersensitivity, dermatitis, edema, fever, lymphadenopathy and muscle pain (Castro *et al.*, 2006).

Due to these side effects, there is an urgent need for the development of new drugs, and a source for these new drugs can be natural products with anti-*T. cruzi* activity (WHO, 2002). Coura and Castro (2002) reported that several herbal products, such as alkaloids, taxoids, estilbenoids, hormones, propolis, naphthoquinones and crude extracts from plants, have activity against *T. cruzi*. In their work, Menezes *et al.* (2005) related that coumarinic compounds with the highest binding affinity, such as chapelin, isolated from *Rutaceae* species promoted the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In this binding study, the affinity of the compounds was determined by their ability to displace NAD⁺ from the enzyme receptor site. The inhibition of this enzyme affects the process of energy generation by glycolysis in the amastigote forms of *T. cruzi*.

Pityrogramma calomelanos (L.) Link. (Pteridaceae), known in Brazil as "avenca-branca" or "avenca-preta" is used as an ornamental and medicinal plant. This fern has several biological properties reported in the literature such as astringent, analgesic, anti-hemorrhagic, anti-hypertensive, anti-pyretic, anti-tussive (Barros and Andrade, 1997). Many compounds from the leaves of *P. calomelanos* that have been isolated are flavonoids, [(8-phenylpropionyl)-5,7-dihydroxydihydroneoflavone] and {8-[3-(4-*p*-methoxyphenyl)propionyl]-7-dihydrodihydroxyneoflavone} named calomelanol (Fujio *et al.*, 1991), terpens, as calomelanolactone (Victor *et al.*, 1978) and chalcones, as 2',6'-dihydroxy-4',4'-dihydroxychalcone (Sukuruman and Ramadasan, 1991). Due to the social and economic importance of Chagas disease and the absence of studies reporting on the effect of this fern against *T. cruzi*, the purpose of this work was to demonstrate the anti-*Trypanosoma* activity of *Pityrogramma calomelanos*.

MATERIALS AND METHODS

Plant Material

Leaves of *Pityrogramma calomelanos* were collected in the rainy season (September, 2009) in the city of Crato, Ceará State, Brazil. The plant material

was identified by Dr. Antonio Álamo Feitosa Saraiva and the voucher specimen was deposited with the identification number 5570 in the Herbarium "Dárdano de Andrade Lima" of University of the Region of Cariri, Crato, CE, Brazil.

Preparation of Ethanol Extract (EEPC) and Hexane Fraction (HFPC) of *Pityrogramma Calomelanos*

Nine hundred fifty grams of leaves were dried and kept at room temperature. The powdered leaf material was macerated using 1 L of 95% ethanol as the solvent for 72 h at room temperature. The mixture was filtered and concentrated under vacuum in a rotary evaporator under 60°C and 760mm/Hg of temperature and pressure, respectively (Brasileiro *et al.*, 2006). Nine hundred fifty grams of aerial parts yielded 50 g of ethanol extract (EEPC). After this, 40 g of EEPC were dissolved in 95% ethanol mixed with silica gel (Merck®) and fractionated with hexane by percolation with the solvent. The hexane fraction (HFPC) was concentrated under vacuum in a rotary evaporator under 60°C and 760 mm/Hg of temperature and pressure, producing 0.56 g of HFPC. The hexane was chosen to extract nonpolar compounds of the EEPC. After this, the ethanol extract and hexane fraction were diluted using 1 mL of DMSO to the assays.

Phytochemical Screening

The qualitative phytochemical assays were performed to detect the presence of secondary metabolites. The tests are based in the visual observation of color modifications or by precipitation after the use of reagents to detect each group of secondary metabolites. The tests were performed according Matos (2009).

Quantification of Phenolics Compounds of Hexane Fraction by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C₁₈ column (4.6 mm × 250 mm) packed with 5 µm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively, following the method described by Laghari *et al.* (2011), with slight modifications. The fern extract was analyzed and dissolved in hexane at a concentration of 3 mg/mL. The presence of six phenolic compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume 40 µL and the wavelengths were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The hexane fraction and mobile phase were filtered through 0.45 µm

membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.020–0.200 mg/mL for kaempferol, quercetin and rutin; and 0.050–0.250 mg/mL for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing retention time with those of reference standards and by DAD spectra (200–400 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

Cell Strains Used

For *in vitro* studies of *Trypanosoma cruzi*, the clone CL-B5 was used (Buckner *et al.*, 1996). The stable parasite, transfected with *Escherichia coli* (*lacZ*) that have the gene coding for β -galactosidase, was kindly provided by Dr. F. Buckner through Instituto Conmemorativo Gorgas (Panama). Epimastigotes were grown at 28°C in liver infusion tryptose broth (Difco, Detroit, MI) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (Reig Jofr'e S.A., Barcelona, Spain), as described previously (Le Senne *et al.*, 2002), and harvested during the exponential growth phase.

Murine J774 macrophages were grown in plastic 25 μ L flasks in RPMI 1640 medium (Sigma®; with glutamine, without bicarbonate and phenol red indicator), supplemented with 20% heat inactivated (30 min, 56°C) fetal bovine serum (FBS) and penicillin G (100 U/mL) and streptomycin (100 μ g/mL) in a humidified 5% CO₂/95% air atmosphere at 37°C. For the experiments, cells in the pre-confluence phase were harvested with trypsin. Cell cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Cell viability was evaluated colorimetrically with resazurin according to a previously described method (Rolon *et al.*, 2006).

Reagents

Chlorophenol red- β -D-galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in 0.9% Triton X-100 (pH 7.4). Penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofre S.A., Barcelona, Spain). Resazurin sodium salt was obtained from Sigma-Aldrich (St Louis, MO) and stored at 4°C protected from light. A solution of resazurin was prepared in 1% phosphate-buffered solution (PBS), pH 7, and filter sterilized prior to use.

Epimastigote Susceptibility Assay

The screening assay was performed in 96-well microplates with cultures that had not reached the stationary phase, as described (Vega *et al.*, 2005). Briefly, epimastigotes were seeded at 1×10^5 mL⁻¹ in 200 μ L of liver tryptose broth medium. The plates were then incubated with EEPC and HFPC (0.1–50 μ g/mL) at 28°C for 72 h, at which time 50 μ L of CPRG solution was added to give a final concentration of 200 μ M. the plates were incubated at 37°C for an additional 6 h and were then read at 595 nm. Nifurtimox was used as the reference drug. Each

concentration was tested in triplicate. Each experiment was performed twice separately. The efficacy of each compound was estimated by calculating the antiepipastigote percent (AE%).

Cytotoxicity Assays

Murine J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottom microplates with 100 μ L of RPMI 1640 medium. The cells were allowed to attach for 24 h at 37°C, 5% CO₂, after which the medium was removed and replaced with medium containing different concentrations of treatment. Macrophages were incubated with treatment for another 24 h. Growth controls were also included. Afterwards, 20 μ L of 2 mM resazurin solution was added and plates were returned to incubator for another 3 h. to evaluate cell viability. The reduction of resazurin was determined by dual wavelength absorbance measurement at 490 nm and 595 nm. Background was subtracted. Each concentration was assayed three times. Blanks include medium and treatment only. The cytotoxicity of each compound was estimated by calculating the cytotoxic percentage (EC₅₀), cells surviving/cells dead.

Statistical Analysis

The statistical analysis was performed using Prism program 5.0. The effective concentration (EC₅₀) was calculated by the linear regression method.

RESULTS AND DISCUSSION

The phytochemicals are shown in Table 1. Two groups of substances were observed in the hexane fraction but not detected in the ethanol extract: flavonols and chalcones.

HPLC Analysis

Due to the results obtained with the hexane fraction, this product was subjected to HPLC analysis for quantification of phenol compounds. HPLC fingerprinting of hexane fraction of *Pityrogramma calomelanos* revealed the presence of the gallic acid ($t_R = 17.83$ min; peak 1), chlorogenic acid ($t_R = 28.14$ min; peak 2), caffeic acid ($t_R = 34.09$ min; peak 3), quercetin ($t_R = 49.78$ min; peak 5) and kaempferol ($t_R = 58.96$ min; peak 6) (Fig. 1 and Table 2). The HPLC analysis, according to the chemical analysis, revealed the presence of flavonoids (quercetin and kaempferol) and phenolic acids (chlorogenic and caffeic acids) in the HFPC.

Our results indicate that HFPC, when tested at a concentration of 50 μ g/mL, was active against epimastigote forms of *T. cruzi*, killing 71% of the parasites with 0% cytotoxicity. This article is the first report relating the chemical composition of *P. calomelanos* with the trypanocidal activity. Hayacibara *et al.* (2005) reported that the chemical composition of propolis (a resinous mixture collected by honey bees from tree buds, flowers or other botanical sources) is

TABLE 1. Phytochemical screening of ethanol extract and hexane fraction of *Pityrogramma calomelanos* (L.) Link.

	METABOLITES														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EEPC	+	-	-	+	+	+	+	+	-	-	+	-	-	-	+
HFPC	-	-	-	-	+	-	+	+	+	-	+	-	+	-	-

1 - Alkaloids; 2 - Anthocyanidins; 3 - Anthocyanins; 4 - Aurones; 5 - Catechins; 6 - Chalcones; 7 - Flavones; 8 - Flavonones; 9 - Flavonols; 10 - Flavononols; 11 - Phenols; 12 - Leucoanthocyanidins; 13 Xanthones - 14 - Pyrogallics Tannins; 15 - Saponins; (+) presence; (-) absence; EEPC (ethanol extract of *Pityrogramma calomelanos*); HFPC (hexane fraction of *Pityrogramma calomelanos*).

rich in phenylpropanoids (chlorogenic and caffeic acids) and Prytyk *et al.* (2003), tested the *in vitro* activity of the propolis extract against *Trypanosoma cruzi*, detecting an interesting activity against epimastigote forms of this parasite. These results are similar to our work, showing a relationship between phenylpropanoids and tripanocidal activity. Chalcones isolated from *Myrcia hiemales* Camb. demonstrated an inhibitory effect against the cruzaine (Deise *et al.*, 2006; Silva, 2007). This is one of the most important cysteine-proteases in *Trypanosoma cruzi*, being essential for the parasite multiplication (Simeonov, 2008).

Several biological activities associated with flavonoids have been reported. Quercetin, the most common flavonoid detected in foods, presented antiprotozoal effects against *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma brucei* and *T. rhodesiense* (Camacho *et al.*, 2002; Williamson and Finnigan,

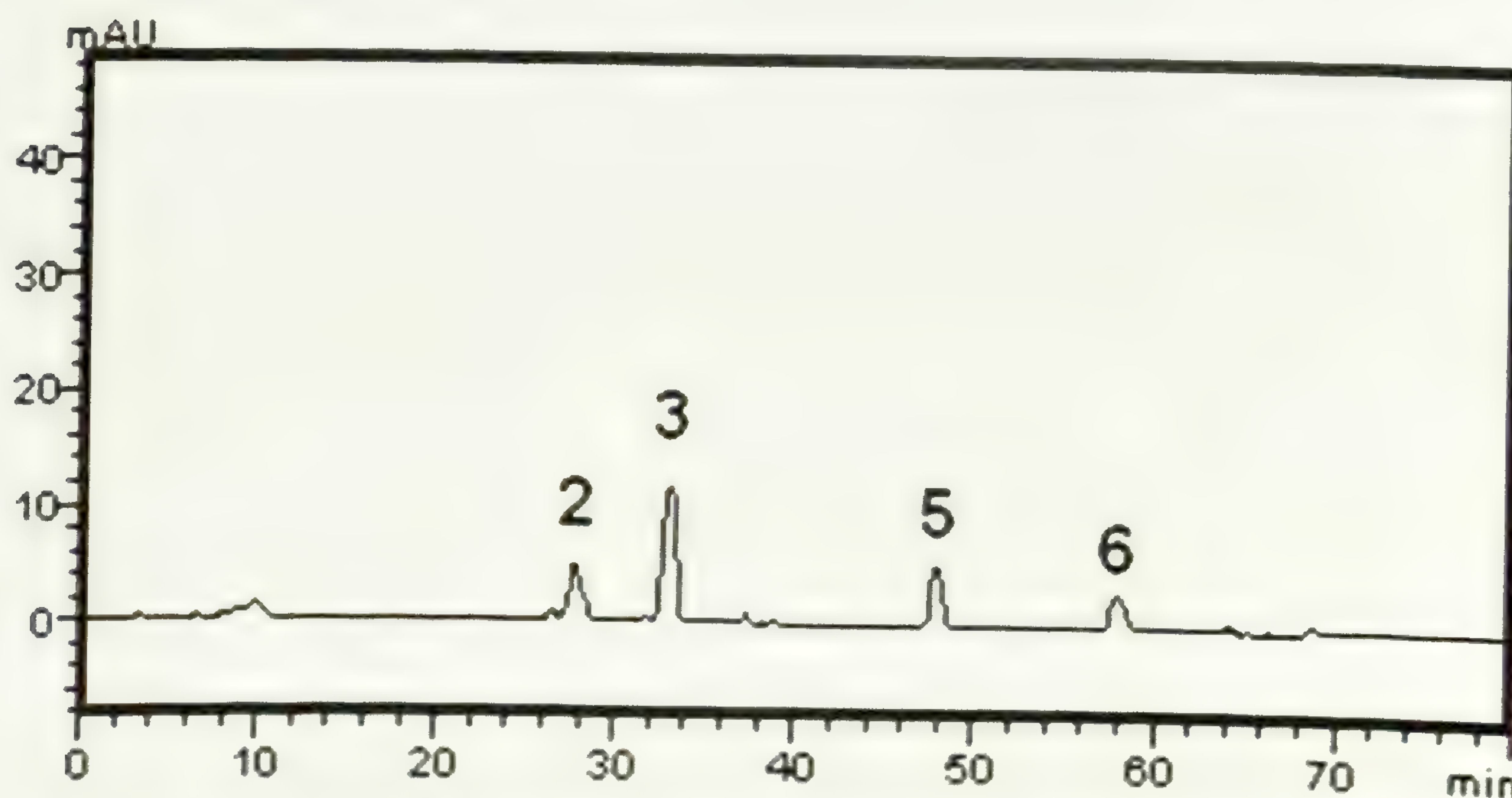


FIG. 1. Representative high performance liquid chromatography profile of (HFPC), detection UV was at 327nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6).

TABLE 2. Phenolics and flavonoids composition of hexane fraction of *Pityrogramma calomelanos* (L.) Link.

Compounds	HFPC	
	mg/g	Percent
Gallic acid	-	-
Chlorogenic acid	1.05 ± 0.01 a	0.10
Caffeic acid	4.59 ± 0.03 b	0.45
Rutin	-	-
Quercetin	1.14 ± 0.02 a	0.11
Kaempferol	0.93 ± 0.01 a	0.09

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.005$.

1978). Ana *et al.* (2010), demonstrated that quercetin and taxifolin, isolated from a hexane fraction of *Rapanea lancifolia* (Mart.) Mez, demonstrated a low trypanocidal activity. Takeara *et al.* (2003) evaluated the quercetin-3-methyl ether isolated of extract from *Lychnophora staavioides* Mart. (Asteraceae), demonstrating a promising potential for the use against *T. cruzi*, not affecting the blood cells.

Trypanosoma cruzi Epimastigote Susceptibility Assay

The trypanocidal activity of EEPC and HFPC is shown in Table 3. The results demonstrated that HFPC was active against the strain CL-B5 strain of *T. cruzi* with a concentration 50 µg/mL, inhibiting 71% for *T. cruzi* ($EC_{50} = 73.57\mu\text{g/mL}$). This is impressive due the fact that an EC_{50} lower than 500 µg/mL is considered clinically relevant (Rosas *et al.*, 2007). However, the ethanol extract of *P. calomelanos* did not show a clinically relevant inhibitory activity against the epimastigote forms due its toxicity against murine macrophages.

Other plants of the Brazilian flora have shown trypanocidal activity, such as extracts and fractions of *Ampelozizyphus amazonicus* Ducke (Rosas *et al.*, 2007), the ethyl acetate fraction of *Camellia sinensis* (L.) Kuntze (Paveto *et al.*, 2004) and polar extracts of *Siphoneugena densiflora* O. Berg (Gallo *et al.*, 2008).

Cytotoxic Activity

Also important in the search for active compounds with trypanocidal activity is the toxicity against mammalian host cells. J774 macrophages were utilized to evaluate the cytotoxicity and determine the selectivity of EEPC and HFPC. The results are presented in Table 2. No toxicity was observed at concentrations of 5, 12, 25, and 50 µg/mL of the hexane fraction. The HFPC showed a low toxicity against macrophages J774 when compared with Roldos *et al.* (2008). In this study, the semi-synthetic compound 1,4-Hydroxylunularin, a derivative hydroxybibenzyl showed a toxic effect against 18.88% of

TABLE 3. Percent of parasite lysis induced by ethanol extract and hexane fraction of *Pityrogramma calomelanos* against the epimastigote forms of *Trypanosoma cruzi* CL-B5 strain.

Extract	Concentration ($\mu\text{g/mL}$)	%AE	%SD	% C	EC ₅₀
EEPC	500	91	3.3	100	55.26
	200	77	3.1	86	
	100	74	2.7	73	
	50	37	1.8	6	
	50	71	1.2	0	
HFPC	25	67	0.7	0	73.57
	12.5	47	0.9	0	
	10	89.1	3.3	-	
	1	54.9	0.7	-	
Nifurtimox	0.5	45.6	4.2	-	0.91

%AE – Percent of inhibition of epimastigote forms; %SD – Standard deviation; % C – cytotoxic effect; EC₅₀ – Concentration with 50% of the maximum activity; EEPC (ethanol extract of *P. calomelanos*), HFPC (hexane fraction of *P. calomelanos*).

macrophages in a concentration of 21.7 $\mu\text{g/mL}$. The hexane fraction (HFPC) of *P. calomelanos* appears to be promising in the development of new drugs to treat *T. cruzi*, mainly due to the low toxic effect *in vitro* and due the antiepipimastigote activity demonstrated in our work, indicating the necessity to proceed with *in vivo* studies.

Conclusion

Our results indicate that *Pityrogramma calomelanos* could be a source of plant-derived natural products with antiepipimastigote activity and low toxicity, representing an interesting alternative to other efforts to combat infectious diseases such Chagas disease.

LITERATURE CITED

- ARAYA, J. E., I. NEIRA, S. SILVA, R. A. MORTARA, P. MANQUE and E. CORDERO. 2003. Diterpenoids from *Azorella compacta* (Umbelliferae) active on *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz 98:413–418.
- BARROS, I. C. L. and L. H. C. ANDRADE. 1997. Pteridófitas medicinais (samambaias, avencas e plantas afins). Recife, Ed. Universitária da Universidade Federal de Pernambuco.
- BRASILEIRO, B. G., V. R. PIZZOLO, D. S. RASLAN, C. M. JAMAL and D. SILVEIRA. 2006. Antimicrobial and cytotoxic activities screening of some Brazilian medicinal plants used in Governador Valadares district. Braz. J. Pharm. Sci. 42:195–202.
- BUCKNER, F. S., C. L. VERLINDE, A. C. LA FLAMME and V. W. C. VAN. 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrob. Agents Chemother. 40:2592–2597.
- CAMACHO, M. D., J. D. PHILLIPSON, S. L. CROFT, D. MARLEY, G. C. KIRBY and D. C. WARHURST. 2002. Assessment of the antiprotozoal activity of *Galphimia glauca* and the isolation of new nor-secofriedelanes and nor-friedelanes. J. Nat. Prod. 65:1457–1461.
- CASTRO, J. A., M. M. DE MECCA and L. C. BARTEL. 2006. Toxic side effects of drugs used to treat Chagas' disease (American tripanosomiasis). Hum. Exp. Toxicol. 25:471–479.

- COURA, J. R. and S. L. CASTRO. 2002. A critical review on Chagas disease chemotherapy. *Mem. Inst. Oswaldo Cruz* 97:3–24.
- CROFT, S. L. and A. H. SUNDAR. 2006. Drug Resistance in Leishmaniasis. *Clin. Microbiol. Rev.* 19:111–126.
- CROFT, S. L., M. P. BARRETT and J. A. URBINA. 2005. Chemotherapy of trypanosomiasis and leishmaniasis. *Trends Parasitol.* 21:508–512.
- DEISE, M. B., M. L. D. C. ALESSANDRA, J. N. RICARDO, O. GLAUCIUS, A. Y. ROSENDO and D. A. ADRIANO. 2006. Síntese e avaliação bioquímica de uma série de Chalconas como inibidores da cruzaina de *Trypanosoma cruzi*. In: 29^ª Reunião Anual da Sociedade Brasileira de Química.
- FUJIO, A., I. MUNEKAZU, T. THOSHIYUKI and M. MIZUO. 1991. Complex flavonoids in farinose exudate from *Pityrogramma calomelanos*. *Phytochemistry* 30:3091–3093.
- GALLO, M. B. C., A. S. F. MARQUES, P. C. VIEIRA, M. F. G. D. SILVA, J. B. FERNANDES and M. SILVA. 2008. Enzymatic Inhibitory activity and trypanocidal effects of extracts and compounds from *Siphoneugena densiflora* O. Berg and *Vitex polygama* Cham. *Z. Naturforsch. C* 63:371–382.
- HAYACIBARA, M. F., H. KOO, P. L. ROSALEN, S. DUARTE, E. M. FRANCO, W. H. BROWEN, M. IKEGAKI and J. A. CURY. 2005. In vitro and vivo effects of isolated fractions of Brazilian propolis on caries development. *J. Ethnopharmacol.* 101:110–115.
- LAGHARI, A. H., S. MEMON, A. NELOFAR, K. M. KHAN and A. YASMIN. 2011. Determination of free phenolic acids and antioxidant activity of methanolic extracts obtained from fruits and leaves of *Chenopodium album*. *Food Chem.* 126:1850–1855.
- LANA, M. and W. L. TAFURI. 2005. *Trypanosoma cruzi* e Doença de Chagas. In: , D. P. NEVES, A. L. MELO, O. GENARO and P. M. LINARD. 2005. *Parasitologia Humana*. 11. ed. São Paulo: Atheneu. cap. 11, 85–108.
- LEITE, A. C., A. P. NETO, A. R. P. AMBROZIN, J. B. FERNANDES, P. C. VIEIRA, M. F. G. F. SILVA and S. ALBUQUERQUE. 2010. Trypanocidal activity of flavonoids and limonoids isolated from *Myrsinaceae* and *Meliaceae* active plant extracts *Rev. Bras. Farmacogn.* 20:1–6.
- LE SENNE, A., S. MUELAS-SERRANO, C. FERNANDEZ-PORTILLO, J. A. ESCARIO and A. GOMEZ-BARRIO. 2002. Biological characterization of a beta-galactosidase expressing clone of *Trypanosoma cruzi* CL strain. *Mem. Inst. Oswaldo Cruz* 97:1101–1105.
- MATOS, F. J. A. 2009. Edições UFC. Introdução à química experimental, 3:4–74.
- MENEZES, I. R. J. C., C. A. LOPES, G. O. MONTANARI, F. M. S. PAVÃO and V. P. C. CASTILHO. 2003. 3D QSAR studies on binding affinities of coumarin natural products for glycosomal GAPDH of *Trypanosoma cruzi*. *J. Comput. Aid. Mol. Des.* 17:277–90.
- PAVETO, C., M. C. GÜIDA, M. I. ESTEVA, V. MARTINO, J. COUSSIO and M. M. FLAWIÁ. 2004. Anti *Trypanosoma cruzi* activity of Green tea (*Camellia sinensis*) Catechins. *Antimicrob. Agents Chemother.* 48:69–79.
- PRATA, A. 2001. Clinical and epidemiological aspects of Chagas disease. *Lancet. Infect. Dis.* 1:92–100.
- PRYTYK, E., A. P. DANTAS, K. SALOMÃO, A. S. PEREIRA, V. S. BANKOVA, S. L. DE CASTRO and N. F. R. AQUINO. 2003. Flavonoids and trypanocidal activity of *Bulgarian propolis*. *J. Ethnopharmacol.* 88:189–193.
- ROLDOS, V., H. NAKAYAMA, M. ROLÓN, A. MONTERO-TORRES, F. TRUCCO and S. TORRES. 2008. Activity of a hydroxybibenzyl bryophyte constituent against *Leishmania spp.* and *Trypanosoma cruzi*: In silico, in vitro and in vivo activity studies. *J. Med. Chem.* 43:1797–1807.
- ROLON, M., E. SECO, C. VEGA, J. J. NOGAL, J. A. ESCARIO and A. GOMEZ-BARRIO. 2006. Selective activity of polyene macrolides produced by genetically modified *Streptomyces* on *Trypanosoma cruzi*. *Int. J. Antimicrob. Agents* 28:104–109.
- ROSAS, L. V., M. S. C. CORDEIRO, F. R. CAMPOS, S. K. R. NASCIMENTO, A. H. JANUÁRIO and S. C. FRANÇA. 2007. In vitro evaluation of the cytotoxic and trypanocidal activities of *Ampelozizyphus amazonicus* (Rhamnaceae). *Braz. J. Med. Biol. Res.* 40:663–670.
- SILVA, P. D. 2007. Estudo fitoquímico e avaliação das atividades antimicrobianas e antiparasitárias dos flavonóides isolados de *Myrcia hiemalis* (Myrtaceae). Universidade Federal da Bahia, Instituto de química, Programa de Pós Graduação em Química (MSc Thesis).
- SIMEONOV, A., A. JADHAV, C. J. THOMAS, Y. WANG, R. HUANG, N. T. SOUTHALL, P. SHINN, J. SMITH, C. P. AUSTIN, D. S. AULD and J. INGLESE. 2008. Fluorescence spectroscopic profiling of compound libraries. *J. Med. Chem.* 51:236–371.

- SIMÕES, C. M. O., E. P. SCHENKEL, G. GOSMANN, J. C. P. MELLO, L. A. MENTZ and P. R. PETROVICK. 2003. *Farmacognosia: da planta ao medicamento*. 5 ed. Porto Alegre/Florianópolis: Editora da UFRGS / Editora UFSC.
- SUKURUMAN, K. and K. RAMADASAN. 1991. Screening of 11 ferns for cytotoxic and antitumor potential with special reference to *Pityrogramma calomelanos*. *J. Ethnopharmacol.* 34:93–96.
- TAKEARA, R., S. ALBUQUERQUE, N. P. LOPES and J. L. C. LOPES. 2003. Trypanocidal activity of *Lychnophora staavioides* Mart. (Vernonieae, Asteraceae). *Phytomedicine* 10:490–493.
- VEGA, C., M. ROLON, A. R. MARTÍNEZ-FERNANDEZ, J. A. ESCARIO and A. GOMEZ-BARRIO. 2005. A new pharmacological screening assay with *Trypanosoma cruzi* epimastigotas expressing beta-galactosidase. *Parasitol. Res.* 95:296–298.
- VICTOR, B., S. M. BALDWIN, H. KEN and C. JON. 1978. Sesquiterpenes from *Pityrogramma calomelanos*. *Phytochemistry* 17:275–277.
- WILLIAMSON, J. and T. J. S. FINNIGAN. 1978. Trypanocidal activity of antitumor antibiotics and other metabolic inhibitors. *Antimicrob. Agents. Chemother.* 13:735–744.
- WORLD HEALTH ORGANIZATION (WHO). 2000. Special Programme for Research and Training in Tropical Disease (TDR). Natural products for parasitic diseases. *TDR News* 62:4.
- WORLD HEALTH ORGANIZATION (WHO). 2002. *Police perspectives on medicines*. Geneva: World Health Organization.

Effects of *Ageratina adenophora* on Spore Germination and Gametophyte Development of *Neocheiropteris palmatopedata*

K. M. ZHANG and J. H. LIU

College of Forest Resources and Environment, Nanjing Forestry University, Nanjing, 210037, Jiangsu, China

X. CHENG

Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650201, Yunnan, China

G. F. ZHANG

Yunnan University, Kunming, 650091, Yunnan, China

Y. M. FANG*

College of Forest Resources and Environment, Nanjing Forestry University, Nanjing, 210037, Jiangsu, China

H. J. ZHANG

Nanjing Forestry University, Nanjing, 210037, Jiangsu, China

ABSTRACT.—As one of the worst invasive alien plant species in China, *Ageratina adenophora* has caused serious economic losses and reduced the diversity of native species, possibly due to allelopathic interactions. However, we have little knowledge of its effects on ferns. In Petri dish bioassays, the effects of the aqueous leachates from roots, stems and leaves of *A. adenophora* on spore germination and gametophyte development of *Neocheiropteris palmatopedata* were investigated. All leachates inhibited spore germination and rhizoid growth of *N. palmatopedata*. Furthermore, the inhibitory effects increased with increasing leachate concentrations, and root leachates exhibited the greatest inhibition. Possible inhibitory causes are discussed in the present study. Additionally, the gametophytes of *N. palmatopedata* treated with the leachates of *A. adenophora* did not show morphological differences compared with the control. This result differs from previous studies investigating morphological changes in other fern species when associated with *A. adenophora*. Varying sensitivity of different fern species to the same allelochemicals of *A. adenophora* may partly be responsible for this difference.

KEY WORDS.—*Ageratina adenophora*, *Neocheiropteris palmatopedata*, spore germination, gametophyte development, allelopathic interaction

At present, there are about 170 species of terrestrial invasive plants in China. *Ageratina adenophora* Sprengel (Synonym: *Eupatorium adenophora*) (Asteraceae) is a very successful invasive species (Sang *et al.*, 2010). It is native to Mexico, where it is common (Cronk and Fuller, 1995). It first invaded Yunnan Province in China in the 1940s. Since then it has intensively colonized southwest China, reduced agricultural production and strongly altered plant community structures (Wang *et al.*, 2011). It survives and proliferates under various conditions and may

* Author for correspondence - jwu4@njfu.com.cn

adversely affect the surrounding vegetation through allelopathic effects in China (He and Liu, 1990; Song *et al.*, 2000; Yu *et al.*, 2004; Yang *et al.*, 2006; Yang *et al.*, 2008).

Most previous studies of *Ageratina adenophora* (Ageratina) have focused on its deleterious effects on spermatophytes (Wan *et al.*, 2010). It excretes allelochemicals into the soil, which hinder the germination and seedling growth of potential competitors (Sang *et al.*, 2010). However, studies of the effects of *A. adenophora* invasion on ferns (including gametophytes and sporophytes) have received comparatively little attention (Zhang *et al.*, 2007; Zhang *et al.*, 2008a, 2008b).

Yunnan is one of the richest regions in species diversity in China (Wang and Wang, 2006), with 762 documented fern species (Kunming Institute of Botany, Chinese Academy of Sciences, 2006). Our field observations showed that some ferns failed to establish in pure patches of *Ageratina adenophora*. Previous studies by Zhang *et al.* (2007, 2008a, 2008b) provide strong evidence in support of the inhibitory effects of *A. adenophora* on three fern species, *Macrothelypteris torresiana* (Gaud.) Ching, *Pteris finotii* Christ and *Cibotium barometz* (L.) J. Sm. Those studies showed that all leachates of *A. adenophora* inhibited spore germination and growth of the first rhizoid of the three fern species, and inhibitory effects increased with increasing leachate concentrations.

Neocheiropteris palmatopedata (Baker) H. Christ (Polypodiaceae) is endemic to China and is distributed in Yunnan, Sichuan and Guizhou. It is well known for its ornamental and medicinal value (Lin, 2000; Ding, 1982). Furthermore, in China, it is rare in the wild (Yu, 1999). Hence, understanding the biology of *N. palmatopedata* is of great importance for conservation purposes.

The distribution of *Neocheiropteris palmatopedata* overlaps with that of *Ageratina adenophora* in Yunnan. Allelopathic activity is more readily observed in the sensitive stage of plant growth (Petersen and Fairbrothers, 1980). In ferns, the activity is easily detected in stages such as spore germination and gametophyte development (Petersen and Fairbrothers, 1980). Hence, this study had two main objectives: (i) to determine the potential effects of *A. adenophora* on spore germination and gametophyte development of *N. palmatopedata*; and (ii) to examine the allelopathic potential of roots, stems and leaves of *A. adenophora*.

MATERIALS AND METHODS

Plant Leachates

The effects of *Ageratina adenophora* leachates on *Neocheiropteris palmatopedata* were evaluated in three ways in this study: 1) rate of rhizoid elongation, 2) rate and percentage of spore germination, and 3) gametophyte morphology. Whole fresh plants of *A. adenophora* were collected from Nanjing Forestry University. The root, stem and leaf leachates were made according to Zhang *et al.* (2008a) and were sterilized by passing them through Duropore[®] PVDF membrane (0.45 µm, Carrigtwohill, Co. Cork, Ireland). For the determination of rhizoid elongation, the sterile leachates were diluted with sterile double-distilled water to give six concentrations (0, 10, 20, 30, 40 and 50% leachate). For trials of spore germination

and gametophyte growth, the sterile leachates were diluted with B₅ basal medium (Gamborg *et al.*, 1968) containing 0.7% agar and 2% sugar to give these six concentrations. In all the trials, B₅ basal medium containing 0.7% agar and 2% sugar was used as a control. All media were added at 6 mL per Petri dish (3.4 cm diameter).

Spore Collection, Incubation, Germination and Gametophyte Growth

Spores of *Neocheiropteris palmatopedata* were collected from 15–20 fertile fronds with mature but closed sporangia from 15 individuals on December 2010 in Yunnan University. The collected fronds were immediately sent to Nanjing. The fronds were unfolded, placed in clean paper bags and air dried at room temperature. Spores were collected and cleared by a mesh with pores 0.088 mm in diameter (Zhejiang Shangyu Yarn and Sieve Factory, Shangyu, China) one week later. About 2 mg of spores was packed into a 1 × 1 cm² bag made of clean filter paper. Thereafter, the spores were immersed into distilled water and washed five times in sterile water after surface-sterilizing in 5% sodium hypochlorite for 4 min. The spore suspension was prepared by adding sterile double-distilled water to the sterilized spores. Spores were inoculated evenly in each Petri dish at a density of 30 spores/cm² for the determination of rhizoid elongation and 400 spores/cm² for the trials of spore germination and gametophyte development. After inoculation, all Petri dishes were put into larger Petri dishes (12 cm diameter) to avoid desiccation and pollution. The larger Petri dishes were placed in the dark at 25 °C for 24 h and then transferred to fluorescent light (photon flux density 1 × 10⁴ μ mol m⁻² s⁻¹) at 25 °C at 12 h light photoperiod. Five visual fields were observed in each of the Petri dishes, through a gridding ocular at a magnification of 20 × 1.5 in a microscope (No. XTS 20130, Beijing Tech Instrument Co., Ltd., Beijing, China).

Germination time was calculated as the time elapsed from sowing to the observance of the first germinated spore (i.e., when signs of a first rhizoid were evident). For each dish, fourteen days after the first observed germination, about 400 spores were selected randomly for the calculation of the germination percentage and also for gametophyte development observation. Thereafter, the germination percentage was calculated every 6 days until no further increase was detected. Data for spore germination and gametophyte assays is presented as the mean value of the three Petri dishes of each treatment.

For the determination of rhizoid elongation, after growing for 2 weeks in medium without leachate, six healthy gametophytes with uniform rhizoids in each Petri dish were selected for further observation. Afterwards, all gametophytes were treated with various leachates. One week later, length of rhizoid was recorded using a crossed ocular micrometer in a dissecting microscope (No. XTS 20130, Beijing Tech Instrument Co., Ltd., Beijing, China).

Statistics

The effects of *Ageratina adenophora* leachates on spore germination and gametophyte development of *Neocheiropteris palmatopedata* were analyzed by ANOVA using the SPSS 10.0 package (SPSS, Chicago, IL, USA).

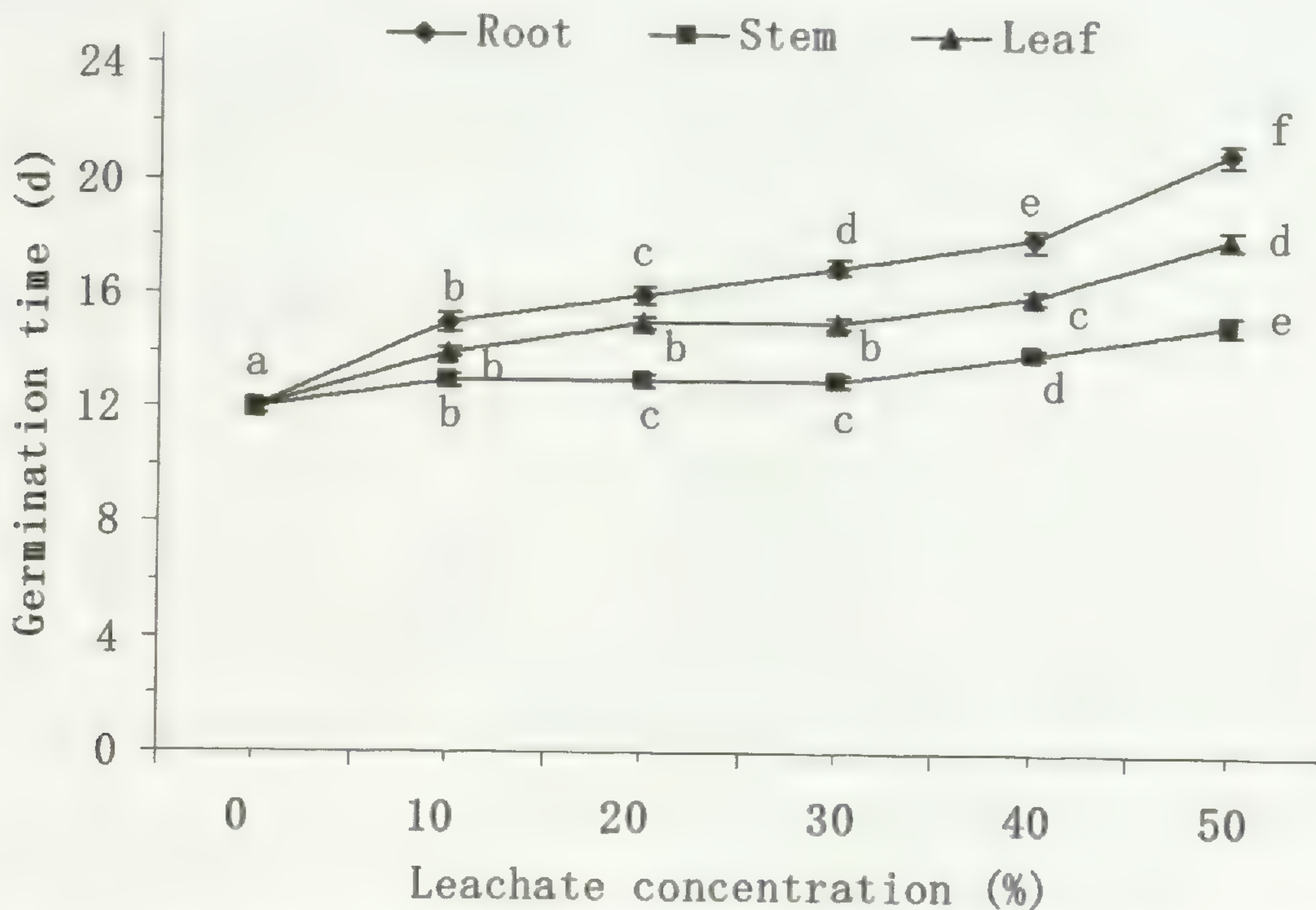


FIG. 1. Germination time of the spores of *Neocheiropteris palmatopedata* in the control and treated with root, stem and leaf leachates of *Ageratina adenophora* after inoculation. Different letters indicate significantly different ($P < 0.05$).

RESULTS

Spore Germination

Spores of *Neocheiropteris palmatopedata* germinated 12 days after inoculation in the control and continued until 35 days. Root, stem and leaf leachates of *Ageratina adenophora* delayed spore germination. The time needed for spore germination differed among treatments. Spores treated with stem leachates germinated faster than those treated with root and leaf leachates (Fig. 1). The inhibition of root, stem and leaf leachates of *A. adenophora* to spore germination percentage of *N. palmatopedata* increased with increasing concentrations ($P < 0.05$) (Fig. 2). As time went by, the germination percentages of *N. palmatopedata* treated with the root, stem and leaf leachates of *A. adenophora* went up and then leveled off ($P < 0.05$) (Fig. 2).

Rhizoid Growth

The root, stem and leaf leachates of *Ageratina adenophora* inhibited the rhizoid growth of *Neocheiropteris palmatopedata* and the inhibitory effect generally increased with increasing leachate concentrations. Root leachates were the most potent inhibitor, followed by the leaf leachates ($P < 0.05$) (Fig. 3).

Gametophyte Development

Gametophyte morphology of *Neocheiropteris palmatopedata* in the control was in agreement with the previous research carried out by Deng *et al.* (2009).

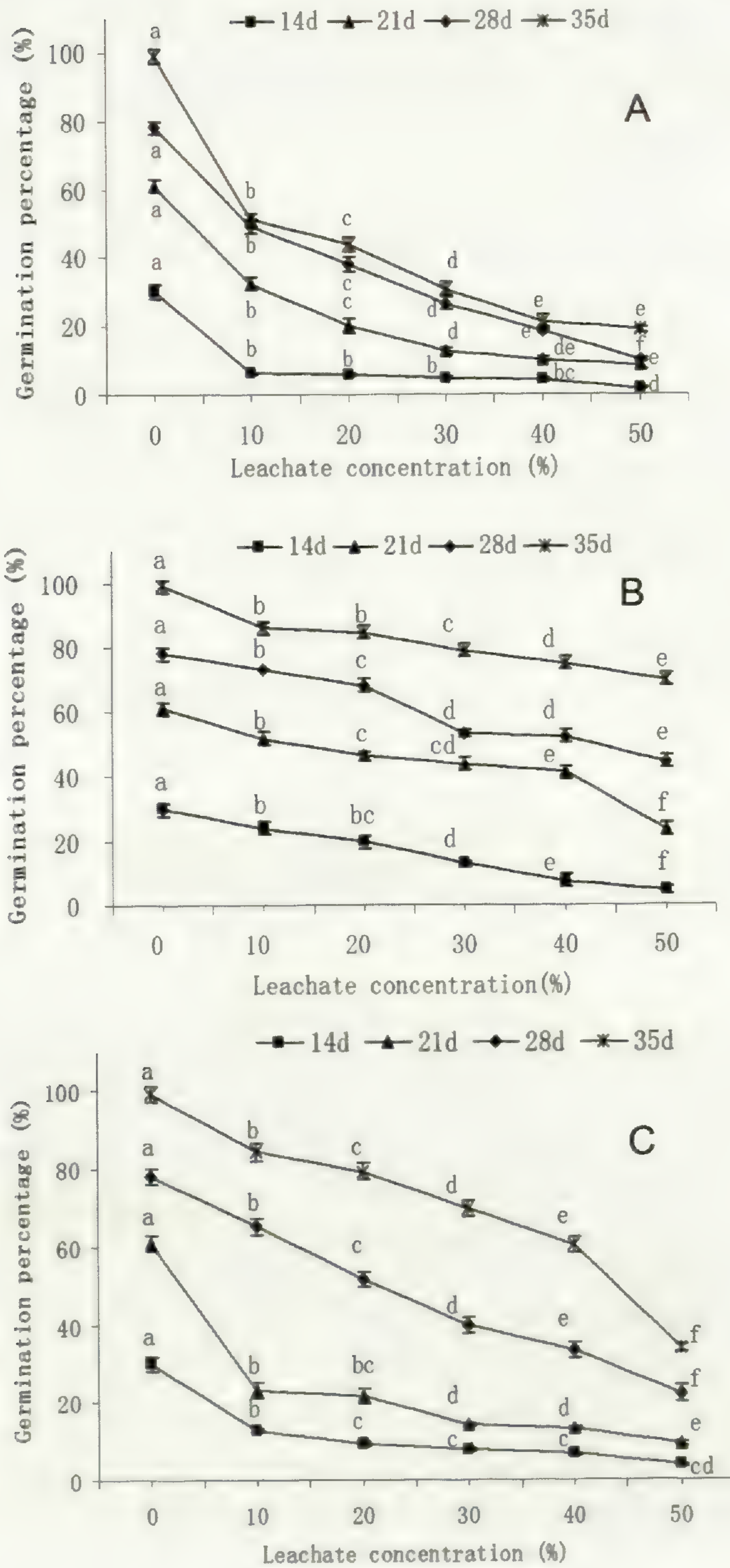


FIG. 2. Germination percentage of the spores of *Neocheiropteris palmatopedata* in the control and treated with root (A), stem (B) and leaf (C) leachates of *Ageratina adenophora*. Statistically significant differences among treatments are indicated by letters above or below lines based on least significant difference (LSD) multiple comparison tests. Two similar letters indicate no significant difference between treatments, and dissimilar letters indicate a significant difference between treatments ($P < 0.05$).

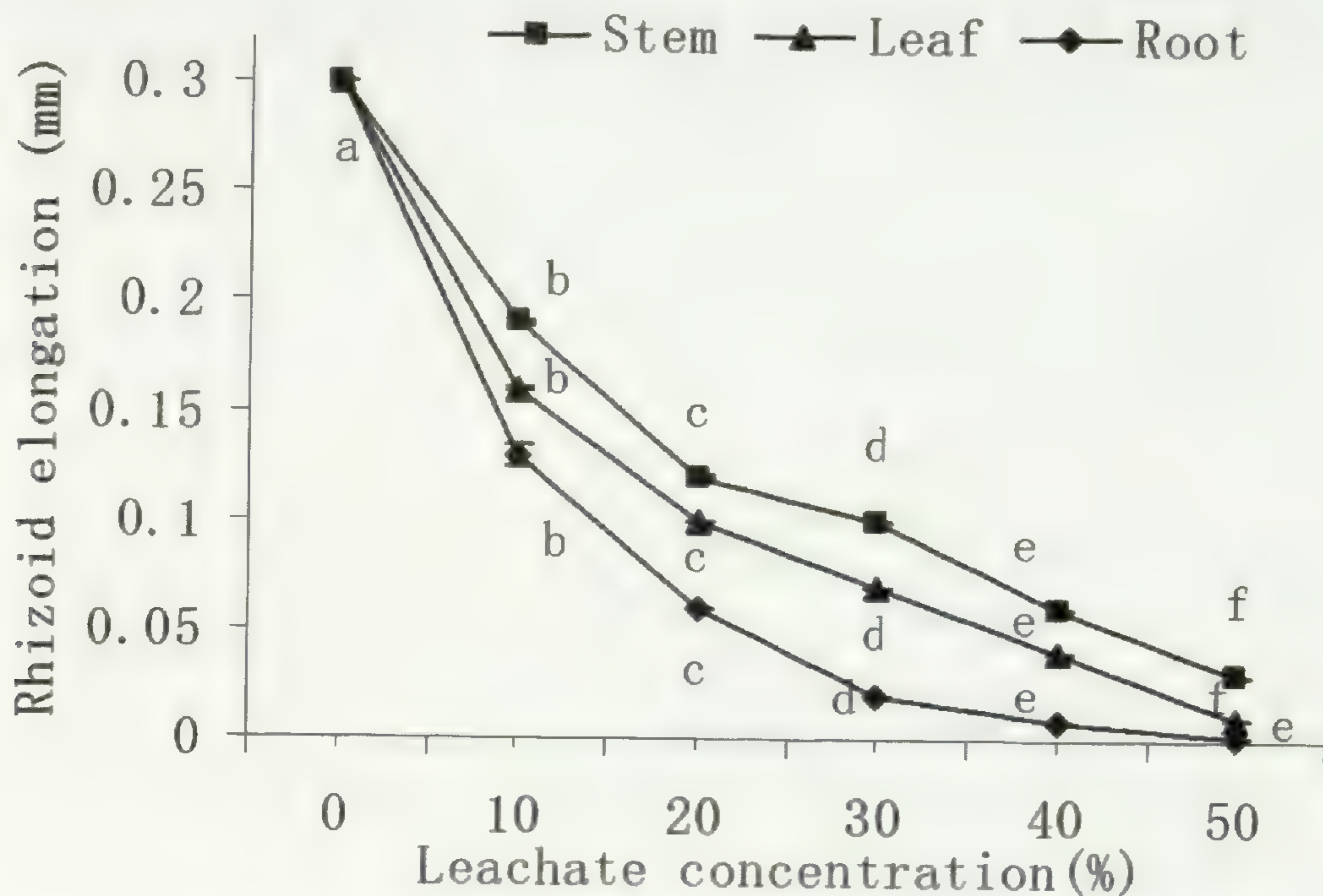


FIG. 3. Mean (\pm SD) of the rhizoid elongation of *Neocheiropteris palmatopedata* in the control and treated with root, stem and leaf leachates of *Ageratina adenophora*. Different letters indicate significantly different ($P < 0.05$).

The gametophytes produced from the spores treated with the leachates of *Ageratina adenophora* did not show morphological differences from the control.

DISCUSSION

Our results indicated that spore germination of *Neocheiropteris palmatopedata* was lower when treated with the root, stem and leaf leachates of *Ageratina adenophora* as compared to the control. Spore germination of *N. palmatopedata* is essential for its sexual reproduction. If this germination inhibition also occurs in nature, where spores of *N. palmatopedata* overlap with plants of *A. adenophora*, these leachates could potentially drastically decrease the abundance of this plant in the community. The delay in germination of *N. palmatopedata* spores could severely affect the competition ability of this fern species for resources.

All leachates of *Ageratina adenophora* inhibited rhizoid growth of *Neocheiropteris palmatopedata*. Furthermore, the inhibitory effects increased with the increasing leachate concentrations. It is generally believed that the rhizoids of fern gametophytes function as organs of uptake and absorption. Inhibition of *N. palmatopedata* rhizoid growth by the root, stem and leaf leachates could reduce the ability of gametophytes to absorb water and nutrients, which could affect the production and potential growth of the future sporophyte and its function in the community. Racusen (2002) demonstrated that rhizoids were the primary site of uptake for monovalent cations in the gametophytes of *Onoclea sensibilis* L. Kamachi *et al.* (2005) showed that *Athyrium* gametophytes had the ability to

accumulate lead in the rhizoids. Therefore, we hypothesize that the active component(s) in the root of *A. adenophora* entered and accumulated in the rhizoids of *N. palmatopedata*, which inhibited the rhizoid elongation of *N. palmatopedata*.

In this study, the gametophytes produced from the spores of *N. palmatopedata* treated with the leachates of *A. adenophora* did not show morphological differences when compared with the control. However, in a study of *Macrotelypteris torresiana*, the rhizoids of the young gametophytes treated with various root leachates of *A. adenophora* were erect, curved, or swollen and the curving and swollen rhizoids increased with the increasing concentrations of *A. adenophora* (Zhang *et al.*, 2008a). The gametophytes from the spores of *Pteris finotii* treated with higher concentrations of root leachates of *A. adenophora* suffered morphological changes (Zhang *et al.*, 2007). This difference might be attributed to differences in sensitivity to the same allelochemicals.

In our experiments, greater inhibitory effects on gametophyte development of *N. palmatopedata* occurred with the root leachates of *A. adenophora*, as compared to stem and leaf leachates. This result was consistent with previous studies, which showed that the root leachates resulted in greater inhibition than the stem and leaf leachates (Zhang *et al.*, 2007; Zhang *et al.*, 2008a; Tripathi *et al.*, 1981). However, He and Liu (1990) observed that the aqueous leaf extracts of *A. adenophora* were the most allelopathic to the seed germination of four plants. Tripathi *et al.* (1981) also reported that the aqueous extracts of different parts of *A. adenophora* caused different effects on other plants. This difference might be due to the differences in the sensitivity to same allelochemicals, concentration of the allelochemicals, or variations in allelochemicals among the tissues. The leachates of *A. adenophora* were identified as phenolic compounds (Yang *et al.*, 2006), which might act as the allelopathic substances (Kim *et al.*, 2005). Further investigation is needed to better understand the chemistry of the allelopathic activity.

Sustainable management of *A. adenophora* in China includes ecological restoration by competitive replacement of *A. adenophora* and biological control agents against *A. adenophora* (Wan *et al.*, 2010). Future efforts will concern the effective control of *A. adenophora* as well as its utilization.

ACKNOWLEDGMENTS

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Jiangsu Planned Projects for Postdoctoral Research Funds (1001078C) and China Postdoctoral Science Foundation funded project (20100481152).

LITERATURE CITED

- CRONK, Q. C. B. and J. L. FULLER. 1995. *Plant invaders: the threat to natural ecosystems*. Chapman and Hall, London, UK.
- DENG, Y. N., X. CHENG, Y. JIAO and G. J. CHEN. 2009. The gametophyte development and endangerment mechanism of *Neochheiropteris palmatopedate* (Polypodiaceae, Fern). *Acta Bot. Yunnanica*. 31:138–144.

- DING, H. S. 1982. *Medicinal spore plants in China*. The Science & Technology Press, Beijing, China.
- GAMBORG, O. L., R. A. MILLER and K. OJIMA. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res.* 50:151–158.
- HE, A. J. and L. H. LIU. 1990. Effects of water extract of *Eupatorium adenophorum* on the germination of several plants. *Chin. J. Weed Sci.* 4:35–38.
- KAMACHI, H., I. KOMORI, H. TAMURA, Y. SAWA, I. KARAHARA, Y. HONMA, N. WADA, T. KAWABATA, K. MATSUDA, S. IKENO, M. NOGUCHI and H. INOUE. 2005. Lead tolerance and accumulation in the gametophytes of the fern *Athyrium yokoscense*. *J. Plant Res.* 118:137–145.
- KIM, Y. O., J. D. JOHNSON and E. J. LEE. 2005. Phytotoxic effects and chemical analysis of leaf extracts from three Phytolaccaceae species in South Korea. *J. Chem. Ecol.* 31:1175–1186.
- KUNMING INSTITUTE OF BOTANY, CHINESE ACADEMY OF SCIENCES. 2006. *Flora of Yunnan*. Science Press, Beijing, China.
- LIN, Y. X. 2000. *Flora Reipublicae Popularis Sinicae*. Science Press, Beijing, China.
- NAYAR, B. K. and S. KAUR. 1971. Gametophytes of homosporous ferns. *Bota Rev.* 37:295–396.
- PETERSEN, R. L. and D. E. FAIRBROTHERS. 1980. Reciprocal allelopathy between the gametophytes of *Osmunda cinnamomea* and *Dryopteris intermedia*. *Am. Fern. J.* 70:73–78.
- RACUSEN, R. H. 2002. Early development in fern gametophytes: interpreting the transition to prothallial architecture in terms of coordinated photosynthetic production and osmotic ion uptake. *Ann. Bot.* 89:227–240.
- SANG, W. G., L. ZHU and C. J. AXMACHER. 2010. Invasion pattern of *Eupatorium adenophorum* Spreng in southern China. *Biol. Invasions.* 12:1721–1730.
- TRIPATHI, R. S., R. S. SINGH and J. P. N. RAI. 1981. Allelopathic potential of *Eupatorium adenophorum*, a dominant ruderal weed of Meghalaya. *Proc. Indian. Acad. Sci.* 47:458–465.
- WAN, F. H., W. X. LIU, J. Y. GUO, S. QIANG, B. P. LI, J. J. WANG, G. Q. YANG, H. B. NIU, F. R. GUI, W. K. HUANG, Z. L. JIANG and W. Q. WANG. 2010. Invasive mechanism and control strategy of *Ageratina adenophora* (Sprengel). *Sci. China Life Sci.* 53:1291–1298.
- WANG, R., J. F. WANG, Z. J. QIU, B. MENG, F. H. WAN and Y. Z. WANG. 2011. Multiple mechanisms underlie rapid expansion of an invasive alien plant. *New Phytol.* 191:828–839.
- WANG, R. and Y. Z. WANG. 2006. Invasion dynamics and potential spread of the invasive alien plant species *Ageratina adenophora* (Asteraceae) in China. *Diver. Distri.* 12:397–408.
- YANG, G. Q., F. H. WAN, W. X. LIU and J. Y. GUO. 2008. Influence of two allelochemicals from *Ageratina adenophora* Sprengel on ABA, IAA and ZR contents in roots of upland rice seedlings. *Allelopathy J.* 21:253–262.
- YANG, G. Q., F. H. WAN, W. X. LIU and X. W. ZHANG. 2006. Physiological effects of allelochemicals from leachates of *Ageratina adenophora* (Spreng.) on rice seedlings. *Allelopathy J.* 18:237–246.
- YU, X. J., D. YU and K. P. MA. 2004. Relationships between allelopathy and invasiveness by *Eupatorium adenophorum* in different sites. *Acta Phytoecol. Sin.* 28:773–780.
- YU, Y. F. 1999. The milestone of the work of protecting wild plant of China - The list of Chinese urgently protected wild plants (the 1st group). *Plants.* 5:3–11.
- ZHANG, K. M., L. SHI, C. D. JIANG and Z. Y. LI. 2007. Influence of root, stem and leaf leachates of *Ageratina adenophora* weed on the gametophyte development of *Pteris finotii*. *Allelopathy J.* 20:203–212.
- ZHANG, K. M., L. SHI, C. D. JIANG and Z. Y. LI. 2008a. Inhibition of *Ageratina adenophora* on spore germination and gametophyte development of *Macrothelypteris torresiana*. *J. Integr. Plant Biol.* 50:559–564.
- ZHANG, K. M., L. SHI, C. D. JIANG and Z. Y. LI. 2008b. Allelopathic effects of *Eupatorium adenophorum* on spore germination and gametophyte development in *Cibotium barometz*. *Acta Pratac. Sinica.* 17:19–25.

Occurrence of Dark Septate Endophytes in the Sporophytes of *Christella dentata*

REBECA GHANTA, SIKHA DUTTA*, and RADHANATH MUKHOPADHYAY

Department of Botany, UGC Centre For Advanced Study, The University of Burdwan,
Burdwan-713104, West Bengal, India

ABSTRACT.—Mycorrhizal fungi form dynamic symbiotic association in the roots of about 80% of the total terrestrial vascular land plants. Among the pteridophytes, mycorrhizal associations are more frequent in the gametophytes than in the sporophytes. Gametophytes of many ferns and lycophytes exhibit obligate mycotrophy. Despite several benefits derived from the mycorrhizal fungi, very little work has been done surveying the distribution and diversity of arbuscular mycorrhizal (AM) fungi in pteridophytes. We intensified our studies in the roots of sporophytes of *Christella dentata* (Forssk.) Brownsey & Jermy (Thelypteridaceae) to understand the distribution of AM fungi in different parts of the main adventitious root and its lateral branches, along with percent root colonization. We also evaluated spore density of AM fungi in the soil and seasonal variations in the hyphal colonization and formation of vesicles and arbuscules. Root colonization was present in the lateral roots only and not on the main adventitious roots. Percent root colonization also varied in different parts of the lateral roots. The results indicated that root colonization percentage of AM mycorrhizal fungi varies not only by the growth stages of host plants but also by season. Soil spore count was highest in the winter and lowest during the rainy season. In addition to AM fungi, another type of root colonizing fungi, dark septate fungi (DSF) or dark septate endophytes (DSE), has also been recorded in the roots of *C. dentata*. Dark septate fungi with melanized hyphae and microsclerotia were documented.

KEY WORDS.—*Christella dentata*, arbuscular mycorrhizae, dark septate fungus, *Glomus* sp

In addition to arbuscular mycorrhizal fungi (AMF), another type of root colonizing fungi, dark septate fungi (DSF), has been reported within the roots of different seedless vascular plants (Cooper, 1976; Berch and Kendrick, 1982; Dhillion, 1993; Jumpponen and Trappe, 1998). Dark septate fungi are defined by Jumpponen (2001) as conidial or sterile fungi that colonize living plant roots without causing any apparent negative effects. The ecology, taxonomic affinities and host range of these DSF are largely unknown, as is their influence on the host and plant communities (Peterson *et al.*, 2004). However, evidence exists showing that DSE fungi can, under some environmental or experimental conditions, enhance host growth and nutrient uptake, hence functioning in a manner typical of mycorrhizal associations (Mandyam and Jumpponen, 2005). If it is accepted that mycorrhizal fungi cause host responses mainly mutualistic with long-term fitness effects, the DSE association should be included when the diversity of mycorrhizal symbioses and responses are considered. Including DSE in mycorrhizal studies would yield valuable information about the importance and frequency of these root colonizers (Jumpponen and Trappe, 1998). Hence, in the present study, an attempt has been made to investigate

* Corresponding author: E mail. Sikha_bu_bot@yahoo.com

the association of DSE and AM in the roots of a common pteridophyte, *Christella dentata* (Forssk.) Brownsey & Jermy to understand the diversity of fungal root endosymbionts in this plant.

MATERIALS AND METHODS

Christella dentata, a common terrestrial fern in the plains of India, grows in broad ecological conditions. Ten young and 10 mature sporophytes were collected from Onda, Bankura district of West Bengal, India at latitude 23°14'N and longitude 87°14'E in each of the three different seasons: winter (November to February), late spring to summer (March to May) and rainy season or monsoon (June to October) for two consecutive years: 2008 and 2009.

Roots were collected by digging and uprooting the whole plant along with the rhizospheric (root region) soil. Root samples were thoroughly washed in running tap water and rootlets were selected. The average root length of the main adventitious root system was measured to be 10.00–11.33 cm. Then, the root system was grouped into two categories: main roots and lateral roots. Each of the root categories was divided into three equal portions: the distal, the middle and the basal portions to determine the variation of mycorrhizal colonization in different regions of the root. Each was cut into small pieces (1 cm in length) and fixed in formaldehyde acetic acid solution (Johansen, 1940) and stored at 4°C.

About 10 g of soil were collected from the rhizosphere of *C. dentata* by digging the soil up to a depth of 15 cm. The soil that adhered to the uprooted roots was separated and added to the soil collection. The total soil collected from around each sporophytic plant of *C. dentata* was kept in separate polythene packets, labeled and stored at 4°C until analysis. Three such soil samples were collected in each season.

Root samples were stained following the method of Phillips and Hayman (1970). For each specimen, 100 root pieces from each of the samples were thoroughly washed in water and boiled at 95°C for 1 hour in 10% KOH. The root segments were washed in distilled water, acidified with 1(N) HCl for 5 minutes and stained in 0.05% trypan blue in acidic glycerol for overnight. The excess colorant was removed by washing with 10% glycerol. Root segments were mounted on slides in acetic acid and glycerol (1:1 V/V) and the edges of the cover slips were sealed with DPX and observed under microscope (Leica, model no. DMLB).

The mycorrhiza type present in the root samples was determined according to the method of Harley and Smith (1983). Arbuscular mycorrhizae were examined in the root samples as percent mycorrhizal association and was calculated as follows:

$$\% \text{Mycorrhizal association} = \frac{\text{No. of mycorrhizae associated segments}}{\text{Total No. of segments scored}} \times 100$$

Ten grams of soil was dissolved in 100 ml of distilled water in a conical flask. The conical flask was shaken for 30 min and then kept in undisturbed condition for 30 min. The soil particles precipitated and the spores floated on the surface of the liquid. Mycorrhizal spores were obtained by wet sieving and decanting technique as followed by Gerdemann and Nicolson (1963).

The soil solution was passed through 350, 300, 53 and 45 μm sieves and the spores were collected from the residue deposited on the sieves. For this, residue present on the sieve was dissolved first in distilled water and then filtered with filter paper. The residue present in the filter paper was taken and mounted on a slide in lactophenol and cotton blue and was examined under a microscope (Leica, model no. DMLB). The decantant was filtered through a filter paper with grid lines. The filter paper was then spread on a glass plate under a dissecting microscope and spores were counted and expressed as spores per 100 g of dry soil.

The prepared root samples (trypan blue stained) were also examined for septate hyphae and microsclerotia corresponding to DSE under a Leica microscope. The mature microsclerotia were allowed to germinate and grow in different media viz. potato dextrose agar medium, Sabouraud medium and malt agar medium. The roots containing mature microsclerotia were surface sterilized and put in potato dextrose agar medium at pH 6.5 and incubated for 30 days for germination of the microsclerotia and to develop the fresh culture of the dark septate endophytic fungus.

RESULTS

The root samples showed the presence of DSE. The septation of the dark septate endophytic fungal hyphae was very prominent and visible even with a very light stain of trypan blue (Fig. 1A). The colonization percentage of DSE was highest in winter to spring and lowest in the rainy season (Table 1). The DSE with microsclerotia (Fig. 1B, C) of different shapes and sizes were highly melanized. The immature microsclerotia (Fig. 1B) took the stain of trypan blue and appeared light blue in color and with maturity the cell wall became thicker and the degree of melanization increased. The microsclerotia were without definite shape, often grew together with the mycelium and remained embedded in the mycelium, bound together with the mycelial strands. Mature microsclerotia (Fig. 1C) did not show well-developed zones of tissue. They were made up of central part made up of pseudoparenchymatous tissue but the hyphal nature exists. Towards the outside of the microsclerotia the hyphae were more loosely arranged.

Upon culturing, branched, dark-colored septate hyphae of the DSE developed, showing small constrictions at branch points. The pure culture of the DSE developed after 30 days of incubation and it was observed that the potato dextrose agar (PDA) medium was most suitable at an optimum pH of 6.5. In culture, microsclerotia developed, the sclerotial initials were found to arise by branching and septation of hyphae. The cells became barrel shaped and considerably wider than the vegetative hyphae, dark brown to reddish – brown in color and was identified as *Rhizoctonia* spp.

Arbuscular mycorrhizae were found in all the lateral root samples studied (Table 1). Both arbuscules (Fig. 2A & B) and vesicles (Fig. 2E) were seen in the roots of *C. dentata*. The number of vesicles was less than the number of arbuscules. The vesicles were large, measuring 38 to 56 μm in diameter, oval to round in shape.

The aseptate hyphae of arbuscular mycorrhizal fungi grew parallel to each other. As the roots were grouped into two categories, main roots and lateral

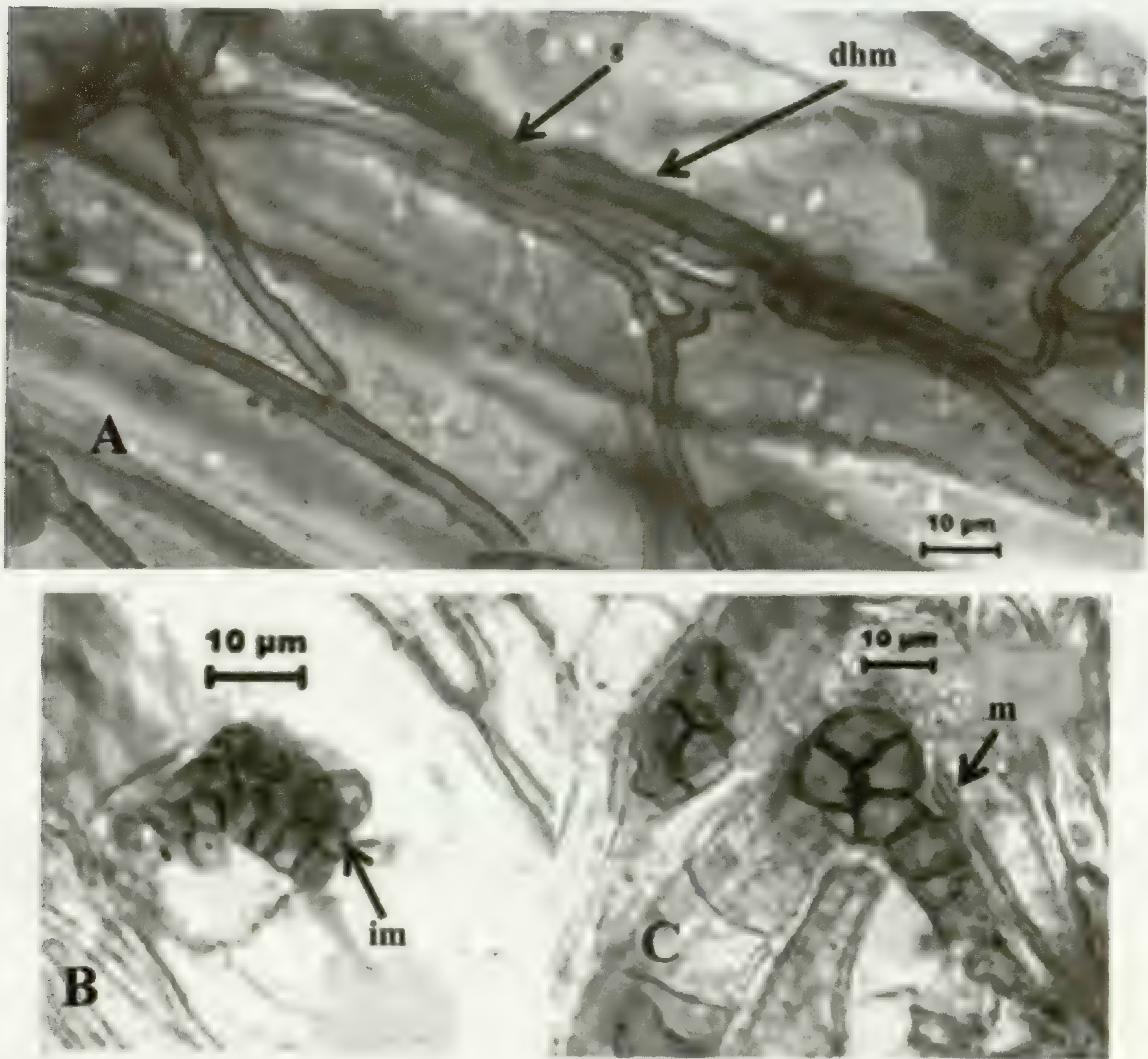


FIG. 1. (A–C) DSE hyphae and microsclerotia within *Christella dentata* A. Septate DSE melanized hyphae B. Immature microsclerotia C. Mature microsclerotia.

roots, they were studied separately and it was evident that the main roots in all the cases showed no mycorrhizal colonization. In contrast, the lateral roots in almost all the cases exhibited mycorrhizal colonization. Percentage of colonization was higher in mature lateral roots (24 to 58.5%) than in young lateral roots (11 to 29%). However, it was noticed that in young plants, the distal portions of the lateral roots had greater mycorrhizal colonization whereas, in mature plants, the basal portions of the lateral roots showed more hyphae and arbuscules. The number of arbuscules and the colonization of aseptate mycorrhizal hyphae was highest in winter and lowest in the rainy season (Table 1). No vesicles were found in young plant roots. Only the mature plant roots possessed vesicles (Fig. 2E) in very low frequency (0.1 to 0.7%).

The root samples of *C. dentata* presented the *Paris*-type of AM colonization (Fig. 2A & B) as the arbuscules, vesicles and intraradical, non-septate hyphae of mycorrhizal fungi were intracellular.

TABLE 1. Colonization of vesicular arbuscular mycorrhizae and dark septate fungi in roots of *Christella dentata*.

Plant growth stage	Season of collection	Portion of lateral root	Mycorrhizal colonization				DSF colonization	
			Percentage of hyphae	Percentage of vesicle	Percentage of arbuscle	Percent root colonization*	Presence or absence of hyphae	Presence or absence of microsclerotia
MATURE PLANT	Winter	Tip	18	0.7	36.3	55 ± 1.11	+	-
		Middle	3	0	40	43 ± 1.32	+	+
	Late spring to summer	Basal	3.5	0	55	58.5 ± 1.10	+	+
		Tip	15	0	30	45 ± 0.89	-	+
	Rainy	Middle	5	0	25.5	30.5 ± 0.90	-	+
		Basal	5	0.1	38.5	43.6 ± 1.22	+	+
		Tip	5	0	20	25 ± 1.50	-	-
		Middle	3	0	21	24 ± 1.45	+	-
		Basal	1.5	0	25	26.5 ± 1.00	+	-
		Tip	19	0	10	29 ± 0.75	+	-
YOUNG PLANT	Winter	Middle	18.5	0	9.1	17.6 ± 0.87	+	-
		Basal	20	0	9	29 ± 0.88	+	-
	Late spring to summer	Tip	20	0	7	27 ± 0.65	-	-
		Middle	21	0	3.5	24.5 ± 0.80	-	-
	Rainy	Basal	18	0	5.5	13.5 ± 1.40	-	-
		Tip	10	0	2	12 ± 1.33	-	-
		Middle	10	0	2.5	12.5 ± 1.22	-	-
		Basal	9	0	2	11 ± 0.95	+	-

*Data are the mean values of ten replicates

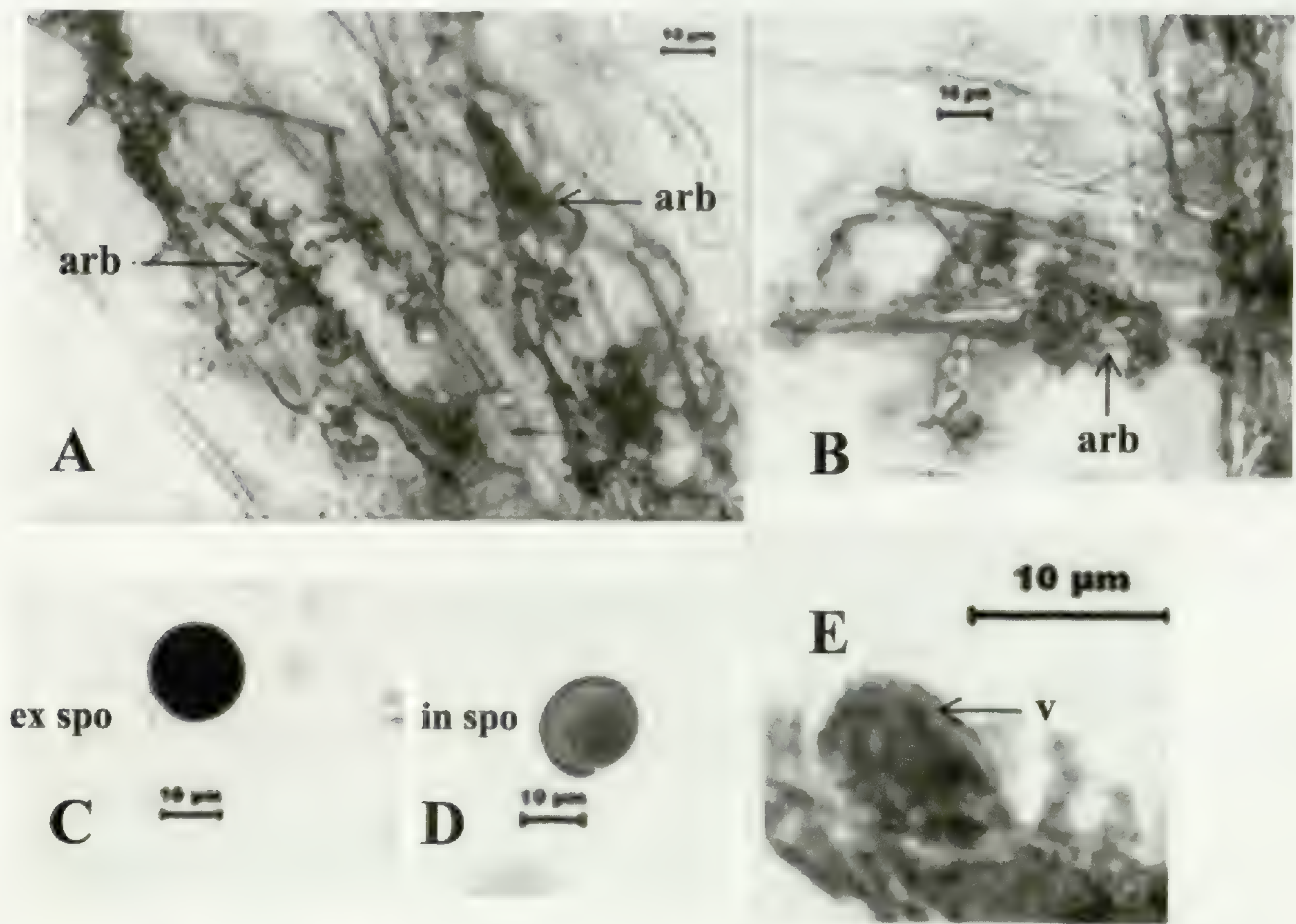


FIG. 2. (A–E) Mycorrhizal arbuscules, vesicle and extra radical spores within roots of *Christella dentata*. A. Arbuscules within *Christella dentata*. B. Arbuscules within *Christella dentata*. C–D. Extra radical spores E. Vesicle within *Christella dentata*.

Spore (Fig. 2C, D) counts per 100 g of soil showed 550 ± 10.44 spores in the winter season, 370 ± 9.89 spores in the late spring to summer, and 210 ± 11.30 spores in the rainy season. The AM fungus has been identified as *Glomus* sp.

DISCUSSION

In this paper, we report occurrence of *Glomus* sp. as arbuscular mycorrhizal fungus in the lateral roots of *Christella dentata* along with dark septate endophytes (DSE), which has been identified as *Rhizoctonia* sp. The arbuscular mycorrhizal fungus *Glomus* was characterized by the formation of large swollen vesicles, well developed arbuscules and extraradical round spores. Though *Glomus* and *Sclerocystis* have been reported earlier by Muthukumar and Udaiyan (2000) as AM in *Christella dentata*, to the best of our knowledge this is the first report of presence of DSE in *Christella dentata* along with microsclerotia.

Mycorrhizal colonization pattern was found to be of *Paris*-type in *Christella dentata* (Fig. 2 A–C). This finding is in conformity with the observations of other studies where this morphotype has been found in other pteridophytes (Zhang *et al.*, 2004; Dickson, 2004; Zubek *et al.*, 2010) and the percent root colonization by AMF varies with the host plants and the habitats have been shown by a number of authors (e.g., Muthukumar and Udaiyan, 2000; Zubek *et al.*, 2010).

Zubek *et al.* (2010) recorded highest AM colonization (M=78%) and arbuscule richness (A = 76%) in *Thelypteris patens* (Sw.) Small and lowest AM colonization and arbuscule abundance in *Sticherus underwoodianus* (Maxon) Nakai (M= 4%, A = 3%). Muthukumar and Udaiyan (2000) showed the intensity of mycorrhizal colonization was significantly influenced by the type of substrate in which the plants grow. Pteridophytes growing in soil (terricolous) had the highest mean colonization levels followed by lithophytic and epiphytic species. As we have studied only one terrestrial member, we are not in a position to compare the result of mycorrhizal colonization in different habitats. However, we have noted that percentage of AM colonization varies not only on the growth stages (young / mature) of host plants but also with the seasonal variations of the year. We found the highest mycorrhizal colonization (58.5 ± 1.10) and spores and arbuscule formations in *Christella dentata* in the winter months and the lowest in the rainy season (Table 1). No vesicles were observed in the rainy season. In summer, there was much less vesicle formation (0.10 ± 0.01) than in winter (0.7 ± 0.1). Mycorrhizal colonization was moderate in the summer months. In young lateral roots mycorrhizal colonization percentage was always less than that of the mature roots (Table 1).

It is assumed that the vesicles, being the storage organs of the AM, are generally produced at comparatively later stages of growth (Powell and Bagyaraj, 1984). Bajwa *et al.* (2001), while surveying AM association in wetland plants, noticed that vesicular infection, in general, started in spring and reached its maximum in summer, autumn or winter depending upon the host species. Another possible reason for the disparity in the pattern of AM development may correspond to variations in stages of completion of life cycles of various AM species involved in forming this association. In a survey on AM association in different vascular plants it was found that colonization remained stable during spring to summer and maximum colonization has been observed during winter (Bajwa *et al.*, 2001). The spore population in association with dicotyledonous species remained consistently high except for a decline in autumn. These results corroborate our present findings on *C. dentata* where AM colonization percentage in the roots and spore number in the soil was highest in winter.

ACKNOWLEDGMENTS

We thank Mr. Kaushik Sarkar for helping in microscopic photography.

LITERATURE CITED

- BAJWA, R., A. YAQOUB and A. JAVAID. 2001. Seasonal variation in VAM in Wetland Plants. *Pakistan Journal of Biological Sciences* 4:464–470.
- BERCH, S. M. and B. KENDRICK. 1982. Vesicular arbuscular mycorrhizae of Southern Ontario ferns and fern allies. *Mycologia* 74:769–776.
- COOPER, K. M. 1976. A field survey of mycorrhizas in New Zealand ferns. *New Zealand J. Bot* 14:169–18.
- DHILLION, S. S. 1993. Vesicular arbuscular mycorrhizae of *Equisetum* species in Norway and USA.: occurrence and mycotrophy. *Mycol. Res.* 97:656–660.

- DICKSON, S. 2004. The Arum-Paris Continuum of mycorrhizal symbioses. *New Phytol.* 163:187–200.
- GERDEMANN, J. W. and T. H. NICOLSON. 1963. Spores of mycorrhizal endogone sp. Extracted from soil by wet-sieving and decanting. *Trans Brit. Mycological Soc.* 46:235–244.
- HARLEY, J. L. and S. E. SMITH. 1983. *Mycorrhizal symbiosis*. Academic Press, New York.
- JOHANSEN, D. A. 1940. *Plant microtechnique*. McGraw Hill: N.Y.
- JUMPPONEN, A. 2001. Dark septate endophytes – are they mycorrhiza? *Mycorrhiza* 11:207–211.
- JUMPPONEN, A. and J. M. TRAPPE. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytol.* 140:295–310.
- MANDYAM, K. and A. JUMPPONEN. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* 53:173–189.
- MUTHUKUMAR, T. and K. UDAIYAN. 2000. Vesicular arbuscular mycorrhizae in pteridophytes of western ghats, southern India. *Phytomorphology* 50:132–142.
- PHILLIPS, M. and D. S. HAYMAN. 1970. Improved procedure for Clearins roots and staining practice and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Brit. Mycological Soc.* 55:158–160.
- POWELL, C. L. I. and D. J. BAGYARAJ. 1984. *In VA mycorrhiza*. CRC Press Inc, Boca Raton, FL.
- PETERSON, R. L., H. B. MASSICOTE and L. H. MELVILLE. 2004. *Mycorrhizas: Anatomy and cell biology*. NRC Research Press, Ottawa.
- ZHANG, Y., L. GUO and R. LIU. 2004. Arbuscular mycorrhizal fungi associated with common pteridophytes in Dujiangyan, southwest China. *Mycorrhiza* 14:25–30.
- ZUBEK, S., K. PIATK, P. NAKS, W. HIESE, M. WAYDA and P. MLECZKO. 2010. Fungal root endophyte colonization of fern and lycophyte species from the Celaque National Park in Honduras. *Amer. Fern J.* 100(2):126–136.

Azolla cristata in the Kashmir Himalaya

BURHAN AHAD*, ZAFAR A. RESHI, and AIJAZ H. GANAIE

Department of Botany, University of Kashmir, Srinagar-190006, Jammu and Kashmir, India

A. R. YOUSUF

Department of Environmental Sciences University of Kashmir, Srinagar-190006,
Jammu and Kashmir, India

ABSTRACT.—The identity of *Azolla* species in the Kashmir Valley has been confusing, with most populations being reported as *Azolla pinnata*. Morphological evidence demonstrates that the species introduced to Kashmir is not *A. pinnata*, but rather *Azolla cristata*, a new species for the Kashmir Valley and a potentially problematic biological invader.

KEY WORDS.—*Azolla caroliniana*, *Azolla cristata*, *Azolla filiculoides*, Kashmir Valley, invasive species

The genus *Azolla* Lam. consists of small, free-floating aquatic ferns, which are invasive in many parts of the world (Garcia-Murillo, 2007), including the Kashmir Valley, where *Azolla* is one of the most prolific invasive alien taxa of aquatic ecosystems (Fig. 1A). The species of *Azolla* that grows in Kashmir has been previously reported as *Azolla pinnata* R. Br. (Mir and Pandit, 2008), referring to the native Indian taxon *A. pinnata* subsp. *asiatica* R.M.K. Saunders & K. Fowler. A detailed study of vegetative and reproductive characters of *Azolla* specimens collected from several water bodies in Kashmir Valley revealed that this species has been incorrectly identified and is, instead, *A. cristata* Kaulf.

MATERIALS AND METHODS

Fresh specimens with sporocarps were collected from five different water bodies of the Kashmir Valley, and deposited in Kashmir University herbarium, KASH (Table 1). Additional material from this herbarium was examined under a stereomicroscope (Zeiss, Discovery V8) and optical trinocular microscope (Leica DMLS2). The branching pattern and leaf trichomes were observed by immersing fresh plants in 95% ethanol for 1 hour. The leaves were then rinsed with water and immersed in concentrated bleaching solution under vacuum for 15 minutes, again rinsed with water, then mounted in lactophenol and observed under the microscope. The structure of glochidia was observed by immersing dry microsporangia in water:ethanol:glycerol (1:1:1) solution for 24 hours. The massulae were then dissociated by gently squashing them under a cover glass. Samples for Scanning Electron Microscopy (SEM) were fixed with 3% glutaraldehyde in 0.2 M phosphate buffer of pH 7.4. The samples

* Corresponding author: burhanahad@gmail.com, burhanahad@rediffmail.com

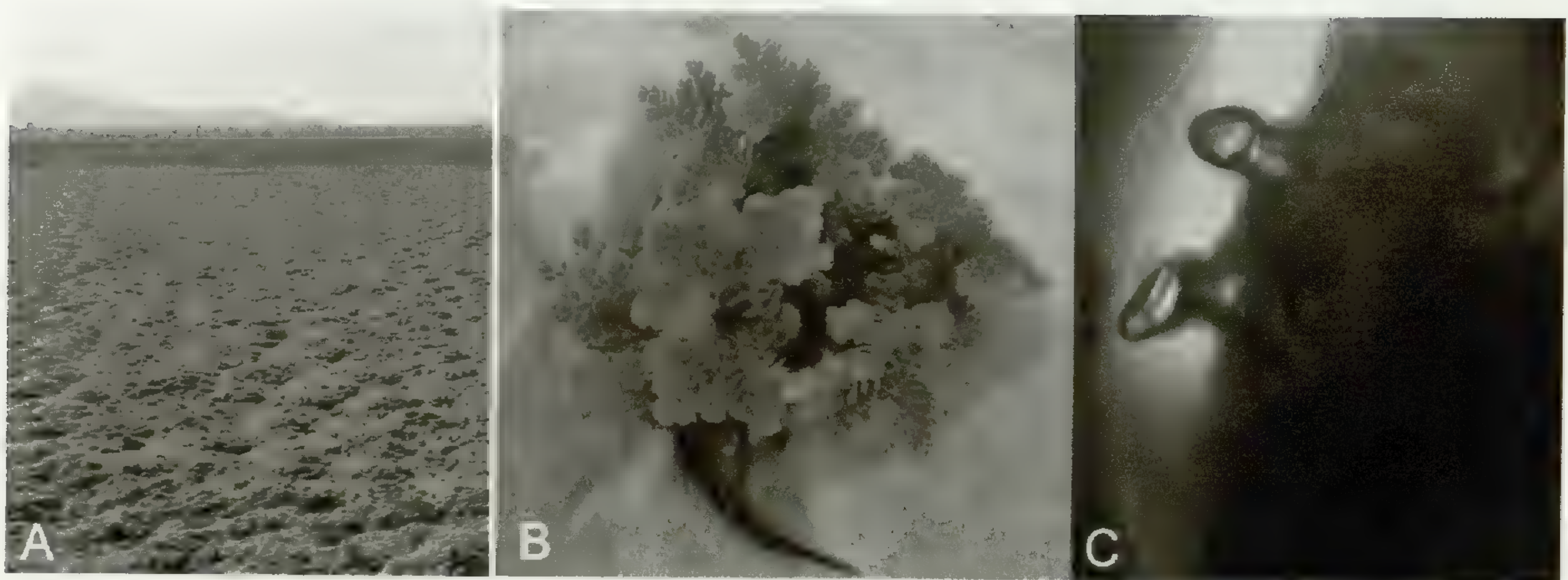


FIG. 1. A. A population of *Azolla* forming a mat in Dal Lake. B) Sporophyte of *Azolla cristata*. Reshi & Ahad 2101 (KASH). C) Bicellular leaf-trichome. Reshi & Ahad 2101 (KASH).

were then fixed in 1% osmium tetroxide, and graded in acetone series. Fixed samples were dried by critical point method in dry carbon dioxide and examined under the SEM (Hitachi S3000-H).

RESULTS AND DISCUSSION

The plants are free-floating, up to 4 cm long, and polygonal in shape. Sporophytes consist of a thin axial stem, bearing small leaves and delicate brown rootlets up to 6 cm long. Leaves are alternate, imbricate, and bilobed; the hyaline ventral leaf lobe and an aerial chlorophyllous dorsal lobe are covered with short bicellular trichomes and bear an extracellular cavity housing filamentous *Anabaena* (Figs. 1B and 1C). Rootlets are solitary, from stem branching points. Sporocarps are borne in pairs at the base of branches. Microspores are aggregated in stalked massulae, massulae with arrow shaped glochidia (Figs. 2A and 2B). Megasporocarps contain a solitary megaspore; megaspore apparatus with three floats, and granular perine (Fig. 2C).

To understand the identification of these plants that were earlier misidentified as *A. pinnata* in the Kashmir valley (a species of section *Rhizosperma*, characterised by hookless glochidia and megaspore apparatus with nine floats) a short background in *Azolla* taxonomy is necessary. One of the earliest classifications of *Azolla* section *Azolla* by Mettenius (1847) recognized four

TABLE 1. Collection information. Elevations are in meters above sea level. Vouchers are deposited at KASH.

Site	Elevation	Coordinates	Voucher
Anchar Lake	1584	34.23°N, 74.90°E	Reshi & Ahad 2103
Dal Lake	1584	34.60°N, 74.80°E	Ahad & Ganaie 2102
Hokersar wetland	1500	34.05°N, 74.43°E	Reshi & Ahad 2105
Manasbal Lake	1584	34.15°N, 74.41°E	Ahad & Ganaie 2104
Wular Lake	1580	34.20°N, 74.44°E	Reshi & Ahad 2101

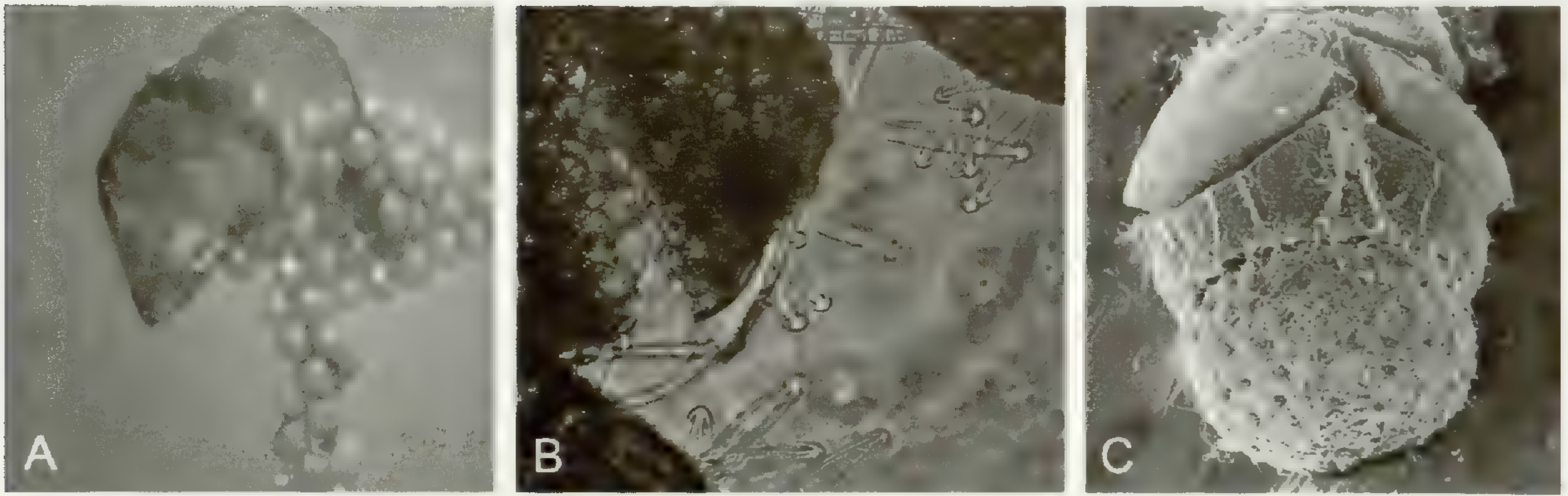


FIG. 2. A. Massulae. *Reshi & Ahad 2101* (KASH). B. Arrow shaped glochidia. *Reshi & Ahad 2101* (KASH). C. SEM of megasporocarp showing perine architecture. *Reshi & Ahad 2101* (KASH).

species: *A. cristata* Kaulf, *A. caroliniana* non Willd., *Azolla magellanica* Willd. (syn. *Azolla filiculoides* Lam.) and *A. microphylla* non Kaulf. Mettenius (1867) reduced the New World species number to two by combining *A. microphylla*, *A. caroliniana* and *A. cristata* into *A. caroliniana* and at the same time placing *A. magellanica* and *A. rubra* into *A. filiculoides* Lam. Strastburger (1873) upheld the later Mettenius taxonomy and recognised only two species of section *Azolla*: *A. caroliniana* and *A. filiculoides*, which he distinguished on the basis of bicellular trichomes and granular perine present in the former species and unicellular trichomes and warty perine present in the latter. The most widely accepted classification of section *Azolla* is currently that of Svenson (1944), however, who returned to a four-species system: *Azolla caroliniana* Willd., *A. filiculoides* Lam., *A. microphylla* Kaulf. and *A. mexicana* Presl. Recent reassessments of species limits in *Azolla* have found that taxonomic treatment to be unsatisfactory (Dunham and Fowler, 1987; Zimmerman *et al.*, 1989, 1991; Pereira *et al.*, 2001; Evrard and van Hove, 2004). These authors have shown that earlier circumscriptions of these species were based on misapplied names, including the fact that the types of *A. caroliniana* and *A. microphylla* are indistinguishable from the oldest name in the group, *A. filiculoides*. The taxon frequently treated as *A. caroliniana* sensu Mettenius, then, needs a name, and the oldest available is *A. cristata*. Compounding this confusion is the natural variation present in these species, and noticeable environmental plasticity among the populations (Zimmerman *et al.*, 1989; Pereira *et al.*, 2001). Zimmerman *et al.* (1989) demonstrated that in section *Azolla*, *A. filiculoides* was easily distinguishable from the other taxa on the basis of leaf trichome morphology and enzyme electrophoresis pattern. Zimmerman *et al.* (1991) suggested that the taxa frequently treated as *A. microphylla*, *A. mexicana* and *A. caroliniana* be regarded as a single species, on the basis of RFLPs, isozymes, phosphorus deficiency symptoms and breeding experiment analysis. These results thus agree perfectly with the existence of two species in section *Azolla*, as advocated by Evrard and Van Hove (2004). One is *A. cristata* Kaulf., which includes *A. caroliniana* non Willd., *A. mexicana* Presl and *A. microphylla* non Kaulf.; the other species is *A. filiculoides* Lam. (including *A. rubra* R. Br., often recognized at the rank of variety).

Following this taxonomy, all plants examined were *Azolla cristata*, on the basis of the presence of bicelled trichomes, hook shaped, multiseptate glochidia, and a 3-float megaspore apparatus with a granular perine surface (Figs. 1C, 2B and 2C). It is possible that *A. filiculoides* is also present in the Kashmir Valley (as an introduction), although we know of no documented occurrences; populations of *Azolla* section *Azolla* should be carefully examined to determine which species is involved.

ACKNOWLEDGMENTS

The authors highly acknowledge the help of Prof. Charles van Hove, Emeritus Professor, Catholic University of Louvain, and Prof. I. Watanabe who confirmed our identifications. The authors also take the opportunity to thank Carl Rothfels (Associate Editor), Christopher Fraser-Jenkins, and an anonymous reviewer for their helpful comments.

LITERATURE CITED

- DUNHAM, D. G. and K. FOWLER. 1987. Taxonomy and species recognition in *Azolla* Lam. Pp. 7–16 in: IIRI (Eds), *Azolla* Utilization. International Rice Research Institute, Los Banos, Laguna, Philippines.
- EVARD, C. and C. VAN HOVE. 2004. Taxonomy of the American *Azolla* species (Azollaceae): a critical review. *Systematics and Geography of Plants* 74:301–318.
- GARCIA-MURILLO, P., R. FERNANDEZ-ZAMUDIO, S. CIRUJANO, A. SOUSA and J. M. ESPINAR. 2007. The invasion of Donana National Park (SW Spain) by the mosquito fern (*Azolla filiculoides* Lam). *Limnetica* 26:243–250.
- METTENIUS, G. 1847. Ueber *Azolla*. *Linnaea* 20:259–282.
- METTENIUS, G. 1867. Filicinae. Pp. 51–54, in *Plantae Tinneanae*. Vienna.
- MIR, S. S. and A. K. PANDIT. 2008. Macrophytic features of Wular Lake (a Ramsar Site) in Kashmir. *J. Res. Dev.* 8:1–11.
- PEREIRA, A. L., G. TEXEIRA, I. SEVNATE-PINTO, T. ANTUNES and F. CARRAPICO. 2001. Taxonomic re-evaluation of the *Azolla* genus in Portugal. *Plant Biosystems* 135:285–294.
- STRASBURGER, E. 1873. Ueber *Azolla*. Jena, Hermann Dabis.
- SVENSON, H. K. 1944. The New World species of *Azolla*. *Amer. Fern J.* 34:69–84.
- ZIMMERMAN, W. J., T. A. LUMPKIN and I. WATANABE. 1989. Isozyme differentiation of *Azolla* Lam. *Euphytica* 42:163–170.
- ZIMMERMAN, W. J., I. WATANABE, T. VENTURA, P. PAYAWAL and T. A. LUMPKIN. 1991. Aspects of the genetic and botanical status of Neotropical *Azolla* species. *New Phytol.* 119:561–566.

SHORTER NOTES

New Country and Regional Records from the Brazilian Side of Neblina Massif.—A list of 37 species is reported here for the first time to the Brazilian side of Neblina Massif: one of the largest sandstone mountains of the western portion of the Guyana Shield along the Venezuelan-Brazilian border. Twenty-seven species represent new additions to the Brazilian Fern Flora (marked with an asterisk) and ten species are the first record to the northwestern portion of the Brazilian Amazon with their closest known Brazilian populations from the Atlantic Rainforests (marked with two asterisks). We provide information on previous geographic ranges and full specimen citation including habit, habitat, and elevation for all taxa.

The specimens were collected by the first author during an expedition to the Cachoeira do Anta (Anta waterfall), Amazonas, Brazil, at the southeastern part of Neblina massif from 24 December 24, 2003 to January 5, 2004. The expedition began at the foot of the Serra da Neblina, from the mouth of the Tucano River ($0^{\circ}37'14''\text{N}$, $65^{\circ}55'29.5''\text{W}$, 50 m elevation) to the top of the Anta waterfall ($0^{\circ}51'20.3''\text{N}$, $65^{\circ}56'30.1''\text{W}$, 2400 m elevation).

The botanical novelties presented here illustrate how the Brazilian side of the Guyana shield is relatively understudied compared to its neighboring areas in Venezuela. All specimens were fixed in 70% ethanol in the field and dried about a month after in an oven at 60°C . A complete set of vouchers is housed at the Herbarium of the Institute for Amazonian Research (INPA) in Manaus, Brazil, with duplicates (when available) sent to the Herbarium of the Federal University of Minas Gerais (BHCB).

*****Asplenium harpeodes* Kunze—Carvalho et al., 351.** Epiphyte. Cloud forests, 2300 m. Distributed from Mexico to Brazil where it was previously known only from the Atlantic Rain Forest in Brazil.

*****Asplenium raddianum* Gaudich.—Carvalho et al., 341.** Epiphyte. Upper montane forest, 1900 m. Previously known from Colombia, Venezuela, Ecuador, Peru, Bolivia and Brazilian Atlantic Forest.

*****Blechnum schomburgkii* (Klotzsch) C. Chr.—Carvalho et al., 380.** Terrestrial. Tepui flat summit near rocks, 2300 m. Distributed from Costa Rica to Bolivia and southeastern Brazil.

*****Ceradenia capillaris* (Desv.) L. E. Bishop—Carvalho et al., 384.** Rocks crevices along Rio Anta. Rocky outcrop, 2400 m. Previously known from Cuba, Jamaica, Hispaniola, Colombia, Venezuela, Ecuador, Peru, Bolivia, and the Atlantic forest in southeastern Brazil.

- **Cochlidium connellii* (Baker ex C. H. Wright) A. C. Sm.—*Carvalho et al.*, 383. Rocky crevices along Rio Anta, growing with mosses. Rocky outcrop, 2300 m. Previously known only from Guyana and Venezuela.
- **Cyathea vel* aff. *aurea* Klotzsch—*Carvalho et al.*, 344. Terrestrial. Fruticose meadows, 2300 m. The range of this taxon is from the Cordillera de la Costa in Venezuela along the northern Andes in Colombia, probably reaching northern Ecuador.
- **Cyathea gracilis* Griseb.—*Carvalho et al.*, 347. Terrestrial. Fruticose meadows, 2200 m. Previous records are from Costa Rica, Panama, Jamaica, Colombia, Ecuador, and northern Peru.
- **Cyathea marginalis* (Klotzsch) Domin—*Carvalho et al.*, 385. On rocks near stream. Rocky outcrop, 2300 m. It is widespread in the Guayana shield from French Guiana to Venezuela.
- **Cyathea neblinae* A.R. Sm.—*Carvalho* 308, 320. Terrestrial. Montane forests, 1300 m. Endemic from Neblina massif. Previous known from Venezuela.
- **Cyathea sipapoensis* (R. M. Tryon) Lellinger—*Carvalho et al.*, 335. Terrestrial. Upper montane forest, 1700 m. Previously known only from Cerro Sipapo (Venezuela), 550 km from Serra da Neblina.
- **Cyathea spectabilis* (Kunze) Domin—*Carvalho et al.*, 249. Terrestrial. Montane forests, 1100 m. Previous records are from Panama, Colombia, Venezuela, Guyana, Suriname, and French Guiana. The closest record of this species is from Serranía de Imataca, Venezuela, about 850 km from Sierra de la Neblina.
- **Cyathea steyermarkii* R. M. Tryon—*Carvalho et al.*, 237. Terrestrial. Montane forest, 1000 m. Previously known only from Cerro Autana (Venezuela), 490 km from Serra da Neblina.
- **Diplazium centripetale* (Baker) Maxon—*Carvalho et al.*, 304. On rocks along stream. Montane forest, 1300 m. Previously known from the Antilles, Venezuela, and Trinidad.
- **Elaphoglossum crispatum* Mickel var. *beitelii* Mickel—*Carvalho et al.*, 293. Epiphyte. Montane forests, 1300 m. Endemic to Serra da Neblina. Previous records from Venezuela.
- **Elaphoglossum wurdackii* Vareschi—*Carvalho et al.*, 337. Epiphyte in fallen branch. Upper montane forests, 1700 m. Previously known only from Venezuela.

- **Enterosora campbellii* Baker subsp. *campbellii*—*Carvalho et al.*, 366. Epiphyte. Cloud forest, 2000 m. Previously known from Jamaica and Venezuela.
- **Enterosora trichosora* (Hook.) L. E. Bishop—*Carvalho et al.*, 354. Epiphyte. Cloud forest, 2300 m. *Enterosora trichosora* is a rarely collected but widespread species with about ten known collections, including three from the Venezuelan side of Serra da Neblina (Alan Smith, pers. comm.). Previously known from Mexico, Guatemala, Costa Rica, Ecuador, and Peru. The nearest published record prior this one is from the Andes in Quito, Ecuador, 1400 km far from Serra da Neblina.
- **Enterosora parietina* (Klotzsch) L. E. Bishop—*Carvalho et al.*, 368. Epiphyte. Cloud forests, 1900 m. Previously known from Central America, Antilles, Colombia, Ecuador, Peru, and Venezuela.
- **Grammitis peritimundi* L. E. Bishop & A. R. Sm.—*Carvalho et al.*, 339. Epiphyte. Upper montane forests, 1800 m. Previously known only from Venezuela.
- **Hymenophyllum karstenianum* J. W. Sturm—*Carvalho et al.*, 338. Epiphyte on fallen branch. Upper montane forests, 1800 m. Previously known only from Venezuela and Peru.
- **Hymenophyllum lindenii* Hook.—*Carvalho et al.*, 355, 361, 394. Epiphyte. Upper montane forests, 1900 m, and Cloud forests, 2300 m. Previously known only from Colombia, Venezuela, and Peru.
- ***Lindsaea arcuata* Kunze—*Carvalho et al.*, 329. Terrestrial. Montane forest, 1500 m. Previously known from Venezuela, Colombia, Guyana, Suriname, French Guiana, and from the Atlantic forests in southern and southeastern Brazil.
- **Lindsaea cultriformis* K. U. Kramer—*Carvalho et al.*, 291. Terrestrial. Montane forest, 1300 m. Previously known only from Colombia and Venezuela. The nearest record of this species is from Cerro Aratitiope about 200 km from Serra da Neblina.
- **Lindsaea stricta* (Sw.) Dryand. var. *jamesoniformis* K. U. Kramer—*Carvalho et al.*, 386. Rock crevices along Rio Anta. Rocky outcrops, 2400 m. Previously known from Porto Rico, Colombia, Venezuela, Guyana, and Suriname.
- **Lycopodiella steyermarkii* B. Øllg.—*Carvalho et al.*, 348. Terrestrial. Fruticose meadows, 2300 m. Previously known from Costa Rica, Panama, Colombia, Venezuela, and Ecuador.
- ***Polyphlebium diaphanum* (Kunth) Ebihara & Dubuisson—*Carvalho et al.*, 396. Epiphyte. Cloud forests, near streams, 2300 m. *Polyphlebium diaphanum*

occurs from Mexico to Brazil. Previous records in Brazil are from the Atlantic forest region.

- **Polypodium aturense* Maury—*Carvalho et al.*, 319. Arching epiphyte. Montane forest, 1300 m. Previously known only from semideciduous forests in Venezuela, 100–400 m.

- **Pterozonium paraphysatum* (A. C. Sm) Lellinger—*Carvalho et al.*, 390. Rock crevices along Rio Anta, erect rhizome. Rock outcrop, 2400 m. Previously known from Venezuela, Guyana, and Suriname.

- **Serpocaulon sessilifolium* (Desv.) A. R. Sm.—*Carvalho et al.*, 377. Epiphyte. Semideciduous forest, 2400 m. Previously known from Costa Rica, Colombia, Venezuela, Ecuador, Peru, and Bolivia. The nearest record is from Cerro Marahuaka, Venezuela, about 300 km from Serra da Neblina.

- **Stenogrammitis jamesonii* (Hook.) Labiak—*Carvalho et al.*, 343, 357. Epiphyte. Upper montane forest, 2000 m; and Cloud forests, 2300 m. Previously known from Guatemala, Costa Rica, Venezuela, Colombia, Ecuador, Peru, and Bolivia.

- **Stigmatopteris longicaudata* (Liebm.) C. Chr.—*Carvalho et al.*, 324. Terrestrial. Montane forest, 1300 m. Previously known from Mexico to Bolivia including Venezuela and French Guiana.

- ***Terpsichore asplenifolia* (L.) A. R. Sm.—*Carvalho et al.*, 363. Epiphyte. Cloud forest, 1900 m. Previously known from Mexico, Antilles, Mesoamerica, Colombia, Venezuela, Ecuador, Peru, Bolivia, and Atlantic forest in northeastern Brazil.

- ***Terpsichore taxifolia* (L.) A. R. Sm.—*Carvalho et al.*, 244. Epiphyte on moss-covered trunk. Montane forest, 1100 m. Previously known from Costa Rica, Panama, Antilles, Colombia, Venezuela, Guyana, Suriname, Ecuador, Peru, Bolivia, and Atlantic forest in Brazil. The nearest record for this species is from Cerro Duida, Venezuela, about 300 km from Serra da Neblina.

- **Trichomanes caliginum* Lellinger—*Carvalho et al.*, 278. On rocks along stream. Montane forests, 1300 m. Previously known from Venezuela, Guyana, and Suriname. The nearest record of this species is from Cerro Marahuaca, about 325 km from Serra da Neblina.

- **Trichomanes dactylis* Sodiro—*Carvalho et al.*, 346. Terrestrial. Cloud forests, 1900 m. Previously known from Panama, Colombia, Venezuela, and Ecuador.

- ***Trichomanes robustum* E. Fourn.—*Carvalho et al.*, 333. Epiphyte on moss-covered trunk. Upper montane forest, 1700 m. Previously known from Antilles,

Venezuela, Colombia, Ecuador, and Brazil. Prior Brazilian records are from the Atlantic Forest, northeastern Brazil.

*****Thelypteris gardneriana*** (Baker) C. F. Reed—*Carvalho et al.*, 358, 395. Terrestrial near stream. Cloud forest, 2300 m. Previously known from Venezuela, Colombia, Ecuador, Peru, Bolivia, and southern and southeastern Brazil.

We thank the Yanomamis from Maturacá who allowed the first author to collect in their area. We thank the following researchers who have helped with the identifications and providing information on rare species: Dr. Alan Smith (*Enterosora*) and Dr. Marcus Lehnert (*Cyathea*). Financial and logistic support was provided by the National Park of Pico da Neblina, ICMBio/MMA/Brazilian Government. The third author received financial support from PROTAX (MCT/CNPq/MEC/CAPES N° 52/2010) during the production of this manuscript.—FERNANDA ANTUNES CARVALHO, Instituto Nacional de Pesquisas da Amazônia, Coordenação de Botânica, Av. André Araújo 2936, Manaus, Amazonas, 69060-001, Brazil; ALEXANDRE SALINO, Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Botânica, Caixa Postal 486, Belo Horizonte, Minas Gerais, 30123-970, Brazil; and CHARLES EUGENE ZARTMAN, Instituto Nacional de Pesquisas da Amazônia, Coordenação de Botânica, Av. André Araújo 2936, Manaus, Amazonas, 69060-001, Brazil.

SHORTER NOTES

New Records Of Ferns From Chiapas, Mexico.—As part of an Inventory of Pteridophytes of the Biosphere Reserves of “El Triunfo” and “La Sepultura” in the Sierra Madre and of the deciduous tropical forest in the Jiquipilas Valley in the Central Depression of Chiapas, Mexico, as well as explorations in other areas of this state between 1998 and 2003, five fern species previously were reported for Chiapas: *Hemionitis levyi* and *Doryopteris concolor* var. *concolor* (Amer. Fern. J. 90:104–111. 2000); *Thelypteris rachiflexuosa* (Sida 20:1309–1313. 2003), *Elaphoglossum ipshookense* and *Anemia guatemalensis* (Amer. Fern J. 93:152–163. 2003). These were determined as new records for the state by comparison to those taxa reported from Chiapas by Mickel and Smith (Mem. New York Bot. Gard. 88:1–1054. 2004). Subsequent explorations of other physiographic regions of Chiapas have resulted in the discovery of the following five additional new records for Chiapas.

These new records are confined to physiographic regions of Chiapas such as the Central Depression, Northern Highlands, and Sierra Madre of Chiapas. The Central Depression is important as a biological corridor to all dry forest in southern Mexico and to the Yucatan Peninsula, according to Rzedowski and Calderon de Rzedowski (Florist. Invent. Trop. Country. New York Bot. Garden: 273.1989), and the Northern Highlands region is important because it is considered part of the Mesoamerican biological corridor (CI & CAP. Northern Region Mesoamer. Biodiver. Hotspot. 2004)

The Central Depression is a great valley located between the Sierra Madre and the Northern Highlands of Chiapas (Müllerried, La Geografia de Chiapas. 1957). This valley is dominated by deciduous tropical forest, and few botanical studies have been conducted in this area (La Veg. Chis. Vol. 1. 7. 1952). It has been considered an important zone for endemic taxa (Reyes, List. Florist. Mex. XVII. Dep. Central Chis. 1997). The Northern Highlands is a mountainous region located between the Central Depression and Gulf Coastal Plain of Chiapas, and is dominated mainly by tropical rain forest and *Quercus* forest. This area is poorly known botanically. The Sierra Madre is located between the Pacific Coastal Plain and the Central Depression (La Geog. Chis. 1957), and is dominated by montane cloud forest. Several floristic studies have shown this area to be rich in vascular plant diversity (Long and Heath, Ann. Inst. Biol. Ser. Bot. 6. 1990; Matuda, Amer. Mid. Nat. 43:195–223. 1950). Unfortunately, all of these areas are becoming heavily deforested and altered due mainly to anthropogenic activities such as cattle ranching, and coffee and maize plantations.

New Records

Adiantopsis seemannii (Hook.) Maxon—Mexico, Chiapas, Mpio. Tonalá, 8 Km N of Ejido Raymundo Flores in the Biosphere Reserve La Sepultura,

16°02'20" N, 93°35'30" W, 900 m, deciduous tropical forest, 5 Sep 1995, *Pérez-Farrera* 741 (HEM). This differs from *Adiantopsis radiata* (L.) Fée, a very common species in deciduous tropical forest, in its ovate, pinnate blades. *Adiantopsis seemannii* is a very rare species at this locality, and grows with *A. radiata* on rock in open-canopy forest. *Adiantopsis seemannii* ranges along the Pacific coast of Mexico between 100–1400 m (Mickel and Smith, 2004). It also has been reported in Guatemala and Belize.

Doryopteris palmata (Willd.) J. Sm.—Mexico, Chiapas, Mpio. Villaflores, between El Ejido Tres Picos and Nueva Independencia, in the Biosphere Reserve La Sepultura, 16°10' N, 93°35' W, 1700 m, montane forest, 8 Aug 1995; Mpio. Villaflores, between El Ejido Tres Picos and Nueva Independencia, in Biosphere Reserve La Sepultura, (16°10' N, 93°35' W), 1700 m montane forest, 10 Aug 1995, *Pérez-Farrera* 672 (CHIP, UAMIZ); *Pérez-Farrera* 2983 (HEM); Mpio. La Concordia, Finca Cuxtepex, 50 Km S of Nueva Independencia, in Biosphere Reserve El Triunfo, 15°43'49" N, 92°57'57" W, 1000 m montane forest, 14 Jun 2006, *Mendez-Guzman* 66 (HEM). This species differs from *D. concolor* (Langsd. et Fisch.) Kuhn var. *concolor* in its areolate venation and finely puberulous stipe grooves. Smith (Fl. Chiapas 2:1–370. 1981), Moran (Flora Mesoamericana 1:129, 1995), and Mickel and Smith (2004) cited *Rovirosa* 1084 (PH, NY and K) as a voucher for Chiapas. This specimen was cited by *Rovirosa* (Pterid. Sur Mex. 142. 1909), but has not been located by anyone since *Rovirosa*, thus the record remains unverified. *Rovirosa* collected in the Northern Highlands of Chiapas in a locality named “El Rosario”, between Bochil and Ixtapa. The present record is the first report of *D. palmata* from the Sierra Madre region and confirms the unseen *Rovirosa* report for Chiapas. This species is very rare and grows on rocky slopes in very wet montane, open-canopy forest.

Selaginella convoluta (Arn.) Spring—Mexico, Chiapas, Mpio. Jiquipilas, El Campanario, 2.5 Km N of Ejido Andrés Quintana Roo, (16° 37' 30" N, 93°34'28" W), 650 m, deciduous forest, 16 Jun 2004, *Gómez-Domínguez* 780 (HEM); El Campanario, 2.5 Km N of Ejido Andrés Quintana Roo, 650 m altitude (16° 37' 30" N, 93°34'28" W), deciduous forest, 16 Jun 2004, *Pérez-Farrera* (CHIP); El Campanario, 2.5 Km N of Ejido San Andrés Quintana Roo, 16° 37' 30" N, 93°34'28" W, 650 m, deciduous tropical forest, 16 Jun 2004, *Farrera-Sarmiento* 440 (CHIP). This species is closely related to *S. lepidophylla* (Hook. et Grev.) Spring, but differs by its peltate lateral leaf bases with a single auricle. This record for Chiapas extends the distribution farther south and west in Mexico. Previously, according to Mickel and Smith (2004) the species was known in Mexico only from Yucatan, but it is widespread in the Neotropics (Guatemala, Honduras, Cuba, Hispaniola, Brazil, Bolivia, Paraguay, and Argentina). *Selaginella convoluta* grows on rocks in open deciduous forests, and is very abundant alongside species of *Selaginella*, *Cheilanthes*, *Lygodium*, and *Hemionitis*.

Asplenium dissectum Sw.—México, Chiapas, Mpio. Cintalapa, in forest, to 3 km NW of Ejido Rafael Cal y Mayor, La Ventana, 50 km to NW of Cintalapa, (16°58.370' N, 94°01.589' W), 942 m, montane tropical forest, 20 Aug 2005, *Martinez-Meléndez N. 1234* (HEM). This species is a member of sect. *Sphenopteris* and is closely related to *A. serra* Langsd. & Fisch., but differs by its thin blade texture and bilacerate pinnae. This record for Chiapas extends the distribution farther southeast in Mexico. Previously, the species was known in Mexico only from Oaxaca, but it is widespread in the Neotropics (Guatemala, Nicaragua, Costa Rica, Panama, Cuba, Jamaica, Hispaniola, Colombia, Venezuela, Ecuador, and Brazil) (Mickel and Smith, 2004). *Asplenium dissectum* grows terrestrially in dense montane tropical forests.

Anemia tomentosa (Savigny) Sw. **var. mexicana** (C. Presl) Mickel—México, Chiapas, Mpio. Cintalapa, to 3 km al Rafael Cal y Mayor 50 km to NW of Cintalapa, (16°55.789' N, 93°59.649' W), 727 m, 20 de aug de 2005, *Martinez-Meléndez N. 1232* (HEM); growing on rocks in *Quercus* forest. This variety is widely distributed in almost all states of Mexico. However it has not been recorded previously from Chiapas or the Yucatan Peninsula. This taxon is distributed in Hispaniola; Colombia, and Venezuela (Mickel and Smith, 2004). In Mexico, its closest relative appears to be *Anemia karwinskyana* (C. Presl) Prantl, from which it is distinguished by its thinner leaf texture and bipinnate-pinnatifid blades. Two other varieties of *A. tomentosa* are distributed in South America (Mickel, Iowa St. J. Sci. 36: 349–482. 1962).

We thank Dr. Christopher Davidson and Sharon Christoph for their financial support for the Floristic inventory of the Triunfo Biosphere Reserve, Chiapas. We also thank Jorge Martinez-Meléndez, Nicolas Méndez-Guzman, and Jeremías López-Chagala for their help in the field and processing of plants. Special thanks to Dr. Alan R. Smith for the verification of specimens and comments on the manuscript.—MIGUEL A. PÉREZ-FARRERA, MA. EVANGELINA LÓPEZ-MOLINA, NAYELY MARTÍNEZ-MELÉNDEZ, and HÉCTOR GÓMEZ-DOMÍNGUEZ, Herbario Eizi Matuda, Facultad de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Libramiento Norte Poniente 1150, Tuxtla Gutiérrez, Chiapas, México, 29039.

Obituary: The Rev. Father Dr. V. S. Manickam S. J. (1944–2012)



Dr. A. BENNIAMIN

Botanical Survey of India, Itanagar

CHRIS FRASER-JENKINS

Kathmandu and Royal Botanic Garden, Edinburgh

With the passing away of Rev. Father Dr. Visuvasam Sousai Manickam, Pteridology in India has lost one of its great stalwarts. Father Manickam died on the 30th March 2012 at St. Mary's Higher Secondary School, Madurai, bringing to an end a life of 68 years spent in spiritual quest and in the pursuit of Pteridology.

Born on 1st June 1944, in the small village of Kamalapuram, Dindigul District of Tamil Nadu, as one of several children, the second son of Visuvasam Paripooranam. After his local school studies he joined the Society of Jesus at Beschi College, Dindigul, in 1961 and studied religion for two years. He then went to Chennai to pursue his college studies and was ordained as a priest at Loyola College, Madras (as Chennai then was) in 1964–1967. He chose an undergraduate course in Botany at Loyola College, which he passed with a high mark and then went on to do a postgraduate course in Botany at St. Joseph's College, Tiruchirappalli (Trichy), earning a first class degree through his dedication and attention to detail.

Following this and his awakening interest in Botanical studies, he was awarded a Ph.D. in Botany in 1975 at the University of Kerala, Thiruvananthapuram (Trivandrum), which he joined in 1971. He studied there under the guidance of Professor C. A. Ninan, in the school of the late Professor A. Abraham, to learn fern cytology. It was under Prof. Ninan's stimulating supervision that he developed his ground-breaking research studying the cytology of South Indian ferns and carried out extensive detailed fieldwork on the Pteridophytes of the Western Ghats from 1969 onwards. He was able to explore the entire Palni Hill range, surveying and collecting some 2500

pteridophytes, and simultaneously pursued his profound religious development and studies. These pteridophyte collections were the basis for the taxonomic part of his Ph.D. work, which he completed at Kerala University from 1971–74. He also studied in detail the ecology of ferns of the Palni Hills, under the guidance of the French Institute, Pondicherry. This included correlating the distribution of ferns in the various phytogeographic zones of the Palni Hills to the micro- and macro-climate. During this work he investigated the cytology of about 35 species of ferns. The results were published in three books, Manickam & Ninan's *Enumeration of ferns of the Palni Hills* (1976) and *Ecological Studies on the Fern Flora of the Palni Hills (S. India)* (1984) and Manickam's *Fern Flora of the Palni Hills (South India)* (1986), as well as a further research-paper on cytology (Manickam, 1984). In these works Father Manickam made every effort to communicate with other botanical authorities internationally and to revise and update the taxonomy and nomenclature to a superior level. It was also a great advantage that unlike his predecessors he documented and numbered his collections conscientiously, providing the numbers and details in his publication, something that no other cytotaxonomists in India were doing at that time. As a result, it has still been possible 35 years later for one of us (CRFJ) to find and photographically record all his cytological voucher-specimens in various herbaria and apply a much changed, more critical modern taxonomy to them, long after he had produced his important cytological results. This has not been possible for the pteridological studies of any other workers from Trivandrum, even recently, who neither kept actual voucher-specimens, nor cited numbers in their publications, and the same applies to many of the earlier publications of the Prof. P.N. Mehra School at Panjab University, Chandigarh, or the work of Prof. T.S. Mahabale at Pune, and others. In South India only Manickam's and Dr. J. Ghatak's cytological work can be properly verified today and applied to modern pteridology.

After his Ph.D. he joined as Lecturer in Botany from 1979–1981 at the Rapinat Herbarium, St. Joseph's College, Trichy, working with the accomplished Botanist, Dr. K.M. Mathew, on the ferns of Karnataka and the whole of the Western Ghats. His extensive herbarium collection from the earlier as well as more recent work is thus mainly housed at Trichy (RHT) and the well-documented, good quality cytological and distributional voucher-specimens he published have been photographed there and when necessary, critically re-identified by CRFJ. But he was not happy at Trichy, and in 1982 he transferred to St. Xavier's College, Palayamkottai, as Lecturer in Botany. From 1982 to 2008 he was settled and worked for nearly 30 years at his beloved St. Xavier's College and continued the bulk of his research career there, also supervising many students and introducing them to the fascinating world of Pteridology, and Botany in general, in a series of major projects. His extensive later herbarium collections are housed at St Xavier's (XCH) and again nearly all the important voucher-specimens of his and his students' have been photographed there for preservation by CRFJ.

During 1984–87, funded by the Department of Science & Technology, he carried out a major project on the Biosystematics of ferns of the Western Ghats

covering an area of 18,000 sq. km., and collecting some 5000 numbers of pteridophytes. Detailed field work was done in all the mountain ranges of Tamil Nadu and Kerala between Kanyakumari and Palghat. Assisted by his accomplished student, Dr. Varaprasatham Irudayaraj (b. 1960), who carried out a spectacular number of well-documented chromosome-counts, he investigated the cytology of 110 ferns collected from the area. The cytological and taxonomic results were published in two books, Manickam and Irudayaraj's *Cytology of ferns of the Western Ghats South India* (1988) (with an addition and correction, Manickam and Irudayaraj, 1989) and the outstandingly useful and major illustrated work, Manickam and Irudayaraj's *Pteridophyte Flora of the Western Ghats - South India* (1992).

In a second project funded by European Jesuits, the Western Ghats were explored from Palghat to Coorg in Karnataka. Detailed fieldwork was carried out in the Nilgiris, Silent Valley, Wyanad and Coorg, collecting 3500 numbers. As a part of this project, five families of ferns were subjected to preliminary phytochemical analysis by his research-scholars. A third related project concentrated on re-exploring several of the higher mountains such as the Anamallays, Palnis and Tirunelveli hills (resulting in a further book, Manickam and Irudayaraj's *Pteridophyte Flora of Nilgiris, South India* (2003)).

In 1987 he crowned his research-career with the opening of a new and modern Research Laboratory at St. Xavier's, the Centre for Biodiversity and Biotechnology (CBB), affiliated to Manonmanium Sundarnar University, Tirunelveli District. He was the founder and Director of the Laboratory, which concentrated on research on Pteridophytes, Angiosperms, and also environmental education to school teachers and children. In all, he supervised 25 Ph.D. students there, who all now have good positions in various organizations. He was successfully able to raise funding grants for the Laboratories' Research-Projects amounting to more than two crore rupees (20 million Indian rupees, or c. 400,000 U.S.D.) from various International and National scientific and educational funding agencies.

Father Manickam was elected a Life Fellow of the Indian Fern Society in recognition of the high quality of his work. He published 10 botanical books and more than 180 papers (a selection of his publications is in *Indian Fern J.* 22(1-2):204-208 (2005)). Later in his life he also wrote a number of spiritually insightful religious discussions with a very practical and down to earth context designed to help young people.

Father Manickam has been honored with the naming of four fern taxa after him, *Polystichum manickamianum* Benniam., Fraser-Jenk. & Irud. (2008), *Thelypteris parasitica* (L.) Tardieu subsp. *manickirudorum* Fraser-Jenk. (2008), *Athyrium x manickamii* Fraser-Jenk. (2008) and *Pteris manickamii* Dom.Rajk., and three Angiosperms, *Memycelon manickamii* Murugan *et al* (2002), *Xanthophyllum manickamii* Murugan (2002) and *Eugenia manickamiana* Murugan (2002). It is much to his credit that unlike other contemporaries he himself did not descend into naming many erroneous and spurious "new species" etc. that has so damaged Indian pteridology.

Unfortunately in the last five years of his life the College wished to take over his grants and therefore sent him to stay at Madurai away from his former students at work. During this time he remained of perfectly sound mind and memory despite the injustice and disappointment of the situation. Even after his unwanted retirement Father Manickam was highly active intellectually. He had learned the Thirukural (of Tamil poetic literature) by heart and spent much time translating and expounding it. He was not only an excellent teacher but also a philosopher who would always guide young students in their chosen direction in life.

In the last year of his life, on 14th Aug. 2011, his village people celebrated a Golden Jubilee for Father Manickam's 50 years since joining the Society of Jesus (1961–2011). It was a most happy occasion, which he enthusiastically enjoyed.

Father Manickam's contribution to science, especially in Pteridology, amounts to a definitive and monumental treatment of the ferns of South India. The loss to science and Botany from his death is immense, as is the loss to humanity of his delightful personality. It is hard indeed to think of his beloved establishment now no longer filled with his jovial laughter, and his lifetime's expertise uniquely guiding his active students along the path of pteridology he trod so splendidly and dedicatedly. May his soul rest in peace.

INFORMATION FOR AUTHORS

Authors are encouraged to submit manuscripts pertinent to pteridology for publication in the *American Fern Journal*. Manuscripts should be sent to the editor at hauk@denison.edu. Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication.

Authors should adhere to the following guidelines; manuscripts not so prepared may be returned for revision prior to review. **Electronic submission is strongly encouraged:** however if it is necessary to submit hard copy, please submit one copy of the manuscript and include a review copy of illustrations and originals of illustrations. After acceptance, please submit final versions of manuscripts via FTP (contact the managing editor for instructions), email, or on diskette or CD ROM (see below for figure formatting). If submitting hard copies, use standard 8.5 by 11 inch paper of good quality, not "erasable" paper. **Double-space manuscripts throughout**, including title, author's names and full addresses, a short, informative abstract, key words, text (including heads and keys), literature cited, tables (separate from text), and figure captions (grouped as consecutive paragraphs separate from figures). Arrange parts of manuscript in order just given. Include author's name and page number in the upper right corner of every sheet, and provide an abbreviated running title. Provide margins of at least one inch (25 mm) all around on typed pages. Do not submit right-justified text, avoid footnotes, and do not break words at end of lines. Make table headings and figure captions self-explanatory. Use S.I. (metric) units for all measures (e.g., distance, elevation, weight) unless quoted or cited from another source (e.g., specimen citations). For nomenclatural matters (i.e., synonymy and typification), use one paragraph per basionym (see *Regnum Veg.* 58:39–40, 1968). Abbreviate titles according to *Botanico-Periodicum-Huntianum* (Lawrence *et al.*, 1968, Hunt Botanical Library, Pittsburgh) and its supplement (1991). References cited only as part of nomenclatural matter are not included in literature cited. For shorter notes and reviews, omit the abstract and put all references parenthetically in text. Use *Index Herbariorum* (*Regnum Veg.* 120:1–693, 1990; or <http://websun.nybg.org/bsci/ih/>) for designations of herbaria. For more detailed instructions on manuscript preparation, see <http://amerfernsoc.org/>.

Illustrations should be proportioned to fit page width (5 inches or 12.5 cm) with caption ultimately to be included on the same page. Halftone and color images should be scanned at a minimum of 300 pixels per inch (ppi). Line art should be scanned at 1200 ppi whenever possible. Please note that nearly all images that are downloaded from the Internet or that are in JPEG or GIF format will be 72 dpi and not acceptable for the printing process. Indicate the file format of the graphics. Please submit image files in TIFF (preferable) or EPS format. Provide margins of at least 25 mm on all illustrations. For continuous-tone illustrations, design originals for reproduction without reduction or by uniform amount. In composite blocks, abut edges of adjacent photographs. Avoid combining continuous-tone and line-copy in single illustrations or blocks. Coordinate sequence and numbering of figures (and tables), with order of citation in text. Explain scales and symbols in figures themselves, not in captions. Include a scale and reference to latitude and longitude in each map.

Instructions for downloading electronic proofs and reprint order forms are sent to authors by the printer. Authors should send proof corrections of corrected proofs to the editor and reprint orders to the printer. Authors will be assessed charges for extensive alterations made after type has been set.

For other matters of form or style, consult recent issues of the *American Fern Journal* and *The Chicago Manual of Style*, 14th ed. (1993, Univ. of Chicago Press, Chicago). Occasionally, departure from these guidelines may be justified. Authors are encouraged to consult the editor for assistance with any aspect of manuscript preparation.

Papers of longer than 32 printed pages may be sent to the Editor of *Pteridologia* (memoir Editor, see journal cover page 2).

PTERIDOLOGIA ISSUES IN PRINT

The following issues of *Pteridologia*, the memoir series of the American Fern Society, are available for purchase:

1. Wagner, David H. 1979. Systematics of *Polystichum* in Western North America North of Mexico. 64 pp. \$10.00 plus postage and handling.

2A. Lellinger, David B. 1989. The Ferns and Fern-allies of Costa Rica, Panama, and the Chocó (Part 1: Psilotaceae through Dicksoniaceae). 364 pp. \$32.00 plus postage and handling.

3. Lellinger, David B. 2002. A Modern Multilingual Glossary for Taxonomic Pteridology. 263 pp. \$28.00 plus postage and handling.

For orders and more information, please contact our authorized agent for sales at: Missouri Botanical Garden Press, P.O. Box 299, St. Louis, MO 63166-0299, tel. 314-577-9534 or 877-271-1930 (toll free). For online orders, visit: <http://www.mbgpress.org>.

AMERICAN FERN JOURNAL ON MICROFICHE

Volumes 1–61 of the *American Fern Journal* are available as archival quality, silver positive microfiches. Single volumes or the entire run may be purchased. The fiches are easily read with 10x or greater magnification (using a dissecting microscope and transmitted illumination or a fiche reader). Silver negative microfiches of vols. 1–50 are also available. The price is \$4.00 per volume or \$244.00 per set of 61 volumes, postpaid.

Send your inquiry or order with a check or money order to: American Fern Society, Inc., % Ecology III, 804 Salem Blvd., Berwick, PA, 18603-9801.

FIDDLEHEAD FORUM

The editor of the Bulletin of the American Fern Society welcomes contributions from members and non-members, including miscellaneous notes, offers to exchange or purchase materials, personalia, horticultural notes, and reviews of non-technical books on ferns.

SPORE EXCHANGE

Ms. Denia Mandt, 12616 Ibbetson Ave., Downey, CA 90242-5050, is Director. Spores exchanged and lists of available spores sent on request. <http://amerfernsoc.org/sporexy.html>

GIFTS AND BEQUESTS

Gifts and bequests to the Society enable it to expand its services to members and to others interested in ferns. Back issues of the Journal and cash or other gifts are always welcomed and are tax-deductible. Inquiries should be addressed to the Membership Secretary.

VISIT THE AMERICAN FERN SOCIETY'S WORLD WIDE WEB HOMEPAGE:

<http://amerfernsoc.org/>

AMERICAN FERN JOURNAL

Volume 102

Number 4

October–December 2012

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

The Distinguished Legacy of DMB: Donald MacPhail Britton (1923–2012)	<i>Kathleen M. Pryer</i>	241
The Life of Barbara Joe Hoshizaki (1928–2012)	<i>Robbin C. Moran</i>	252
Low Within Population Genetic Variation and High Among Population Differentiation in <i>Cytromium falcatum</i> (L.f.) C. Presl (Dryopteridaceae) in Southern Korea: Inference of Population-Establishment History	<i>Mi Yoon Chung, Jordi López-Pujol, Jae Min Chung, Ki-Joong Kim, and Myong Gi Chung</i>	256
SEM Studies on Tracheids of Lycopodiaceae; Observations on Adaptations in <i>Phylloglossum</i>	<i>Sherwin Carlquist, Edward L. Schneider, and Kevin F. Kenneally</i>	273
<i>Phlegmariurus changii</i> (Huperziaceae), a New Hanging Firmoss from Taiwan	<i>Tung-Yu Hsieh, Kent A. Hatch, and Yuan-Mou Chang</i>	283
Lectotypification of <i>Marsilea quadrifolia</i> L. (Marsileaceae)	<i>Duilio Iamónico</i>	289
Referees for 2012		293
Table of Contents for Volume 102		294

The American Fern Society

Council for 2012

KATHLEEN PRYER, Dept. of Biology, Duke University, P.O. Box 90338, Durham, NC 27708.	<i>President</i>
JAMES E. WATKINS, JR., Dept. of Biology, Colgate University, Hamilton, NY 13346.	<i>President-Elect</i>
MARY STENSVOLD, USDA Forest Service, 204 Siginaka Way, Sitka, AK 99835.	<i>Secretary</i>
JAMES D. CAPONETTI, Div. of Biology, University of Tennessee, Knoxville, TN 37996-0830.	<i>Treasurer</i>
GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.	<i>Membership Secretary</i>
JAMES D. MONTGOMERY, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.	<i>Curator of Publications</i>
WARREN D. HAUK, Dept. of Biology, Denison University, Granville, OH 43023.	<i>Journal Editor</i>
R. JAMES HICKEY, Dept. of Botany, Miami University, Oxford, OH 45056.	<i>Memoir Editor</i>
JOAN N. E. HUDSON, Dept. of Biological Science, Sam Houston State University, Huntsville, TX 77341-2116.	
DAVID SCHWARTZ, 9715 Christey Way, Bakersfield, CA 93312-5617.	<i>Bulletin Editors</i>

American Fern Journal

EDITOR

WARREN D. HAUK Dept. of Biology, Denison University, Granville, OH 43023,
ph. (740)587-5758, e-mail: hauk@denison.edu

ASSOCIATE EDITORS

NAN CRYSTAL ARENS Dept. of Geoscience, Hobart & William Smith Colleges, Geneva, NY 14456
JOSE MARÍA GABRIEL Y GALÁN Dept. de Biología Vegetal I. Universidad Complutense de Madrid,
Madrid, Spain 28040
GERALD J. GASTONY Dept. of Biology, Indiana University, Bloomington, IN 47405-6801
GARY K. GREER Biology Dept., Grand Valley State University, Allendale, MI 49401
CHRISTOPHER H. HAUFLER Dept. of Ecology and Evolutionary Biology, University of Kansas,
Lawrence, KS 66045-2106
R. JAMES HICKEY Dept. of Botany, Miami University, Oxford, OH 45056
PAULO LABIAK Universidade Federal do Paraná, Dept. de Botânica, 81531-980, Curitiba-PR, Brasil
ROBBIN C. MORAN New York Botanical Garden, Bronx, NY 10458-5126
NATHALIE NAGALINGUM Royal Botanical Garden Sydney, Sydney, NSW, Australia
CARL ROTHFELS Botany Dept., Duke University, Durham, NC 27708
EMILY SESSA Botany Dept., University of Wisconsin-Madison, Madison, WI 53706
MICHAEL SUNDUE Pringle Herbarium, The University of Vermont, Burlington, VT 05405
JAMES E. WATKINS, JR. Dept. of Biology, Colgate University, Hamilton, NY 13346

The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, % Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

Claims for missing issues, made 6 months (domestic) to 12 months (foreign) after the date of issue, and orders for back issues should be addressed to Dr. James D. Montgomery, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.

Changes of address, dues, and applications for membership should be sent to the Membership Secretary.

General inquiries concerning ferns should be addressed to the Secretary.

Back volumes are available for most years as printed issues or on microfiche. Please contact the Back Issues Curator for prices and availability.

Subscriptions:

Society Membership - USA, Canada, Mexico (includes Journal and Fiddlehead Forum)	\$30
Society Membership - All other countries (includes Journal and Fiddlehead Forum)	\$40
Society Life Membership - \$360 (add \$120 mailing surcharge for outside USA, Canada, Mexico)	
Regular Membership - USA, Canada, Mexico (includes Fiddlehead Forum)	\$15
Regular Membership - All other countries (includes Fiddlehead Forum)	\$22
Institutional Membership - \$40 to USA, Canada, Mexico; \$50 elsewhere (-\$3 agency fee)	

POSTMASTER: Send address changes to American Fern Journal, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

The Distinguished Legacy of DMB: Donald MacPhail Britton (1923–2012)

KATHLEEN M. PRYER

Department of Biology, Duke University, Durham, NC 27708-0339, USA
e-mail: pryer@duke.edu

In a letter dated 23 May 1995, Donald M. Britton wrote to me, “... things are moving along at a leisurely pace and my lifestyle is changed – No pipe, No coffee, No chocolate peppermints, No stress, No strain. ...Dan [Brunton] and I are still banging away at *Isoetes* ...it is nice to have a hobby project so the old neurons do not completely short-circuit ...I noticed when I passed 70 that mail eased up considerably. I guess workers feel that either you are retired, or should be! One looks a bit furtively at the obits to see who has made the list”.

Fortunately for pteridology, Don Britton remained active in research throughout his “retirement” (Brunton, 2012a, 2012b; Brunton and Catling, 2012) and he published nearly 20 more papers after the aforementioned letter (Appendix 1). Most of these were with his longtime friend and collaborator Daniel F. Brunton. From 1995 to 2006 “Brunton and Britton” and “Britton and Brunton” described seven new North American species and hybrids of *Isoetes* (Appendix 2), and also published studies clarifying the distribution, status, and taxonomy of several more in this notoriously difficult genus (Appendix 3).

Donald MacPhail Britton (Fig. 1) was born on March 6, 1923 in Toronto, Canada, the youngest son of Arthur Britton and Marjorie Spence. He attended University of Toronto Schools (UTS) and was awarded a J. S. McLean Scholarship in Science to the University of Toronto in 1942. Britton was a hard-working and successful student, receiving the I. M. Gilchrist Prize in Botany (1944) and graduating with first class honors in science and biology (1946). That fall, he entered the graduate program at the University of Virginia under the auspices of a Philip Francis du Pont Fellowship. His time at UVA involved a semiannual migration, with the academic year spent at the Miller School of Biology (Charlottesville, VA), and the summer working at Blandy Experimental Farm (Boyce, VA). In 1950, Britton completed his Ph.D. with a dissertation entitled “*Cytogenetic studies on the Boraginaceae*” and received an honorable mention from the Virginia Academy of Science. The following year he married Mary Cronyn, whom he had met at the University of Toronto.

With Ph.D. in hand, Britton pursued a postdoctoral fellowship at the Department of Plant Science at the University of Alberta. Following this, he worked several years as an Assistant Professor of Horticulture at the University of Maryland, where he specialized in the cytogenetics and breeding of *Rubus* and other flowering plants. In 1958 he moved to the University of Guelph and, in 1971, became a Full Professor in the Department of Botany and Genetics. He spent the remainder of his academic career at Guelph.

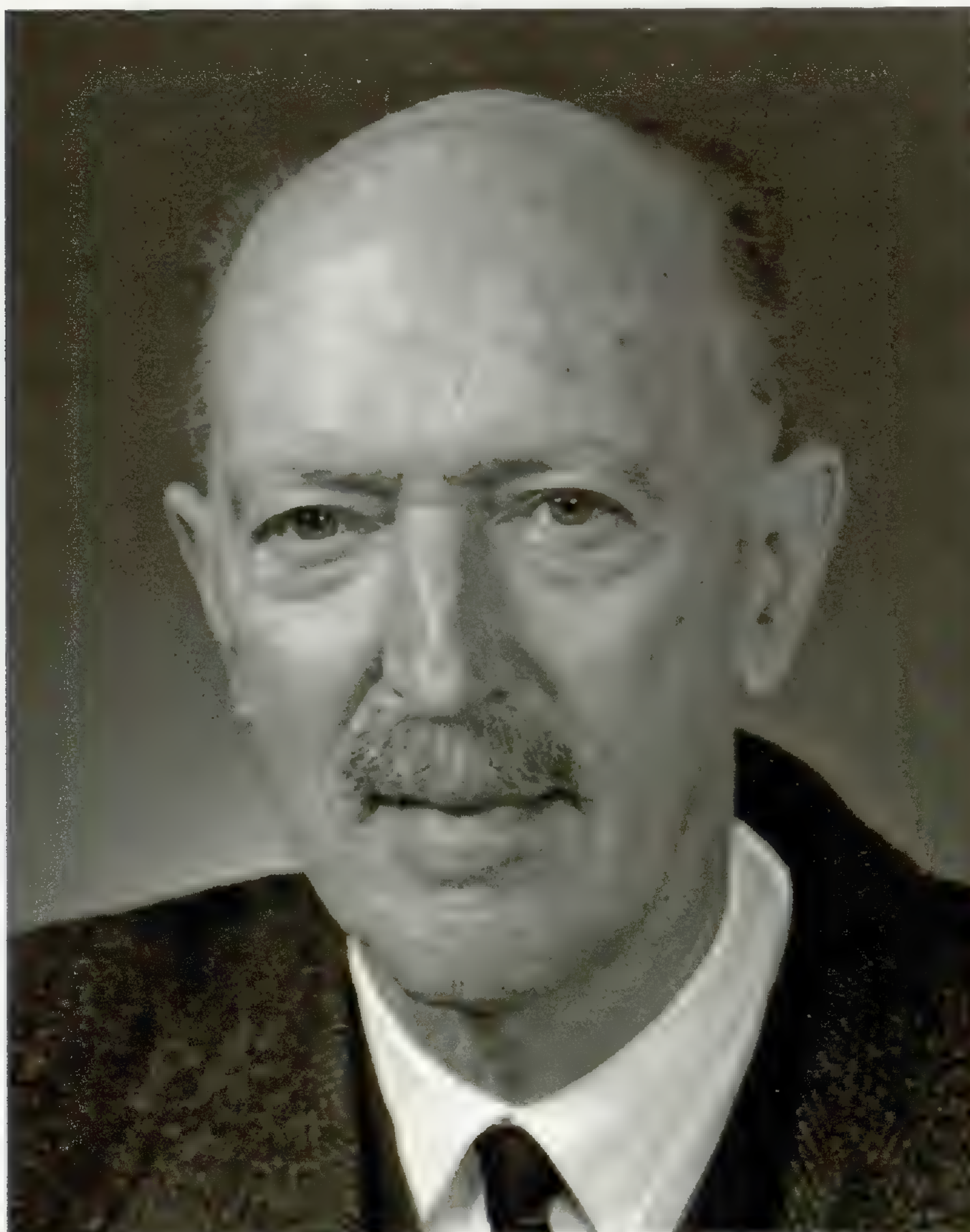


FIG. 1. Photograph of Donald M. Britton taken in 1996 on the occasion of the *Richard and Minnie Windler Award* presented by the Southern Appalachian Botanical Society for the publication: Brunton, D. F., D. M. Britton, and T. F. Wieboldt. 1996. Taxonomy, identity, and status of *Isoetes virginica* (Isoetaceae). *Castanea* 61:145–160.

Showing an early interest in ferns, Britton became a member of the American Fern Society in 1946. Later in his career, he would be awarded an honorary membership in this Society—a special category for persons who have made outstanding contributions to the study of ferns. Britton's early emphasis on angiosperms eventually gave way to a career-long focus on ferns. Building on his strong interest in cytogenetics, his first fern paper, entitled "*Chromosome studies on ferns*," was published in the *American Journal of Botany* in 1953. This was a landmark paper following up on Manton's (1950, see also 1973) methodological breakthrough combining acetocarmine staining with a squash technique that flattened dividing cells so that their chromosomes could be photographed in one focal plane. Prior to the introduction of this technique, the only method available to count chromosome numbers was to compare camera lucida drawings based on serial microtome sections of paraffin-

embedded material. Britton's paper (1953) provided chromosome numbers for 25 species of ferns collected in southern Ontario. Because many of the species were also native to the British flora studied by Manton (1950), his work provided vital corroboration of Manton's results, along with additional evidence of polyploidy in ferns.

While at Guelph, Britton supervised four graduate students in pteridology: Jane Rigby (M.Sc. 1969, *Pellaea*), Laima Kott (M.Sc. 1972, *Polypodium*; Ph.D. 1980, *Isoetes*), Ruth Hersey (M.Sc. 1979, *Lycopodium*), and me, Kathleen Pryer (M.Sc. 1981, *Gymnocarpium*), all of whom published their work with him (Appendix 1). I remember how he would frequently come into the lab to read us letters (while smoking his pipe with tobacco from the local Wiff'n Puff) that he received from scientists all over the world who sought his opinion and shared new information with him. What a great way for students to learn about ferns and the kinds of research questions being asked at the time! This was well before Chris Haufler (who Britton referred to as the "wunderkind in Kansas") took the fern world by storm with isozymes.

Britton also took his students to important fern meetings, including the famous New England Fern Conference that was held in Petersham, Massachusetts at Harvard Forest. These meetings were critical for fostering communication among botanists working on ferns, but in diverse disciplines. One of these trips included an introduction to the herbaria at Harvard and the wonderful hospitality of Alice and Rolla Tryon at their residence within a stone's throw from the herbarium (where we feasted on the best fiddlehead appetizers ever...!).

I never called him Don, always Dr. Britton. But after I graduated with my M.Sc. from Guelph in 1981, I addressed him by his initials in correspondence and that is how I have always referred to him since—DMB. DMB was extremely generous with his time and very patient with everyone (students, colleagues, and amateur enthusiasts, alike), and through his example showed us how to put in the long hours to get those almost-perfect chromosome squashes, and to locate those hard-to-find ferns when doing fieldwork. DMB's connection with those outside the academic world was particularly evident in how he was always so welcoming to anyone interested in his area of study. His quiet encouragement and the confidence it instilled in those working with natural history and regional conservation organizations were both effective and appreciated, as acknowledged when he was awarded an Honorary Membership by the Ottawa Field-Naturalists' Club in recognition of both his scholarly and conservation contributions (Brodo et al., 2001).

Doing fieldwork with DMB was a treat (Fig. 2). It was a natural talent for him—it was as though he had special radar in the field for finding the ferns he was after. One does not learn how to do that from books, but by watching and observing, if you are fortunate to be with someone who has the "know how". Field trips with DMB were meticulously planned—everything happened on schedule, ALWAYS with good humor, and without a hitch.

Except for one trip, a trip that is a favorite memory that still makes me smile, to a special *Gymnocarpium* collecting site in Wellington County, near Guelph,



FIG. 2. DMB in 1979, collecting ferns in northwestern Ontario (near Thunder Bay).

to which we returned a few times to collect chromosome and spore material. It meant driving about 20 minutes from campus, parking on the side of the road, then trudging our way through a few acres of pasture with all our collecting gear in tow, to get to a woodlot that had the patch of ferns we were after. On one occasion, as we were making our way through the pasture, I spotted a bull facing us.... and he seemed to be pawing at the ground. I squinted for a better look, and nervously whispered to DMB "What is one supposed to do when you think a bull might be getting ready to charge?" Not getting an answer, I glanced over my shoulder and there was DMB, in the distance, with his great long legs hightailing it over the fence. I bolted after him at the speed of light, and learned another important lesson; don't waste time asking questions, just watch and learn...

After his official retirement at age 65, DMB was awarded the title of Professor Emeritus by the University of Guelph. For nearly 20 more years, he enjoyed

going to his office every day, socializing with the younger members of his department, and continuing his scientific studies. The year following his retirement from Guelph, DMB coauthored a book with William J. Cody from Agriculture Canada entitled “Ferns and Fern Allies of Canada” (1989). Nearly 25 years later, it still is (and long will be) the definitive reference book on the ferns of Canada. In 1991, DMB was awarded the *Lawson Medal* for “outstanding scientific achievement over the period of a career” from the Canadian Botanical Association. He was awarded the *Richard and Minnie Windler Award* for his publication on *Isoetes virginica* with Brunton et al. (1996) in the journal *Castanea*. In 2007, the Field Botanists of Ontario awarded him the inaugural *John Goldie Award* for his dedicated service to the field of botany. The three fern taxa named in his honor (Appendix 4) provide further tribute to DMB’s contribution to our knowledge of ferns.

The last time I saw DMB was when I visited him in Guelph in June 2000 and we spent two days together racing over the countryside collecting as many different species of *Equisetum* as possible. It was a very productive effort—9 of the 15 species of *Equisetum* that are known worldwide can be found within a short distance from Guelph and he led me to all of them. This resulted in a phylogenetic publication in 2003 (Des Marais et al.) that was DMB’s first and only publication to include molecular DNA sequence data (or what he would call “the O. J. solution”). A paper presenting a molecular phylogeny of Cystopteridaceae (including *Cystopteris* and *Gymnocarpium*, two of DMB’s favorite ferns), and currently in press at *Systematic Botany* (Rothfels et al. 2013), is dedicated to the memory of DMB.

With a “second retirement” at age 80 (see tributes by: Brunton, 2003; Catling, 2003; Ceska and Ceska, 2003; Pryer, 2003; Reznicek, 2003), DMB’s world contracted, especially after being diagnosed with Alzheimer’s disease in 2008 and the death of his wife Mary in 2010. DMB was hospitalized on May 15, 2012 with pneumonia and died peacefully in hospice on May 18. A private family funeral service was held on May 19, followed by a memorial service to celebrate his life on July 28 at St. George’s Anglican Church in Guelph. He leaves behind his son Robert, and two daughters, Anne (Terry Greenlay) and Barbara, as well as two grandsons, Scott and Ben, of whom he was very proud.

To me, DMB embodies all that is essential to being a great scientific advisor, including a wry sense of humor and the ability to get students to move beyond their comfort zone. He is the one I have always tried to measure up to in my own scientific interactions, especially with graduate students. Several people contacted me this summer to say how very sorry they were to hear about our loss of DMB. They all described him as “a larger-than-life guy”. I will be forever grateful to DMB for the guidance and opportunities that he provided. His influence in my life has been pervasive—I think of him every time I interact with my own graduate students, every time I do fieldwork, and every time I see a bull in a pasture...

ACKNOWLEDGEMENTS

I am very grateful to DMB’s children (Robert, Anne, and Barbara), Dan Brunton, Usher Posluszny, Dean Whittier, and Michael Windham for their encouragement, insightful comments,

and help with preparing this tribute. David Barrington made useful comments in review, and George Yatskievych provided information regarding DMB's American Fern Society membership.

LITERATURE CITED

- BRITTON, D. M. 1953. Chromosome studies on ferns. *Amer. J. Bot.* 40:575–583.
- BRODO, I. M., C. HANRAHAN, S. DARBYSHIRE and S. THOMSON. 2001. The Ottawa Field-Naturalists' Club Awards April 2001, Honorary Membership: Donald M. Britton. *Can. Field-Naturalist* 115:728.
- BRUNTON, D. F. 2003. Don Britton at 80. *Botanical Electronic News (BEN)* 305. Retrieved 30 August 2012, from <http://bomi.ou.edu/ben/ben305.html>.
- BRUNTON, D. F. 2012a. Donald M. Britton (1923–2012). *Botanical Electronic News (BEN)* 458. Retrieved 30 August 2012, from <http://www.ou.edu/cas/botany-micro/ben/ben458.html>.
- BRUNTON, D. F. 2012b. A tribute to Donald M. Britton (1923–2012), Canada's premier pteridologist. *Can. Field-Naturalist* 126: in press.
- BRUNTON, D. F. and P. M. CATLING. 2012. A tribute for Donald M. Britton (1923–2012), a wonderful botanist! *Can. Bot. Assoc. Bull.* 45:104–105.
- BRUNTON, D. F., D. M. BRITTON and T. F. WIEBOLDT. 1996. Taxonomy, identity, and status of *Isoetes virginica* (Isoetaceae). *Castanea* 61:145–160.
- CATLING, P. M. 2003. Donald M. Britton—1991 Recipient of the Lawson Medal. *Botanical Electronic News (BEN)* 304. Retrieved 30 August 2012, from <http://bomi.ou.edu/ben/ben304.html>.
- CESKA, O. and A. CESKA. 2003. *Isoetes minima*—guess who identified it! *Botanical Electronic News (BEN)* 304. Retrieved 30 August 2012, from <http://bomi.ou.edu/ben/ben304.html>.
- CODY, W. J. and D. M. BRITTON. 1989. Ferns and fern allies of Canada. Agriculture Canada Research Branch Publication 1829/E, Ottawa. 430 pp.
- DES MARAIS, D. L., A. R. SMITH, D. M. BRITTON and K. M. PRYER. 2003. Phylogenetic relationships and evolution of extant horsetails, *Equisetum*, based on chloroplast DNA sequence data (*rbcL* and *trnL-F*). *Int. J. Pl. Sci.* 164:737–751.
- HERSEY, R. E. 1979. Study of variation in three species of *Lycopodium* L. (section *Complanata* Vict.) in Ontario. M.Sc. thesis, University of Guelph, ON, Canada.
- KOTT, L. S. 1972. Morphological study of the cytotypes of *Polypodium virginianum* L. in southern Ontario. M.Sc. thesis, University of Guelph, ON, Canada.
- KOTT, L. S. 1980. The taxonomy and biology of the genus *Isoetes* L. in northeastern North America. Ph.D. dissertation, University of Guelph, ON, Canada.
- MANTON, I. 1950. Problems of cytology and evolution in the Pteridophyta. University Press, Cambridge.
- MANTON, I. 1973. Explanations: How it all began. *Brit. Fern Gaz.* 10:285–292.
- PRYER, K. M. 1981. Systematic studies in the genus *Gymnocarpium* Newm. in North America. M.Sc. thesis, University of Guelph, ON, Canada.
- PRYER, K. M. 2003. Don Britton (DMB) turns 80? *Botanical Electronic News (BEN)* 304. Retrieved 30 August 2012, from <http://bomi.ou.edu/ben/ben304.html>.
- RESNICEK, A. A. 2003. Don Britton – Appreciation. *Botanical Electronic News (BEN)* 304. Retrieved 30 August 2012, from <http://bomi.ou.edu/ben/ben304.html>.
- RIGBY, S. J. 1969. Investigation of *Pellaea glabella* Mett. ex Kuhn and *Pellaea atropurpurea* (L.) Link and their relationships. M.Sc. thesis, University of Guelph, ON, Canada.
- ROTHFELS, C. R., M. D. WINDHAM and K. M. PRYER. 2013. A plastid phylogeny of the cosmopolitan fern family Cystopteridaceae (Polypodiopsida). *Syst. Bot.* 38: in press.

APPENDIX 1. Fern bibliography (including abstracts and reviews) of Donald M. Britton, arranged first by decade, and then alphabetically within each decade.

1950s

BRITTON, D. M. 1953. Chromosome studies on ferns. *Amer. J. Bot.* 40:575–583.

TRYON, A. F. and D. M. BRITTON. 1958. Cytotaxonomic studies on the fern genus *Pellaea*. *Evolution* 12:137–145.

1960s

BRITTON, D. M. 1960. Recent fern literature—Ferns of Alberta. *Amer. Fern J.* 50:156–157.

BRITTON, D. M. 1960. Report on the Rougemont field trip. *Amer. Fern J.* 50:216–218.

BRITTON, D. M. 1961. The problems of variation in North American *Dryopteris*. *Amer. Fern J.* 51:23–20.

BRITTON, D. M. 1962. *Dryopteris dilatata* (Hoffm.) A. Gray in North America. *Rhodora* 64:207–212.

BRITTON, D. M. 1964. Chromosome numbers of ferns in Ontario. *Canad. J. Bot.* 42:1349–1354.

BRITTON, D. M. 1965. Hybrid wood ferns in Ontario. *Michigan Botanist* 4:3–9.

BRITTON, D. M. 1966. Review of *Flore Laurentienne* by Frère Marie-Victorin. *Amer. Fern J.* 56:84–86.

BRITTON, D. M. 1967. Diploid *Dryopteris dilatata* from Quebec. *Rhodora* 69:1–4.

BRITTON, D. M. 1968. The spores of four species of spinulose wood ferns (*Dryopteris*) in eastern North America. *Rhodora* 70:340–347.

BRITTON, D. M. and S. J. RIGBY. 1969. In search of the purple cliff brake. *Ontario Nature* 8(5–7):12.

BRITTON, D. M. and J. H. SOPER. 1966. The cytology and distribution of *Dryopteris* species in Ontario. *Canad. J. Bot.* 44:63–78.

BRITTON, D. M., A. LEGAULT and S. J. RIGBY. 1967. *Pellaea atropurpurea* (L.) Link and *Pellaea glabella* Mett. in Quebec. *Naturaliste Canad.* 94:761–763.

KNOBLOCH, I. W. and D. M. BRITTON. 1963. The chromosome number and possible ancestry of *Pellaea wrightiana*. *Amer. J. Bot.* 50:52–55.

TRYON, R. M. and D. M. BRITTON. 1966. A study of variation in the cytotypes of *Dryopteris spinulosa*. *Rhodora* 68:69–92.

WIDÉN, C.-J. and D. M. BRITTON. 1969. A chromatographic and cytological study of *Dryopteris dilatata* in eastern North America. *Canad. J. Bot.* 47:1337–1344.

1970s

BRITTON, D. M. 1972. Spinulose woodferns (*Dryopteris*) in western North America. *Canad. Field-Naturalist* 86:241–247.

BRITTON, D. M. 1972. Spore ornamentation in the *Dryopteris spinulosa* complex. *Canad. J. Bot.* 50:1617–1621.

BRITTON, D. M. 1972. The spores of *Dryopteris clintoniana* and its relatives. *Canad. J. Bot.* 50:2027–2029.

BRITTON, D. M. 1974. The significance of chromosome numbers in ferns. *Ann. Missouri Bot. Gard.* 61:310–317.

BRITTON, D. M. 1976. The distribution of *Dryopteris spinulosa* and its relatives in eastern Canada. *Amer. Fern J.* 66:69–74.

BRITTON, D. M. 1976. Two decades of cytotaxonomic research on Canadian ferns [Pteridophyta]. Pp. 116–126 in P. Kachroo, ed., *Recent Advances in Botany* (Prof. P. N. Mehra Commemorative Volume).

BRITTON, D. M. 1977. “A Note: Solicited review of The origin of *Dryopteris campyloptera* by M. Gibby, *Canad. J. Bot.* 55:1419–1428.” *Amer. Fern J.* 67:121–122.

BRITTON, D. M. 1977. The fern *Woodsia obtusa* (Spreng) Torrey in Ontario. *Canad. Field-Naturalist* 91:84–85.

BRITTON, D. M. 1978. Review of “Cytotaxonomical Atlas of the Pteridophyta. Cytotaxonomical Atlases, Volume III by A. Löve, D. Löve, and R. E. G. Pichi Sermolli. Germany: J. Cramer”. *Quart. Rev. Biol.* 53:64–65.

BRITTON, D. M. 1978. Review of “Evolutionary Patterns and Process in Ferns, by J.D. Lovis In: *Advances in Botanical Research, Volume 4*”. *Bull. Torrey Bot. Club* 105:245–246.

- BRITTON, D. M. 1979. Review of "Ferns of the Ottawa District, by W. J. Cody, Supply and Services, Canada". *Canad. Field-Naturalist* 93:345.
- BRITTON, D. M. and A. C. JERMY. 1974. The spores of *Dryopteris filix-mas* and related taxa in North America. *Canad. J. Bot.* 52:1923–1926.
- BRITTON, D. M. and C.-J. WIDÉN. 1974. Chemotaxonomic studies on *Dryopteris* from Quebec and eastern North America. *Canad. J. Bot.* 52:627–638.
- BRITTON, D. M., C.-J. WIDÉN, D. F. BRUNTON and P. A. KEDDY. 1975. A new hybrid woodfern, *Dryopteris* × *algonquinensis* D. M. BRITTON, from Algonquin Park, Ontario. *Canad. Field-Naturalist* 89:163–171.
- CAMPBELL, C. A. and D. M. BRITTON. 1977. Pteridophytes of the Regional Municipality of Waterloo, Ontario. *Canad. Field-Naturalist* 91:262–268.
- RIGBY, S. J. and D. M. BRITTON. 1970. The distribution of *Pellaea* in Canada. *Canad. Field-Naturalist* 84:137–144.
- WIDÉN, C.-J. and D. M. BRITTON. 1971. A chromatographic and cytological study of *Dryopteris dilatata* in North America and eastern Asia. *Canad. J. Bot.* 49:247–258.
- WIDÉN, C.-J. and D. M. BRITTON. 1971. A chromatographic and cytological study of *Dryopteris filix-mas* and related taxa in North America. *Canad. J. Bot.* 49:1589–1600.
- WIDÉN, C.-J. and D. M. BRITTON. 1971. Chemotaxonomic investigations on *Dryopteris fragrans*. *Canad. J. Bot.* 49:989–992.
- WIDÉN, C.-J. and D. M. BRITTON. 1971. Chemotaxonomic investigations on the *Dryopteris cristata* complex in North America. *Canad. J. Bot.* 49:1141–1154.
- WIDÉN, C.-J., D. M. BRITTON, W. H. WAGNER, JR. and F. S. WAGNER. 1975. Chemotaxonomic studies on hybrids of *Dryopteris* in eastern North America. *Canad. J. Bot.* 53:1554–1567.
- 1980s**
- BRITTON, D. M. 1981. Review of "Ferns of Northwestern Himalayas, by K. K. Dhir, 1980". *Canad. Field-Naturalist* 95:119–120.
- BRITTON, D. M. 1981. Review of "Vascular Plants of Continental Northwest Territories, Canada, by A. E. Porsild and W. J. Cody, National Museum of Canada, 1980". *Amer. Fern J.* 71:68.
- BRITTON, D. M. 1983. Review of "Ferns and Allied Plants with Special Reference to Tropical America, by R. M. Tryon and A. F. Tryon, New York: Springer-Verlag, 1982". *Canad. Field-Naturalist* 97:361.
- BRITTON, D. M. 1984. Biosystematic studies on pteridophytes in Canada: progress and problems. Pp. 543–560 in W. F. Grant, ed., *Plant Biosystematics*. Academic Press, Toronto.
- BRITTON, D. M. 1984. Checklist of Ontario pteridophytes. Part One: Fern Allies. *Pl. Press* 2(4):95–99.
- BRITTON, D. M. 1985. Checklist of Ontario pteridophytes. Part Two: Ferns. *Pl. Press* 3(1):14–23.
- BRITTON, D. M. 1986. Review of "A Field Manual of the Ferns and Fern-Allies of United States and Canada". *Pl. Press* 4(1):34–35.
- BRITTON, D. M. and A. B. ANDERSON. 1986. The ferns of Manitoulin Island. Notes and a new record. *Pl. Press* 4(2):60–61.
- BRITTON, D. M. and D. F. BRUNTON. 1989. A new *Isoetes* hybrid (*Isoetes echinospora* × *riparia*) for Canada. *Canad. J. Bot.* 67:2995–3002.
- BRITTON, D. M., H. L. DICKSON and D. WHITE. 1983. Rare species of Ophioglossaceae. In G. W. Argus and D. J. White, eds., *Atlas of Rare Vascular Plants of Ontario/Atlas des plantes vasculaires rares de l'Ontario*, Part 2. Ottawa: National Museum of Natural Sciences.
- BRITTON, D. M., W. G. STEWART and W. J. CODY. 1985. *Cystopteris protrusa* (Creeping Fragile Fern), an addition to the flora of Canada. *Canad. Field-Naturalist* 99:380–382.
- BRITTON, D. M., H. L. DICKSON, K. M. PRYER and D. J. WHITE. 1987. Rare species of Ophioglossaceae (additions). In K. M. Pryer and G. W. Argus, eds., *Atlas of Rare Vascular Plants of Ontario/Atlas des plantes vasculaires rares de l'Ontario*, Part 4. Ottawa: National Museum of Natural Sciences.
- BRITTON, D. M., P. M. CATLING, H. L. DICKSON, K. M. PRYER and D. J. WHITE. 1987. Rare species of Aspleniaceae (additions). In K. M. Pryer and G. W. Argus, eds., *Atlas of Rare Vascular Plants of Ontario/Atlas des plantes vasculaires rares de l'Ontario*, Part 4. Ottawa: National Museum of Natural Sciences.

- CODY, W. J. and D. M. BRITTON. 1985. *Dryopteris filix-mas* (Male Fern), a phytogeographically important discovery in northern Saskatchewan. *Canad. Field-Naturalist* 99:101–102.
- CODY, W. J. and D. M. BRITTON. 1989. Ferns and fern allies of Canada. Agriculture Canada Research Branch Publication 1829/E, Ottawa. 430 pp.
- CRANFILL, R. and D. M. BRITTON. 1983. Typification within the *Polypodium virginianum* complex (Polypodiaceae). *Taxon* 32:557–560.
- HAUFLER, C. H., M. D. WINDHAM, D. M. BRITTON and S. J. ROBINSON. 1985. Triploidy and its evolutionary significance in *Cystopteris protrusa*. *Canad. J. Bot.* 63:1855–1863.
- HERSEY, R. E. and D. M. BRITTON. 1981. A cytological study of three species and a hybrid taxon of *Lycopodium* (section *Complanata*) in Ontario. *Canad. J. Gen. Cytology* 23:497–504.
- KOTT, L. S. and D. M. BRITTON. 1980. Chromosome numbers for *Isoetes* in northeastern North America. *Canad. J. Bot.* 58:980–984.
- KOTT, L. S. and D. M. BRITTON. 1982. A comparative study of spore germination of some *Isoetes* species of northeastern North America. *Canad. J. Bot.* 60:1679–1687.
- KOTT, L. S. and D. M. BRITTON. 1982. A comparative study of sporophyte morphology of the three cytotypes of *Polypodium virginianum* in Ontario. *Canad. J. Bot.* 60:1360–1370.
- KOTT, L. S. and D. M. BRITTON. 1982. Comparison of chromatographic spot patterns of some North American *Isoetes* species. *Amer. Fern J.* 72:15–18.
- KOTT, L. S. and D. M. BRITTON. 1983. Spore morphology and taxonomy of *Isoetes* in northeastern North America. *Canad. J. Bot.* 61:3140–3163.
- KOTT, L. S. and D. M. BRITTON. 1985. Role of morphological characteristics of leaves and the sporangial region in the taxonomy of *Isoetes* in northeastern North America. *Amer. Fern J.* 75:44–55.
- PRYER, K. M. and D. M. BRITTON. 1983. Spore studies in the genus *Gymnocarpium*. *Canad. J. Bot.* 61:377–388.
- PRYER, K. M., D. M. BRITTON and J. MCNEILL. 1983. A numerical analysis of chromatographic profiles in North American taxa of the fern genus *Gymnocarpium*. *Canad. J. Bot.* 61:2592–2602.
- PRYER, K. M., D. M. BRITTON and J. MCNEILL. 1983. Systematic studies in the genus *Gymnocarpium* Newm. (Aspleniaceae) in North America. *Amer. J. Bot.* 70(5):60. (Abstract).
- PRYER, K. M., D. M. BRITTON and J. MCNEILL. 1984. Hybridization in the fern genus *Gymnocarpium* Newman (Aspleniaceae) in North America. *Amer. J. Bot.* 71(5):142. (Abstract).
- SARVELA, J., D. M. BRITTON and K. M. PRYER. 1981. Studies on the *Gymnocarpium robertianum* complex in North America. *Rhodora* 83:421–431.
- WIDÉN, C.-J. and D. M. BRITTON. 1985. Phloroglucinol derivatives of *Dryopteris tokyoensis* and the missing genome in *D. cristata* and *D. carthusiana*. *Amer. J. Bot.* 72(6):929. (Abstract).
- WIDÉN, C.-J. and D. M. BRITTON. 1985. Phloroglucinol derivatives of *Dryopteris tokyoensis* and the missing genome in *D. cristata* and *D. carthusiana*. *Ann. Bot. Fenn.* 22:213–218.
- WIDÉN, C.-J., J. SARVELA and D. M. BRITTON. 1983. On the location and distribution of phloroglucinols (filicin) in ferns. New results and review of the literature. *Ann. Bot. Fenn.* 20:407–417.
- 1990s**
- BRITTON, D. M. 1991. A hybrid *Isoetes*, *I. × harveyi*, in northeastern North America. *Canad. J. Bot.* 69:634–640.
- BRITTON, D. M. and D. F. BRUNTON. 1991. The spores and affinities of *Isoetes taiwanensis* (Isoetaceae: Pteridophyta). *Fern Gaz.* 14:73–81.
- BRITTON, D. M. and D. F. BRUNTON. 1992. *Isoetes × jeffreyi*, *hyb. nov.*, a new *Isoetes* (*Isoetes macrospora × Isoetes riparia*) from Quebec, Canada. *Canad. J. Bot.* 70:447–452.
- BRITTON, D. M. 1993. *Isoetes reticulata* R. S. Hill 1987 (*Acheringa* 12: 158) is an illegitimate name. *Amer. Fern J.* 83:128.
- BRITTON, D. M. and D. F. BRUNTON. 1993. *Isoetes × truncata*: a newly considered pentaploid hybrid from western North America. *Canad. J. Bot.* 71:1016–1025.
- BRITTON, D. M. and D. F. BRUNTON. 1995. *Isoetes × marensis*, a new interspecific hybrid from western Canada. *Canad. J. Bot.* 73:1345–1353.
- BRITTON, D. M. and D. F. BRUNTON. 1996. *Isoetes × pseudotruncata*, a new triploid hybrid from western Canada and Alaska. *Canad. J. Bot.* 74:51–59.

- BRITTON, D. M. and D. F. BRUNTON. 1999. Rush quillwort (*Isoetes junciformis*, sp. nov.), a new pteridophyte from Southern Georgia. *Amer. Fern J.* 89:187–197.
- BRITTON, D. M. and J. P. GOLTZ. 1991. *Isoetes prototypus*, a new diploid species from eastern Canada. *Canad. J. Bot.* 69:277–281.
- BRITTON, D. M., D. F. BRUNTON and S. S. TALBOT. 1997. *Isoetes* in Alaska, the Yukon, and the Aleutians - a classical biosystematic study and its limitations. *Amer. J. Bot.* 84:S161. (Abstract).
- BRITTON, D. M., D. F. BRUNTON and S. S. TALBOT. 1999. *Isoetes* in Alaska and the Aleutians. *Amer. Fern J.* 89:133–141.
- BRITTON, D. M., P. M. CATLING, J. NORRIS and S. VARGA. 1991. Engelmann's quillwort, *Isoetes engelmannii*, an addition to the Flora of Canada. *Canad. Field-Naturalist* 105:67–70.
- BRUNTON, D. F. and D. M. BRITTON. 1993. *Isoetes prototypus* (Isoetaceae) in the United States. *Rhodora* 95:122–128.
- BRUNTON, D. F. and D. M. BRITTON. 1996. Noteworthy collections: Alabama and Georgia. *Castanea* 61:398–399.
- BRUNTON, D. F. and D. M. BRITTON. 1996. Taxonomy and distribution of *Isoetes valida*. *Amer. Fern J.* 86:16–25.
- BRUNTON, D. F. and D. M. BRITTON. 1996. The status, distribution, and identification of Georgia quillwort (*Isoetes georgiana*; Isoetaceae). *Amer. Fern J.* 86:105–113.
- BRUNTON, D. F. and D. M. BRITTON. 1997. A new Appalachian *Isoetes* species in the eastern United States. *Amer. J. Bot.* 84:S161. (Abstract)
- BRUNTON, D. F. and D. M. BRITTON. 1997. Appalachian quillwort (*Isoetes appalachiana*, sp. nov.; Isoetaceae), a new pteridophyte from the eastern United States. *Rhodora* 99:118–133.
- BRUNTON, D. F. and D. M. BRITTON. 1998. *Isoetes microvela* (Isoetaceae), a new quillwort from the coastal plain of the southeastern United States. *Rhodora* 100:261–275.
- BRUNTON, D. F. and D. M. BRITTON. 1999. Maritime quillwort, *Isoetes maritima* (Isoetaceae), in the Yukon Territory. *Canad. Field-Naturalist* 113:641–645.
- BRUNTON, D. F., D. M. BRITTON and W. C. TAYLOR. 1994. *Isoetes hyemalis*, sp. nov. (Isoetaceae): A new quillwort from the southeastern United States. *Castanea* 59:12–21.
- BRUNTON, D. F., D. M. BRITTON and T. F. WIEBOLDT. 1996. Taxonomy, identity, and status of *Isoetes virginica* (Isoetaceae). *Castanea* 61:145–160.
- SHARP, M. J. and D. M. BRITTON. 1991. *Isoetes tuckermanii*, Tuckerman's quillwort, an addition to the flora of Ontario. *Canad. Field-Naturalist* 105:283–285.
- TAYLOR, W. C., N. T. LUEBKE, D. M. BRITTON, R. J. HICKEY and D. F. BRUNTON. 1993. Isoetaceae Reichenbach—Quillwort Family. Pp. 64–75 in FNA Editorial Committee, eds. *Flora of North America North of Mexico, Volume 2*. Oxford: Oxford University Press.
- WHITTIER, D. P. and D. M. BRITTON. 1995. Gametophytes of *Diphasiastrum* × *habereri*. *Amer. Fern J.* 85:89–94.

2000s

- BRITTON, D. M. 2002. "In His Element". A reminiscence within "Obituary: Warren H. Wagner, Jr. (1920–2000)". *Amer. Fern J.* 92:46.
- BRUNTON, D. F. and D. M. BRITTON. 2000. *Isoetes* × *echtuckerii*, hyb. nov., a new triploid quillwort from northeastern North America. *Canad. J. Bot.* 77:1662–1668.
- BRUNTON, D. F. and D. M. BRITTON. 2006. *Isoetes melanopoda* spp. *silvatica* (subsp. nov.), a new quillwort (Isoetaceae) from eastern North America. *Castanea* 71:15–30.
- BRUNTON, D. F. and D. M. BRITTON. 2006. *Isoetes* × *novae-angliae* (Isoetaceae), an additional hybrid quillwort from New England. *Rhodora* 108:228–241.
- DES MARAIS, D. L., A. R. SMITH, D. M. BRITTON and K. M. PRYER. 2003. Phylogenetic relationships and evolution of extant horsetails, *Equisetum*, based on chloroplast DNA sequence data (*rbcL* and *trnL-F*). *Int. J. Pl. Sci.* 164:737–751.

APPENDIX 2. Pteridophyte taxa authored or co-authored by D. M. Britton.

Family	Taxon name	Citation
Dryopteridaceae	<i>Dryopteris</i> × <i>algonquinensis</i> D. M. Britton	Canad. Field-Naturalist 89: 165. 1975.
Isoetaceae	<i>Isoetes appalachiana</i> D. F. Brunt. & D. M. Britton	Rhodora 99: 129. 1997.
Isoetaceae	<i>Isoetes</i> × <i>echtuckeri</i> D. F. Brunt. & D. M. Britton	Canad. J. Bot. 77: 1667. 2000.
Isoetaceae	<i>Isoetes hillii</i> D. M. Britton	Amer. Fern J. 83: 128. 1993.
Isoetaceae	<i>Isoetes</i> × <i>jeffreyi</i> D. M. Britton & D. F. Brunt.	Canad. J. Bot. 70: 451. 1992.
Isoetaceae	<i>Isoetes junciformis</i> D. F. Brunt. & D. M. Britton	Amer. Fern J. 89: 193. 1999.
Isoetaceae	<i>Isoetes</i> × <i>marensis</i> D. M. Britton & D. F. Brunt.	Canad. J. Bot. 73: 1352–1353. 1995.
Isoetaceae	<i>Isoetes melanopoda</i> J. Gay & Durieu subsp. <i>silvatica</i> D. F. Brunt. & D. M. Britton	Castanea 7: 26. 2006.
Isoetaceae	<i>Isoetes</i> × <i>novae-angliae</i> D. F. Brunt. & D. M. Britton	Rhodora 108: 238. 2006.
Isoetaceae	<i>Isoetes prototypus</i> D. M. Britton	Canad. J. Bot. 69: 278. 1991.
Isoetaceae	<i>Isoetes</i> × <i>pseudotruncata</i> D. M. Britton & D. F. Brunt.	Canad. J. Bot. 74: 58. 1996.

APPENDIX 3. Pteridophyte taxa redefined/recircumscribed by D. M. Britton and D. F. Brunton.

Family	Taxon name	Citation
Isoetaceae	<i>Isoetes</i> × <i>dodgei</i> A. A. Eaton pro sp.	Canad. J. Bot. 67: 3001. 1989.
Isoetaceae	<i>Isoetes</i> × <i>harveyi</i> A. A. Eaton pro sp.	Canad. J. Bot. 69: 640. 1991.
Isoetaceae	<i>Isoetes</i> × <i>truncata</i> (A. A. Eaton) Clute pro sp.	Canad. J. Bot. 71: 1024. 1993.
Isoetaceae	<i>Isoetes hyemalis</i> D. F. Brunt.	Castanea 59: 13. 1994.
Isoetaceae	<i>Isoetes microvela</i> D. F. Brunt.	Rhodora 100: 270. 1998.
Isoetaceae	<i>Isoetes valida</i> (Engelm.) Clute	Amer. Fern J. 86: 23. 1996.
Isoetaceae	<i>Isoetes virginica</i> Pfeiffer	Castanea 61: 154. 1996.

APPENDIX 4. Pteridophyte taxa named in honor of D. M. Britton.

Family	Taxon name	Citation
Cystopteridaceae	<i>Gymnocarpium</i> × <i>brittonianum</i> (Sarvela) K. M. Pryer & Haufler	Syst. Bot. 18: 168. 1993.
Dryopteridaceae	<i>Dryopteris filix-mas</i> (L.) Schott subsp. <i>brittonii</i> Fraser-Jenk. & Widén	Advances Forest. Res. India 29. 2006.
Isoetaceae	<i>Isoetes</i> × <i>brittonii</i> D. F. Brunt. & W. C. Taylor	Amer. Fern J. 80: 85. 1990.

The Life of Barbara Joe Hoshizaki (1928–2012)

ROBBIN C. MORAN

The New York Botanical Garden, Bronx, NY 10458-5126, USA, e-mail: rmoran@nybg.org

Barbara Joe Hoshizaki (Fig. 1), past president and life member of the American Fern Society, died on 30 May 2012. She was one of the country's leading fern horticulturists. Besides the American Fern Society, Barbara served as president of the Southern California Horticultural Institute and the Los Angeles International Fern Society. She had also served as vice-president of the Pacific Horticultural Foundation. Furthermore, she was an honorary member of the Los Angeles International Fern Society and the Tropical Fern and Exotic Plant Society, Inc. Throughout her career she collaborated with researchers in academia and with commercial and amateur horticulturalists. Traveling widely, she studied ferns in their native habitats in North and South America, the Pacific Islands, Southeast Asia, Australia, New Zealand, and Africa. She introduced many fern species into cultivation and wrote scientific and popular papers on ferns. An avid taxonomist, she corrected the scientific names of many ferns that had been misidentified in the horticultural trade.

Born June 14, 1928, Barbara attended public schools in Los Angeles. In 1951 she received a BS from the University of California, Los Angeles. There she met Mildred Mathias, a professor of botany, who became Barbara's mentor and encouraged her to study ferns. In 1954 Barbara received an MS from UCLA and soon afterwards became a professor of biology at Los Angeles City College where she taught for 28 years. She was also Curator of Ferns at the UCLA Herbarium.

In 1967 Barbara spent eight weeks in Costa Rica on a fern course sponsored by the Organization for Tropical Studies (OTS). The course, taught by Warren H. Wagner, Jr., and John T. Mickel, introduced her to the diversity of tropical ferns and lycophytes, an experience she never forgot. She requested that, after her death, donations in her name be sent to OTS.

Barbara was best known for her book *Fern Growers Manual* (Knopf, 1975). This work, which treated about 390 species, served as a standard reference for ferns cultivated in the United States and Canada. A revised edition, which included about 700 species, was later published with Robbin C. Moran as co-author (Timber Press, 2001). It described and illustrated nearly all of the ferns and lycophytes commonly found in the horticultural trade in North America.

A personal reminiscence: While revising the *Fern Grower's Manual*, I spent three days with Barbara and her husband, Takashi, a plant physiologist, at their home in Los Angeles. Their greenhouse and yard encompassed a superb collection of living ferns, which at its peak harbored about 1000 species. The purpose of my visit was to compare our descriptions of fern species in the revised manuscript with the living ferns in her garden. At the end of the three days, we had not completed the task. The collection was so extensive that we



FIG. 1. Barbara Joe Hoshizaki at her home in Los Angeles, California (1999). (Photo by Robbin C. Moran.)

were able to examine only about four-fifths of the outdoor ferns—and we never made it to the greenhouse! During the visit, it was apparent that Barbara’s first-hand knowledge of ferns in her garden was remarkable. She often pointed out subtle distinctions between related species such as differences in shades of green of the leaves, how the leaves oriented themselves above ground, and seasonal timing in the production of leaves. I remember her telling me—much to my amazement as a “northerner”—that her biggest problem growing ferns was not cold weather but the fiercely hot and dry Santa Ana winds that barreled down from the San Bernardino mountains in the fall, sucking the moisture out of the soil and plants. Besides serving for research, Barbara’s garden was the source of plants that she generously sent to whoever requested a particular species.

Barbara will be greatly missed. A warm, gentle, easy-going person, she readily helped others with all aspects of fern horticulture. She did much to popularize ferns, especially as a sought-after public speaker for horticultural societies and garden clubs. The fern world has lost a dear friend.

BIBLIOGRAPHY OF BARBARA JOE HOSHIZAKI (1928–2012)

HOSHIZAKI, B. J. 1962. Summer fern foray in Oregon. *Amer. Fern J.* 52:166–167.

- HOSHIZAKI, B. J. 1968. Ferns and fertilizer. *Amer. Fern J.* 58:49–53.
- HOSHIZAKI, B. J. 1970. The genus *Adiantum* in cultivation (Polypodiaceae). *Baileya* 17:97–19, 145–191.
- HOSHIZAKI, B. J. 1970. Rhizome scales of *Platynerium*. *Amer. Fern J.* 60:144–160.
- HOSHIZAKI, B. J. 1972. Morphology and phylogeny of *Platynerium* species. *Biotropica* 4:93–117.
- HOSHIZAKI, B. J. 1973. A tree fern cultivar. *Amer. Fern J.* 63:21.
- HOSHIZAKI, B. J. 1973. Fern growing in southern California. *Calif. Hort. J.* 34:47–54.
- HOSHIZAKI, B. J. 1973. Ferns for subtropical gardens. *Fairchild Trop. Gard. Bull.* 28:4–9.
- HOSHIZAKI, B. J. 1975. A staghorn fern (*Platynerium*) hybrid. *Amer. Fern J.* 65:99–101.
- HOSHIZAKI, B. J. 1975. On growing staghorn ferns. *Fiddlehead Forum* 2:2.
- HOSHIZAKI, B. J. 1975. Fern propagation for the home grower. *Calif. Hort. J.* 36:158–162.
- HOSHIZAKI, B. J. 1975. Larry L. Kiefer (1935–1975). *Amer. Fern J.* 65:120–121.
- HOSHIZAKI, B. J. 1975. *Fern Grower's Manual*. Knopf, Inc, New York.
- HOSHIZAKI, B. J. 1976. Maidenhair ferns of California. *Fremontia* 4:17–20.
- HOSHIZAKI, B. J. 1977. Staghorn ferns today and tomorrow. *Garden's Bulletin*, Singapore 30:13–15.
- HOSHIZAKI, B. J. 1979. Getting started on ferns. *Pacific Hort. Winter*, pp. 44–46.
- HOSHIZAKI, B. J. 1980. Maidenhair ferns outdoors. *Pacific Hort. Winter*, pp. 43–44.
- HOSHIZAKI, B. J. 1981. The fern genus *Davallia* in cultivation (Davalliaceae). *Baileya* 21:1–42.
- HOSHIZAKI, B. J. 1981. *Davallia* relatives in cultivation: *Araiostegia*, *Davallodes*, *Humata*, and *Scyphularia* (Davalliaceae). *Baileya* 21:43–50.
- HOSHIZAKI, B. J. 1981. The genus *Pyrrosia* in cultivation (Polypodiaceae). *Baileya* 21:53–76.
- HOSHIZAKI, B. J. 1981. Review of "Trees and shrubs for dry California landscapes – plants for water conservation," by Bob Perry. *Crossosoma* 7:5–6.
- HOSHIZAKI, B. J. 1981. The genus *Polypodium* in cultivation (Polypodiaceae). *Baileya* 22:1–52.
- HOSHIZAKI, B. J. 1982. The genus *Polypodium* in cultivation (Polypodiaceae). *Baileya* 22:1–98.
- HOSHIZAKI, B. J. 1982. New Humatas in cultivation (Polypodiaceae). *Baileya* 22:99–100.
- HOSHIZAKI, B. J. 1984. Tree ferns. *Pacific Hort. Spring*, pp. 27–30.
- HOSHIZAKI, B. J. 1985. Review of "Ferns to know and grow," by John T. Mickel. *Quart. Rev. Biol.* 60:359–360.
- HOSHIZAKI, B. J. 1987. Review of "Encyclopedia of ferns," by David L. Jones. *Amer. Fern J.* 77:141–142.
- HOSHIZAKI, B. J. 1987. Ferns of the future. *Greenhouse Grower*. May, pp. 33–35.
- HOSHIZAKI, B. J. 1989. Review of "Pteridophyte flora of Oaxaca, Mexico," by John T. Mickel. *Pacific Hort. Winter*, pp. 16–17.
- HOSHIZAKI, B. J. 1990. Tree ferns. Pp. 162–166, In: G. Waters and N. Harlow, eds. *The Pacific horticulture book of western gardening*. David Godine, Inc.
- HOSHIZAKI, B. J. 1990. Allergic to ferns? *Fiddlehead Forum* 17:31.
- HOSHIZAKI, B. J. 1991. An "intergeneric" hybrid: *Aglaomorpha* × *Drynaria*. *Amer. Fern J.* 81:37–43.
- HOSHIZAKI, B. J. 1991. A *Goniophlebium* (*Polypodium*) hybrid. *Amer. Fern J.* 81:89–94.
- HOSHIZAKI, B. J. 1991. Scyphularias in cultivation. *South Florida Fern Society, Inc. March Bulletin*, 7–10.
- HOSHIZAKI, B. J. 1992. A *Pteris* hybrid. *L. A. I. F. S. [Los Angeles International Fern Society]* 19:47.
- HOSHIZAKI, B. J. 1992. Scyphularias in cultivation. *L. A. I. F. S. [Journal of the Los Angeles International Fern Society]* 19:48–52.
- HOSHIZAKI, B. J. 1992. *Nephrolepis* 'Monstrosum' finds its identify. *L. A. I. F. S. [Journal of the Los Angeles International Fern Society]* 19:64–65.
- HOSHIZAKI, B. J. 1992. The potential for new fern introductions. Pp. 97–103, In: Jennifer M. Ide, A. Clive Jermy and Alison M. Paul (eds.). *Fern Horticulture: Past, Present, and Future Perspectives*. Intercept, Andover, United Kingdom.
- HOSHIZAKI, B. J. 1993. Review of "Index to distribution maps of pteridophytes in Asia," ed. 2, by T. Nakaike. *Amer. Fern J.* 93:166–168.
- HOSHIZAKI, B. J. 2009. Review of "Illustrated flora of ferns and fern allies of South Pacific Islands," edited by T. Nakamura and S. Matsumoto. *Amer. Fern J.* 99:59–60.
- HOSHIZAKI and M. G. PRICE. 1990. *Platynerium* update. *Amer. Fern J.* 80:53–69.

- HOSHIZAKI and K. WILSON. 1999. *Dryopteris* in cultivation in the United States. *Amer. Fern J.* 89:1–100.
- JOE, B. 1958. *Pteris* species cultivated in California. *Lasca Leaves* 8:26–29.
- HOSHIZAKI, B. J. The fern ball in cultivation. *Amer. Fern J.* 48:72–74.
- HOSHIZAKI, B. J. 1959. Ferns cultivated in California: *Phyllitis*, *Doodia*, *Pyrrosia*, *Llavea*. *Lasca Leaves* 9:1–14.
- HOSHIZAKI, B. J. 1959. Ferns cultivated in California: *Woodwardia*, *Aglaomorpha*, *Pityrogramma*. *Lasca Leaves* 9:60–65.
- HOSHIZAKI, B. J. 1959. Ferns cultivated in California: *Cyrtomium*, *Onychium*, *Stenochlaena*. *Lasca Leaves* 9:61–67.
- HOSHIZAKI, B. J. 1959. Ferns cultivated in California: *Hypolepis*. *Lasca Leaves* 9:74–75.
- HOSHIZAKI, B. J. 1959. Ferns cultivated in California: *Lygodium*. *Lasca Leaves* 10:86–87.
- HOSHIZAKI, B. J. 1960. Ferns cultivated in California: *Sadleria*. *Lasca Leaves* 11:8–14.
- HOSHIZAKI, B. J. 1960. Species of *Blechnum* in cultivation. *Baileya* 8:102–117.
- HOSHIZAKI, B. J. 1961. Ferns cultivated in California: *Blechnum*, *Ctenitis*. *Baileya* 8:102–117.
- HOSHIZAKI, B. J. 1962. Species of *Microlepia* cultivated in California. *Lasca Leaves* 12:8–12.
- HOSHIZAKI, B. J. 1963. Species of *Thelypteris* cultivated in California. *Baileya* 11:99–110.
- HOSHIZAKI, B. J. 1963. Species of *Dryopteris* cultivated in California. *Baileya* 11:117–130.
- HOSHIZAKI, B. J. 1964. Notes and news: a new locality for *Asplenium vespertinum*. *Madroño* 17:172.
- HOSHIZAKI, B. J. 1964. Ferns cultivated in California: *Asplenium*. *Baileya* 12:19–27.
- HOSHIZAKI, B. J. 1964. Ferns cultivated in California: *Tectaria*. *Baileya* 12:47–51.
- HOSHIZAKI, B. J. 1964. A review of the species of *Platycterium* (Polypodiaceae). *Baileya* 12:137–146.
- HOSHIZAKI, B. J. 1964. Cultivated tree ferns of the genus *Cibotium* (Dicksoniaceae). *Baileya* 12:69–126.
- HOSHIZAKI, B. J. 1965. Cup ferns (*Dennstaedtia*) cultivated in California. *Amer. Fern J.* 55:58–62.
- KIEFER, L. L. and B. JOE. 1967. Check list of California pteridophytes. *Madroño* 3:65–73.
- MATHIAS, M. E., F. H. LEWIS and B. HOSHIZAKI. 1977. Key to the vascular plant families of southern California. University of California, Los Angeles.

Low Within Population Genetic Variation and High Among Population Differentiation in *Cyrtomium falcatum* (L.f.) C. Presl (Dryopteridaceae) in Southern Korea: Inference of Population-Establishment History

MI YOON CHUNG

Department of Biology, Gyeongsang National University, Jinju 660-701, Republic of Korea

JORDI LÓPEZ-PUJOL

BioC-GReB, Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona, Barcelona 08028, Spain

JAE MIN CHUNG

Department of Plant Resources Conservation, Korea National Arboretum, Pocheon 487-821, Republic of Korea

KI-JOONG KIM

School of Life Sciences, Korea University, Seoul 136-701, Republic of Korea

MYONG GI CHUNG¹

Department of Biology and the Research Institute of Natural Science, Gyeongsang National University, Jinju 660-701, Republic of Korea

ABSTRACT.—In the Korean Peninsula, the current distribution of the warm-temperate and subtropical vegetation (including many homosporous ferns) is limited to southern coastal areas. Paleoecological data suggest that during the Last Glacial Maximum this vegetation retreated to glacial refugia putatively located in southern Japan and/or southern China, followed by a post-glacial recolonization. Two broad scenarios of post-glacial recolonization could be hypothesized: extant Korean populations are derived from multiple source populations (i.e., from multiple refugia); alternatively, they originate from a single refugium. To test which of these scenarios is more likely, we surveyed patterns of genetic diversity in eight ($n = 307$) populations of *Cyrtomium falcatum* from southern Korea. We found extremely low levels of allozyme variation within populations coupled with high among-population differentiation. These data best support the second hypothesis, and indicate that the current genetic diversity may be a consequence of post-glacial long-distance dispersal events and subsequent founder effects. In addition, restricted gene flow among the discontinuous populations of *C. falcatum* in southern Korea has likely contributed to the high degree of among-population genetic differentiation. From a conservation perspective, several populations should be targeted for both *in situ* and *ex situ* conservation, as *C. falcatum* exhibits a high degree of divergence among populations.

KEY WORDS.—Dryopteridaceae, *Cyrtomium*, allozymes, conservation, founder effect, glacial refugia, homosporous fern, gametophytic selfing, population history, population structure

Genetic diversity patterns of plant species are shaped by interacting historical, biological, ecological, and demographic factors (Nevo *et al.*, 1984;

¹ Author for correspondence. Tel: +82 055 772 1343, Fax: +82 055 772 1349, email: mgchung@nongae.gsnu.ac.kr

Hamrick and Godt, 1989; Gray, 1996; Duminil *et al.*, 2007). From a historical viewpoint, the Quaternary glacial-interglacial oscillations played an increasingly recognized role in shaping the current distribution of plant species and thus, their contemporary levels and partitioning of genetic diversity within and among populations (Hewitt, 1999, 2000; Hu *et al.*, 2009). For example, populations/species that occurred in formerly glaciated regions usually show lower levels of genetic diversity than those from unglaciated areas (e.g., glacial refugia) through founder effects and bottlenecks as a result of multiple stepwise colonization events (Hewitt, 1996; Widmer and Lexer, 2001; Jiménez *et al.*, 2010). Thus, the patterns of genetic diversity maintained by the species (especially the spatial distribution of genotypes) are often used to infer the location of refuges and the post-glacial migration routes from these, and this has been particularly fruitful in Europe and North America (Soltis *et al.*, 2006; Weiss and Ferrand, 2007, Hu *et al.*, 2009; Hewitt, 2011).

Ferns have some life-history traits that are strikingly different from seed plants and that have potentially significant effects on patterns of population genetic variation. First, fern dispersal occurs via haploid spores. Second, their gametophytic generation is independent from the maternal sporophytes. Third, owing to their small size, fern spores tend to be dispersed much farther by wind compared to most seeds (Tryon, 1970, 1972), although this feature is analogous to the tiny, dust-like seeds of orchids (Arditti and Ghani, 2000). As in many seed plants, however, the majority of propagules fall around the immediate vicinity of the parent (Peck *et al.*, 1990). Fourth, since spermatozoids require transport in water, male gamete dispersal distance of ferns tends to be very limited (within a few centimeters; Peck *et al.*, 1990). Finally, in many homosporous ferns, in the absence of genetic load a single spore could produce a sporophyte via intragametophytic selfing (self-fertilization of a haploid gametophyte), enabling the successful colonization of new sites (Lloyd, 1974; Flinn, 2006; Edgington, 2007; Wubs *et al.*, 2010). Intragametophytic selfing results, in a single generation, in completely homozygous sporophytes (Klekowski, 1972; Vogel *et al.*, 1999a), a situation without analogue in seed plants. Thus, it has been suggested that genetically polymorphic populations could be attributed to the occurrence of multiple independent spore dispersal and establishment events over time, whereas genetically monomorphic homosporous fern populations are more likely to have arisen from single colonists (i.e., single spores; Pryor *et al.*, 2001).

Habitat specificity and recurrent gene flow of homosporous ferns should be regarded as factors determining the degree of population differentiation. Soltis *et al.* (1989) hypothesized that xeric or rock dwelling ferns would exhibit higher among-population differentiation than would ferns occurring in mesic habitats, due to limited gene flow among isolated rocky habitats. Since then, several population-genetics studies have supported this hypothesis (Pryor *et al.*, 2001 and references therein).

In Korea, many ferns are characteristic of the warm-temperate and subtropical vegetation, such as *Cyrtomium falcatum* (L.f.) C. Presl (Dryopteridaceae), a rock dwelling homosporous fern native to southern and eastern

Asia, which is taken here as a case study. This vegetation belt currently occurs in a narrow zone along the southeastern and southern coast (Yi, 2011). The few available pollen and spore records suggest, however, that this warm-temperate vegetation was likely absent from the Korean Peninsula during the Last Glacial Maximum (LGM, ca. 21,000 yr ago; e.g., Choi, 1998; Chung *et al.*, 2006; Chung, 2007; Chung *et al.*, 2010; Yi and Kim, 2010), a scenario consistent with regional vegetation reconstructions (e.g., Adams and Faure, 1997; Harrison *et al.*, 2001; Hope *et al.*, 2004; Prentice *et al.*, 2011). On the southern coast of Korea, the onset of the Holocene (ca. 11,000 years BP) and the accompanying climatic amelioration were marked by a sudden increase in abundance of ferns in the family Polypodiaceae, and an abrupt decline in herbaceous taxa, together with the expansion of cool temperate deciduous broad-leaved forests (Chung *et al.*, 2010). The first appearance after the LGM of broad-leaved evergreen vegetation in the Korean Peninsula was approximately 8,500 years ago (Chung, 2011) and somewhat earlier in Jeju Island (ca. 12,000–10,000 yr BP; Chung, 2007), which also coincided with a rise of fern spores, indicating warmer and more humid conditions (Chung *et al.*, 2010). These paleovegetation studies suggest that post-glacial colonization either from southern Japan (e.g., Kyushu; Fig. 1) or southern China, which harbored glacial refugia for warm-temperate vegetation (e.g., Hope *et al.*, 2004; Gotanda and Yasuda, 2008; López-Pujol *et al.*, 2011; Qiu *et al.*, 2011), would be much more plausible than persistence of warm-temperate and subtropical vegetation in Korean refugia during the Pleistocene glaciations.

Cyrtomium falcatum is an evergreen homosporous fern that usually grows on coastal rocky slopes in the warmer parts of south to northeastern Asia (India, Vietnam, eastern and southern China, Taiwan, southern Korea, and Japan; Iwatsuki, 1992). However, it has become naturalized in many parts of the world (including Hawaii, North America, Australia, western and southern Europe, Réunion Island, and South Africa) because it escaped from gardens (Roux, 2011). The species, 10–60 cm tall, has a short, erect rhizome, and thus, it is highly likely that proximally located individuals within populations are distinct genets. In southern Korea, *C. falcatum* usually grows on crevices in steep cliffs, rocks, and man-made vertically oriented stone walls near seashores, and thus, populations occur discontinuously. Chromosome numbers of $n = 41$ (diploid) or $n = 82$ (tetraploid) have been reported for *C. falcatum* in Japan (Iwatsuki, 1992).

Based on the life-history and ecological traits of homosporous ferns, together with the information available on the paleoecology of the Korean Peninsula, we hypothesize two broad scenarios for the origin of current populations of warm-temperate homosporous fern species in southern Korea. If contemporary populations were derived from multiple source populations (i.e., from multiple glacial refugia), presumably from southern Japan and/or southern China, we would expect high levels of within-population genetic variation as consequence of the admixture of genetically divergent lineages arriving from different refugia (i.e., the ‘melting pot’ effect that has been described for many European trees and shrubs; Petit *et al.*, 2003). Regarding among-population

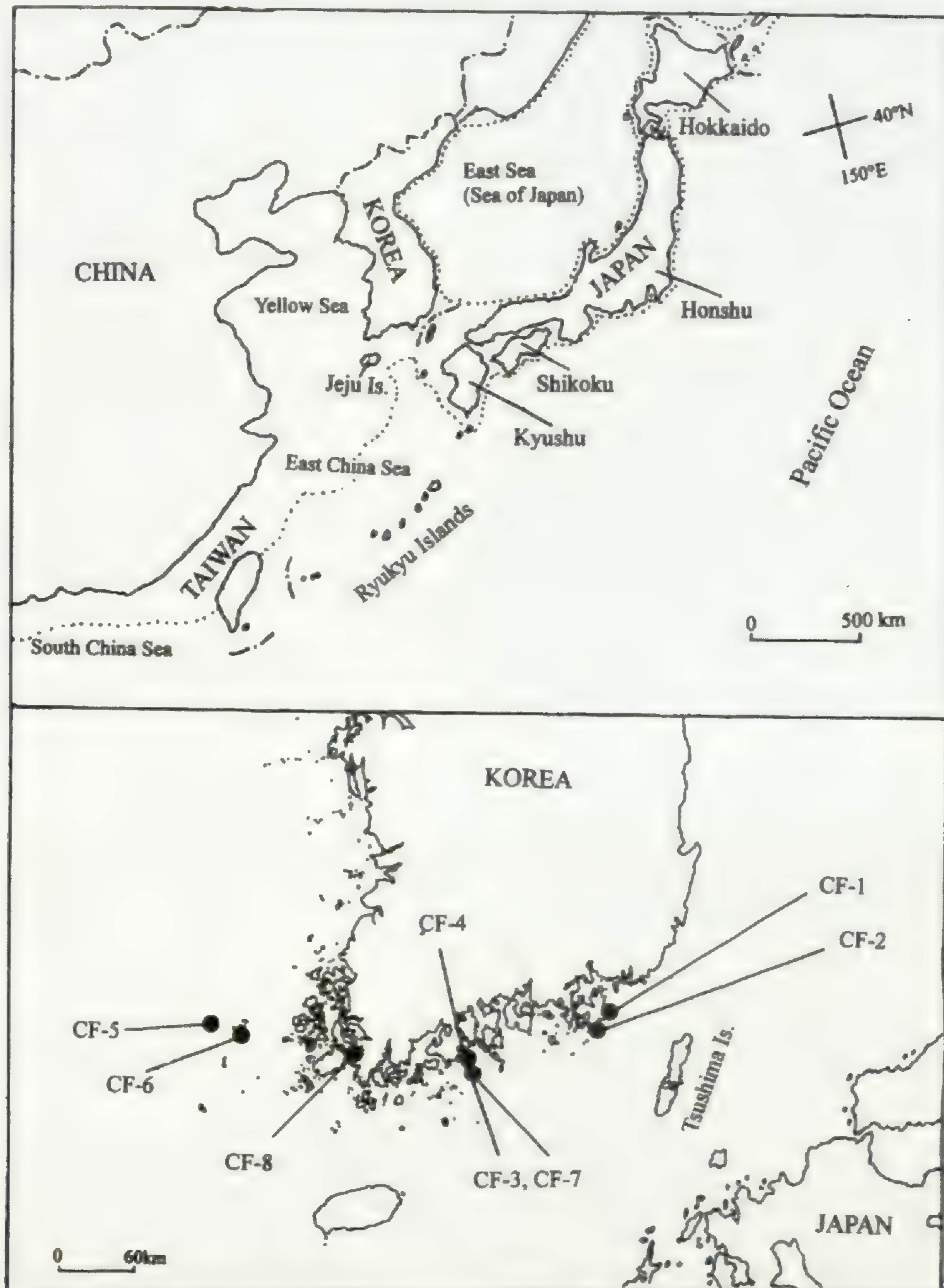


FIG. 1. Upper figure: map of northeastern Asia. Dotted line indicates exposed coastal lines during the Last Glacial Maximum (Shi, 2002; Xu *et al.*, 2010). Lower figure: collection sites of *Cyrtomium falcatum* in southern Korea; CF-1 and CF-2 in Geojae Island. CF-3 and CF-7 (each separated by ca. 1.5 km) in Oenaro Island; CF-4 in Naenaro Island; CF-5 in Hong Island; CF-6 in Heuksan Island; and CF-8 in Haenam-gun (mainland Korea).

genetic differentiation, either low or high values would be exhibited depending on ecological factors (Hamrick and Nason, 1996). Large populations that are continuously distributed should exhibit low inter-population variation probably due to high recurrent gene flow between adjacent populations. In contrast, high genetic divergence would be expected among small disjunct populations because of low rates of gene flow between isolated

TABLE 1. Summary of genetic diversity measures and mean fixation values (F_{IS}) observed in eight populations of *Cyrtomium falcatum*.

Population	<i>n</i>	% <i>P</i>	<i>A</i>	<i>AR</i>	H_o (SE)	H_e (SE)	F_{IS}
CF-1	36	14.3	1.14	1.14	0.044 (0.053)	0.043 (0.042)	-0.071
CF-2	36	14.3	1.14	1.12	0.039 (0.042)	0.046 (0.049)	0.154
CF-3	51	9.5	1.10	1.08	0.024 (0.036)	0.019 (0.028)	-0.270 ^a
CF-4	42	14.3	1.14	1.14	0.028 (0.026)	0.027 (0.025)	-0.037
CF-5	23	28.6	1.29	1.28	0.050 (0.041)	0.071 (0.050)	0.299 ^a
CF-6	18	4.8	1.05	1.05	0.019 (0.030)	0.018 (0.030)	-0.008
CF-7	76	9.5	1.10	1.10	0.044 (0.057)	0.044 (0.049)	0.001
CF-8	25	0.0	1.00	1.00	0.000 (0.000)	0.000 (0.000)	na
Average	38	11.9	1.12	1.11	0.031 (0.006)	0.034 (0.008)	0.030 ^b
Pooled samples	307	38.1	1.38		0.033 (0.018)	0.069 (0.034)	
Homosporous ferns ^c		36.1	1.63			0.132	

Abbreviations: *n*, sample size; %*P*, percentage of polymorphic loci; *A*, mean number of alleles per locus; *AR*, mean allelic richness based on a minimum sample size of 18 individuals; H_o , observed heterozygosity; H_e , Hardy-Weinberg (H-W) expected heterozygosity or gene diversity; SE, standard error; F_{IS} , fixation index within populations; na, not available (because of monomorphism across all the loci examined in this population).

^a Denotes significance ($P < 0.05$) based on permutation (999 replicates) under the null hypothesis of $F_{IS} = 0$.

^b Non-significant Weir and Cockerham (1984) estimate of F_{IS} over populations.

^c Allozyme-based genetic data from Tables 7 and 8 in Li and Haufler (1999).

populations. Alternatively, if extant populations were established from colonizers coming from a single source (i.e., a single refugium), then within-population genetic variation would be low because of long-distance dispersal associated bottlenecks (e.g., Hewitt, 1996, 2000). Genetic differentiation among populations would be high or low depending on rates of contemporary gene flow among the Korean populations. To date, these colonization hypotheses have not been empirically tested for the warm-temperate and subtropical homosporous ferns native to the Korean Peninsula. In this study, we surveyed the levels and distribution of allozyme-based genetic diversity in *C. falcatum* to test which of the post-glacial colonization hypotheses is most likely. Achieving a better understanding of the genetic structure of this currently rare fern in the Korean Peninsula, in addition, will provide guidelines for its recovery and management.

MATERIALS AND METHODS

Sample collection.—We collected one leaf segment (pinna) from each individual to minimize damage to the plants. A total of 307 individuals were sampled from eight populations of *C. falcatum* from southern Korea, including several islands (Fig. 1 and Table 1). All sampled leaf tissue was kept on ice until its transportation to the laboratory, where it was stored at 4 °C until enzyme extraction.

Allozyme electrophoresis.—We extracted enzymes by finely cutting leaf samples, adding an extraction buffer (Mitton *et al.*, 1979), and then crushing them with a mortar and pestle. Enzyme extracts were absorbed onto chromatography wicks and stored in microtiter plates in an ultra-cold (-70°C) freezer until analyzed. We conducted electrophoresis on 13% starch gels, with three buffer systems. We used a modification (Haufler, 1985) of system 6 of Soltis *et al.* (1983) to resolve alcohol dehydrogenase (*Adh*), diaphorase (*Dia-1*, *Dia-2*), fluorescent esterase (*Fe-1*, *Fe-2*), and cathodal peroxidase (*Cpx*). We used system 11 of Soltis *et al.* (1983) to resolve glyceraldehyde-3-phosphate dehydrogenase (*G-3-pdh-1*, *G-3-pdh-2*), hexokinase (*Hk-1*, *Hk-2*), isocitrate dehydrogenase (*Idh*), phosphoglucosomerase (*Pgi-1*, *Pgi-2*), phosphoglucosomutase (*Pgm-1*, *Pgm-2*, *Pgm-3*), and shikimate dehydrogenase (*Skdh*). In addition, we used the morpholine-citrate buffer system (pH 6.1) of Clayton and Tretiak (1972) to resolve fructose-1,6-diphosphatase (*F1,6*) and malate dehydrogenase (*Mdh-1*, *Mdh-2*, *Mdh-3*). We followed stain recipes from Soltis *et al.* (1983) except for diaphorase (Cheliak and Pitel, 1984). We designated putative loci sequentially, with the most anodally migrating isozyme designated as 1, the next 2, and so on. We also designated different alleles within each locus sequentially by *a*, the next *b*, and so on. The observed enzyme banding patterns were consistent with their typical subunit structure and subcellular compartmentalization in diploid plants (Weeden and Wendel, 1989).

Data analysis.—We considered a locus to be polymorphic when two or more alleles were observed, regardless of their frequencies. We estimated the genetic diversity parameters within populations using the programs POPGENE (Yeh *et al.*, 1999) and FSTAT (Goudet, 1995): percent polymorphic loci ($\%P$), mean number of alleles per locus (*A*), allelic richness (*AR*) corrected by minimum sample size ($n = 18$ at CF-6, the population with the smallest sample size), observed heterozygosity (H_o), and Hardy-Weinberg (H-W) expected heterozygosity or Nei's (1978) gene diversity (H_e). Except for *AR* and H_o , these parameters were also estimated for the total samples as a whole (i.e., at the species level). To test for recent decreases in effective population size (bottlenecks), we evaluated differences across loci between the H-W H_e and the equilibrium heterozygosity (H_{eq}) expected assuming mutation-drift equilibrium. H-W H_e is not very sensitive to the fate of low frequency alleles, whereas H_{eq} is relatively sensitive to population bottlenecks, and declines as a result of the loss of such alleles. These differences ($H_e - H_{eq}$ calculated for a number of independent loci) were evaluated using a sign test and a Wilcoxon sign-rank test under an infinite allele model using the program BOTTLENECK (Piry *et al.*, 1999). Since allelic diversity is generally lost more rapidly than H_e (Nei *et al.*, 1975), recently bottlenecked populations will exhibit an excess of H-W H_e relative to H_{eq} (Cornuet and Luikart, 1996; Luikart *et al.*, 1998).

We used the program SPAGeDi (Hardy and Vekemans, 2002) to calculate population-level F_{IS} (inbreeding) and its significance level by 999 permutations under the null hypothesis of $F_{IS} = 0$. To measure deviations from H-W equilibrium at each polymorphic locus, we calculated averages of Wright's

TABLE 2. Results of statistical tests for evidence of recent population bottlenecks in *Cyrtomium falcatum*. Numbers reported are *P*-values of sign and Wilcoxon sign-rank tests conducted using the program BOTTLENECK.

Population	Sign test	Wilcoxon sign-rank test
CF-1	0.406	0.188
CF-2	0.442	0.188
CF-3	0.659	0.875
CF-4	0.605	0.938
CF-5	0.445	0.578
CF-6	0.519	0.250
CF-7	0.162	0.125
CF-8	na	na

(1965) F_{IS} and F_{ST} (deviations from H-W equilibrium of individuals relative to their local populations and local populations relative to the total population, respectively) following Weir and Cockerham (1984). Using FSTAT, we constructed 95% bootstrap confidence intervals (CI; 999 replicates) around means of F_{IS} and F_{ST} , and considered the observed F_{IS} and F_{ST} to be significant when the 95% CI did not overlap zero.

To test the overall pattern of genetic structure at the regional scale (i.e., isolation-by-distance effects), we conducted a Mantel test (Mantel, 1967) with 999 replicates, between all pairwise $F_{ST}/(1 - F_{ST})$ (F_{ST} was calculated following Weir and Cockerham, 1984) and the corresponding logarithm pairwise geographical distance (Rousset, 1997) under the null hypothesis of no spatial genetic structure (regression slope, $\beta = 0$). Finally, to determine the degree of genetic divergence among populations of *C. falcatum*, we calculated Nei's (1978) unbiased genetic identity (I) and distance (D) between pairs of populations. Using Nei's D values, we clustered populations into a phenogram following unweighted pair-group method using arithmetic averages (UPGMA).

RESULTS

Allozyme variation within populations.—Of the 21 putative loci resolved for *C. falcatum*, eight were polymorphic (*Dia-1*, *F1,6*, *Fe-1*, *Fe-2*, *Hk-2*, *Idh*, *Pgm-2*, and *Pgm-3*). Allozyme variation within populations was extremely low across the eight studied populations: mean percentage of polymorphic loci within populations ($\%P$) was 11.9, mean number of alleles per locus (A) was 1.12, and mean genetic diversity (H_e) was 0.034 (Table 1). Population CF-5 harbored the highest allelic richness and genetic diversity ($AR = 1.28$ and $H_e = 0.071$; Table 1), whereas no allozyme variation was found in CF-8 (Table 1). Slightly higher levels of genetic diversity were estimated from pooled samples over all populations ($n = 307$): $\%P = 38.1$; $A = 1.38$; and $H_e = 0.069$ (Table 1). Although we did not conduct any bottleneck test on CF-8 because it had no allozyme polymorphism, we found no significant indications of recent bottlenecks in any of the remaining seven populations (Table 2).

TABLE 3. Allele frequencies for the three loci with the highest degree of population differentiation (*F1,6*, *Pgm-2*, and *Pgm-3*).

Population	Allele frequency					
	<i>F1,6</i>		<i>Pgm-2</i>		<i>Pgm-3</i>	
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
CF-1	0.000	1.000	0.194	0.806	0.931	0.069
CF-2	0.000	1.000	0.514	0.486	0.292	0.708
CF-3	0.000	1.000	0.980	0.020	0.765	0.235
CF-4	0.000	1.000	0.083	0.917	0.083	0.917
CF-5	0.565	0.435	0.109	0.891	0.000	1.000
CF-6	0.750	0.250	1.000	0.000	0.000	1.000
CF-7	0.000	1.000	0.704	0.296	0.480	0.520
CF-8	1.000	0.000	0.000	1.000	0.000	1.000

Population genetic structure.—Except for CF-3 and CF-5, population-level F_{IS} estimates were not significantly different from zero at the 0.05 level (Table 1). These results, as well as the non-significant multi-population-level F_{IS} ($F_{IS} = 0.030$; Table 1 and 95% CI = -0.254 to 0.401), indicated that populations were generally at H-W equilibrium. Deviations from H-W expectations due to allele frequency differences between populations were, in contrast, significantly high ($F_{ST} = 0.543$, 95% CI = 0.218 to 0.703). This level of among-population differentiation was largely due to skewed allele frequencies at the three loci *F1,6*, *Pgm-2*, and *Pgm-3* (Table 3).

Pairwise Nei's (1978) I values between populations were high, ranging from 0.878 (CF-3 vs. CF-8) to 0.997 (CF-2 vs. CF-7) and with a mean of 0.951 ± 0.011 (SD), which is comparable with the average values reported for other conspecific populations of homosporous pteridophytes (average $I = 0.911 \pm 0.086$, $N = 16$; Soltis and Soltis, 1989) and of plants overall (average $I = 0.950 \pm 0.059$, $N = 1,572$; van der Bank *et al.*, 2001). The apparent discordance between the high values of F_{ST} and the high values of I in *C. falcatum* is simply due to the fact that only polymorphic loci are used for the calculation of F_{ST} , whereas both monomorphic and polymorphic loci are employed for estimating pairwise Nei's I . The UPGMA phenogram showed that the eight populations were clustered largely in accordance with their geographical locations: CF-1/CF-2 and CF-5/CF-6/CF-8 (which are located in the eastern and western extremes of southern part of Korea, respectively) were clustered separately (Fig. 2). However, we found no significant correlation between pairwise genetic differentiation estimates and their corresponding between-population logarithm pairwise geographical distance ($\beta = 0.069$, $R^2 = 0.016$, $P = 0.277$; Fig. 3), indicating that most variation (ca. 98%) in genetic differentiation was due to factors other than geographic distance.

DISCUSSION

Genetic diversity and structure.—Levels of within-population genetic diversity are extremely low in *C. falcatum* (mean population-level estimates;

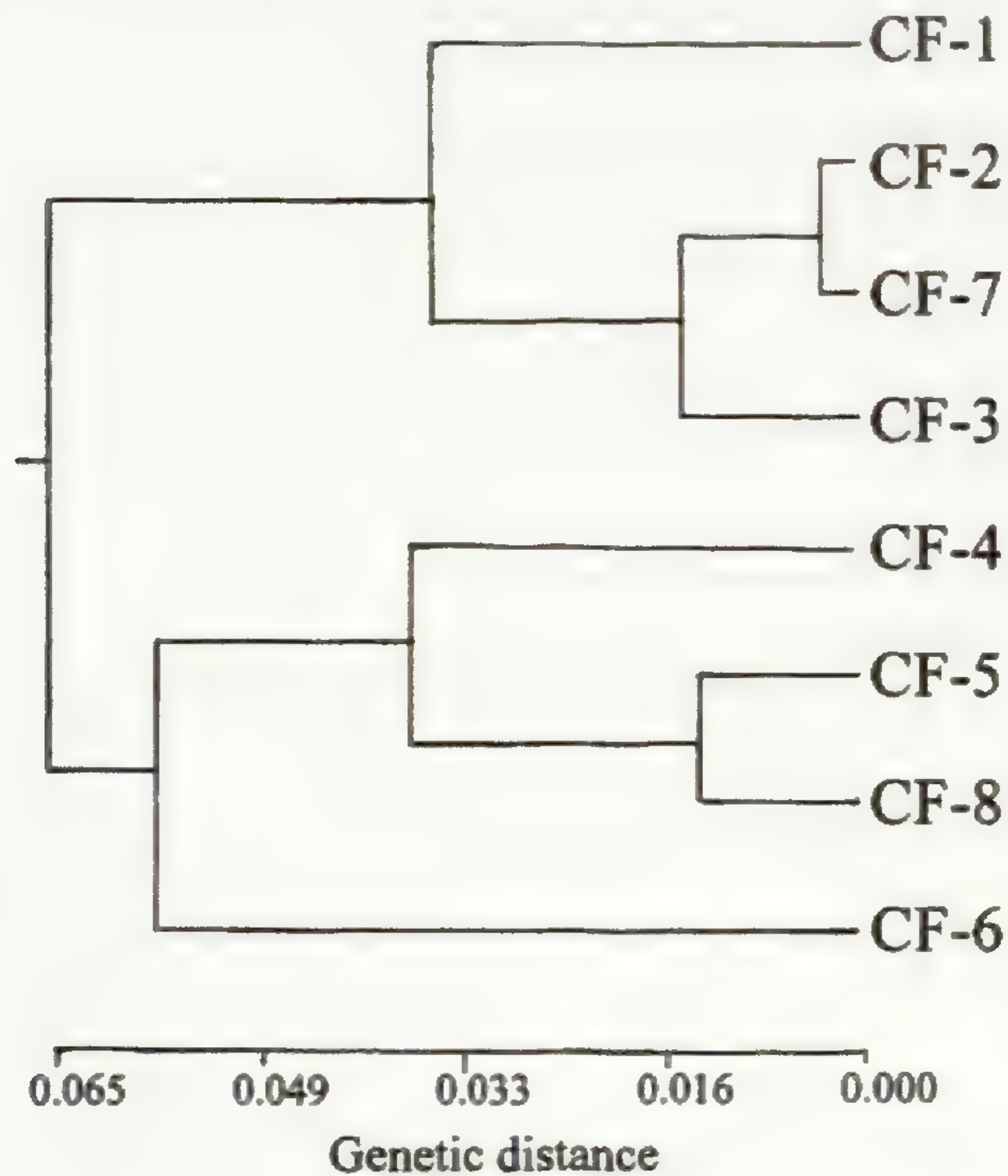


FIG. 2. UPGMA phenogram based on Nei's genetic distances between populations of *Cyrtomium falcatum*.

$%P = 11.9$, $A = 1.12$, $H_e = 0.034$). Although slightly higher values for these genetic diversity measures were obtained from pooled samples over all populations ($%P = 38.1$, $A = 1.38$, $H_e = 0.069$), A and H_e are still lower than expected for homosporous ferns (mean species-level estimates; $%P = 36.1$, $A = 1.63$, $H_e = 0.132$; Li and Haufler, 1999). The low levels of genetic variation in the southern Korean populations may be a consequence of post-glacial long-distance dispersal events and subsequent founder effects (see below for a detailed discussion).

Populations of *C. falcatum* were generally at H-W equilibrium (multi-population level $F_{IS} = 0.030$), a relatively unexpected finding since many homosporous ferns have potential for intragametophytic selfing (Klekowski and Baker, 1966), which could cause a substantial deviation from H-W equilibrium (i.e., a deficit of heterozygotes) within populations. Consistent with this expectation, a considerable excess of homozygotes has been found within populations of species of *Botrychium* and *Mankyua* (Ophioglossaceae), which have subterranean gametophytes that obligately self-fertilize via intragametophytic selfing (McCauley *et al.*, 1985; Soltis and Soltis, 1986; Watano and Sahashi, 1992; Hauk and Haufler, 1999; M. Y. Chung *et al.*, 2010). However, many diploid homosporous ferns exhibit high outbreeding rates (as inferred from inbreeding coefficients; Soltis and Soltis, 1989, 1992; Ranker and Geiger, 2008), and some studies have suggested that they possess mechanisms that promote outcrossing in natural populations (Klekowski, 1973; Haufler and Gastony, 1978; Haufler and Ranker, 1985; Wubs *et al.*, 2010). Some of these mechanisms promote the formation of functionally unisexual gametophytes

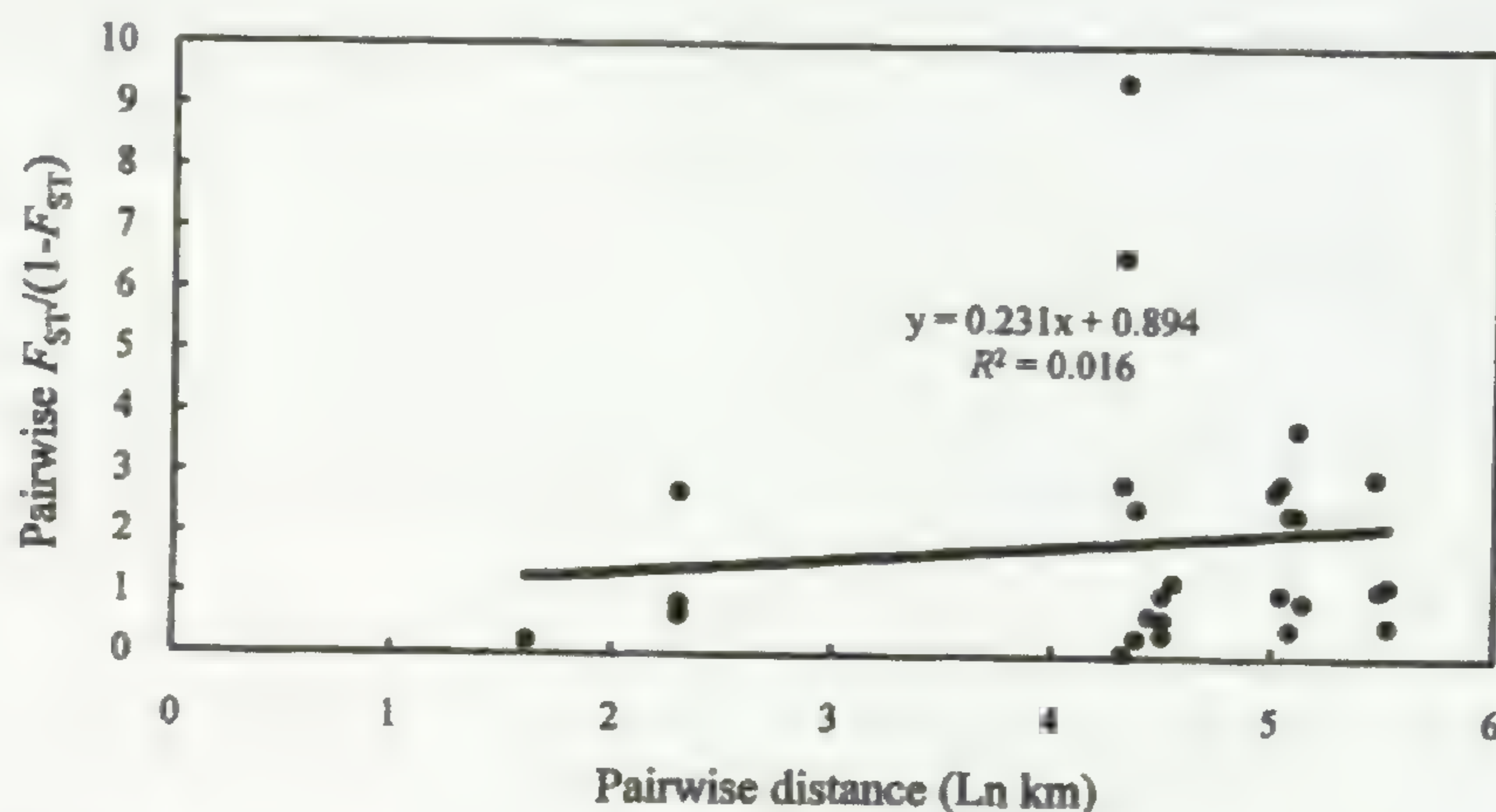


FIG. 3. Differentiation between populations of *Cyrtomium falcatum*. Multilocus estimates of pairwise differentiation of $F_{ST}/(1 - F_{ST})$ are plotted against pairwise logarithm (Ln) geographical distances in kilometers according to Rousset (1997). There was a non-significant positive relationship between pairwise $F_{ST}/(1 - F_{ST})$ and pairwise Ln geographical distance ($r = 0.126$, $P = 0.277$).

through the asynchronous maturation of male and female gametes and the control of antheridia initiation by the pheromone antheridiogen produced by maturing female gametophytes (Döpp, 1950; Lloyd, 1974; Haufler and Welling, 1994; Pajarón *et al.*, 1999). This seems to apply for populations of *C. falcatum*, although we do not know which of the above-mentioned mechanisms is promoting outcrossing in the Korean populations of this fern.

Outcrossing plant species usually maintain most of their genetic variation within rather than among populations, whereas selfing species show the reverse trend (Brown, 1979; Hamrick *et al.*, 1979). Thus, because populations of *C. falcatum* exhibit high inter-population divergence ($F_{ST} = 0.543$), factors other than mating system are probably important in shaping genetic structure among populations of *C. falcatum*. A high degree of genetic differentiation among populations has been observed in other homosporous ferns, including *Adiantum capillus-veneris* (Pryor *et al.*, 2001), *Asplenium csikii* (Vogel *et al.*, 1999b), *Asplenium ruta-muraria* (Schneller and Holderegger, 1996), *Asplenium septentrionale* (Holderegger and Schneller, 1994), *Asplenium trichomanes* subsp. *quadrivalens* (Suter *et al.*, 2000), *Cheilanthes gracillima* (Soltis *et al.*, 1989), and *Sadleria cyatheoides* and *S. pallida* (Ranker *et al.*, 1996). For all these cases, patchiness of suitable habitat (which caused restricted gene flow) has been proposed as a major driver of population divergence. This habitat trait may also account for the high among-population differentiation found in *C. falcatum* in southern Korea, which is primarily due to allele frequency differences at three loci (Table 3). For example, six of eight populations were monomorphic at *F1,6*; of these, the CF-8 population was fixed for the allele *a*, whereas the other five populations were fixed for the allele *b*. At *Pgm-2*, CF-6 was fixed for the allele *a*, whereas CF-8 was fixed for the alternative allele *b*. Apart from the low levels of gene flow, genetic drift would have been enhanced by small population sizes. Although current populations are of moderate size (M. Y. Chung and M. G. Chung, pers. observ.)

and we did not find any indications of recent bottlenecks (BOTTLENECK is only able to detect those bottlenecks that have occurred within approximately the past $2N_e$ – $4N_e$ generations; Piry *et al.*, 1999), the possibility of older bottlenecks should not be dismissed.

Inference of colonization history of C. falcatum in the southern Korean Peninsula.—Since *C. falcatum* is a member of the warm-temperate and subtropical vegetation community, and the Korean populations are at the northern edge of the species' geographic range, one may hypothesize that it endured the Quaternary glacial periods at more southerly latitudes. Glacial refugia for many elements of warm-temperate and subtropical flora have been proposed to occur in southern Kyushu and also in southern Honshu, Japan (see Fig. 1; Tsukada, 1984; Hattori, 1985; Matsuoka and Miyoshi, 1998; Aoki *et al.*, 2004; Gotanda and Yasuda, 2008). Fern spores usually have a high dispersal potential; ca. 500 ~ 800 km and even 3,200 km are suggested as maximum spore-dispersal distances (Tryon, 1970, 1972). The Tsushima (Korean) Strait was only about 10–20 km wide during the LGM and remained relatively narrow until ca. 14,000–12,000 yr BP (Park *et al.*, 2000; Lee *et al.*, 2008) being therefore easily passable. Even during the Holocene the 200 km channel width would have not constituted an insurmountable barrier for spore dispersal. Current populations of *C. falcatum* could also have arrived from the more distantly located southern regions, as the East China Sea (ECS) was largely exposed until at least 10,000 yr BP (Xu *et al.*, 2010). Therefore, migrations from southern China, Taiwan or even from some locations offshore in the southern part of the ECS cannot be ruled out (see Harrison *et al.*, 2001; Hope *et al.*, 2004; Prentice *et al.*, 2011).

The low within-population genetic variation for *C. falcatum* argues against the multiple-refugia hypothesis and supports the second hypothesis that the contemporary Korean populations of *C. falcatum* are descendant from colonizers from a single glacial refugium, presumably from southern Japan and/or southern China. However, we should bear in mind that these two proposed scenarios (multiple vs. single source populations) are the two extremes of a spectrum of possibility (e.g., some of the extant Korean populations could come from a single source, whereas others could originate from the admixture of several lineages). Moreover, many factors could have altered and/or modeled these “ideal” patterns, such as the number of colonization events, the number of propagules arriving at each colonization event, and the occurrence of genetic bottlenecks. For example, if population sizes have been historically small, random genetic drift since the post-glacial colonization events would have lead to low levels of intrapopulation genetic diversity even if the populations originated from multiple sources. In this latter case, patterns of genetic variation will be hardly distinguishable from those expected for species that immigrated from a single refugium. Clearly, more species (especially those continuously distributed) should be studied to draw firm conclusions about the post-glacial colonization history of warm-temperate homosporous fern populations currently occurring in Korea.

A similar scenario of glacial survival in remote refugia and post-glacial recolonization has been proposed for the homosporous fern *Dryopteris*

aemula. Jiménez *et al.* (2009) reported a total lack of allozyme variation ($H_T = 0.000$) of this fern in the Iberian Peninsula, which was attributed to founder effects during the Holocene expansion. Later, using five microsatellite loci and adding one population from the Macaronesian archipelago of Azores, Jiménez *et al.* (2010) found low levels of genetic variation within populations (total heterozygosity, $H_T = 0.447$) and a high degree of population genetic differentiation ($F_{ST} = 0.520$) in *D. aemula*. Interestingly, the Macaronesian population was much more variable than the Iberian ones and, based on these findings, the authors suggested that the Azores acted as a glacial refugium from which *D. aemula* spread northeastward and recolonized mainland Europe (Jiménez *et al.*, 2010). The role of glacial refugia as sources of plant diversity for the post-glacial recolonization in Europe of the Macaronesian Islands has been acknowledged in recent years (e.g., Caujapé-Castells, 2011; Fernández-Palacios *et al.*, 2011; Hutsemékers *et al.*, 2011).

In sum, southern Korean populations of *C. falcatum* exhibit low within-population genetic variation, which may be a consequence of post-glacial long-distance dispersal events, presumably from a single glacial refugium, and subsequent founder effects. In addition, restricted gene flow among the highly specific rock habitats on which *C. falcatum* occurs discontinuously in southern Korea would have contributed to the high degree of among-population genetic differentiation.

Conservation implications.—An understanding of how genetic diversity is partitioned within and among populations is critical to design adequate plant conservation plans (Godt and Hamrick, 2001; Sun and Wong, 2001). In order to preserve a representative sample of the genetic variation, species with high population differentiation require the conservation of more populations *in situ*, and also a more extensive population sampling for *ex situ* conservation. Since *C. falcatum* exhibits a high degree of divergence among populations, a relatively large number of populations should be targeted for both *in situ* and *ex situ* conservation. Using the formula proposed by Ceska *et al.* (1997), $P = 1 - (F_{ST})^n$ (where P is the proportion of genetic variation desired to be preserved and n is the number of populations to be sampled/protected), we should protect/sample at least four populations in order to conserve $\geq 90\%$ of the genetic diversity found in *C. falcatum*. Considering allelic richness, allele frequencies, and the UPGMA phenogram, we suggest that the populations CF-1 and CF-7 from one of the clusters and CF-5 and CF-6 from the other cluster deserve both *in situ* preservation and *ex situ* conservation in southern Korea. Thus, these populations should be protected by law (e.g., by designing plant reserves), whereas spores should be collected and deposited in spore storage facilities (e.g., by cryoconservation; Ballesteros *et al.*, 2012).

ACKNOWLEDGMENTS

The authors thank Cheol Hwan Kim for helping us in locating populations of *Cyrtomium falcatum* in southern Korea, and B. J. Shim, E. J. Im, M. S. Park, and C. H. Chung for field and laboratory assistance. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (R05-2004-000-11055-0) to MGC.

LITERATURE CITED

- ADAMS, J. M. and H. FAURE. 1997. Review and atlas of palaeovegetation: preliminary land ecosystem maps of the world since the Last Glacial Maximum. Oak Ridge National Laboratory, Oak Ridge (TN). [cited 2012 May 12]. Available at: <http://www.esd.ornl.gov/projects/qen/adams1.html>.
- AOKI, K., T. SUZUKI, T.-W. HSU and N. MURAKAMI. 2004. Phylogeography of the component species of broad-leaved evergreen forests in Japan, based on chloroplast DNA variation. *J. Pl. Res.* 117:77–94.
- ARDITTI, J. and A. K. A. GHANI. 2000. Tansley review No. 110: Numerical and physical properties of orchid seeds and their biological implications. *New Phytol.* 145:367–421.
- BALLESTEROS, D., E. ESTRELLES, C. WALTERS and A. M. IBARS. 2012. Effects of temperature and desiccation on *ex situ* conservation of nongreen fern spores. *Amer. J. Bot.* 99:721–729.
- BROWN, A. H. D. 1979. Enzyme polymorphism in plant populations. *Theor. Populat. Biol.* 15:1–42.
- CAUJAPÉ-CASTELLS, J. 2011. Jesters, red queens, boomerangs and surfers: A molecular outlook on the diversity of the Canarian endemic flora, Pp. 284–324, *In*: D. Bramwell and J. Caujapé-Castells, eds. *The biology of island floras*. Cambridge University Press, London.
- CESKA, J. F., J. M. AFFOLTER and J. L. HAMRICK. 1997. Developing a sampling strategy for *Baptisia arachnifera* based on allozyme diversity. *Conservation Biol.* 11:1131–1139.
- CHELIAK, W. M. and J. P. PITEL. 1984. Technique for starch gel electrophoresis of enzyme from forest tree species. *Information report PI-X-42*. Petawawa National Forestry Institute, Chalk River, Ontario, Canada.
- CHOI, K.-R. 1998. The post-glacial vegetation history of the lowland in Korean Peninsula. *Korean J. Ecol.* 21:169–174.
- CHUNG, C.-H. 2007. Vegetation response to climate change on Jeju Island, South Korea, during the last deglaciation based on pollen record. *Geosci. J.* 11:147–155.
- CHUNG, C.-H. 2011. Holocene vegetation dynamics and its climatic implications inferred from pollen record in Boseong area, South Korea. *Geosci. J.* 15:257–264.
- CHUNG, C.-H., H. S. LIM and H. I. YOON. 2006. Vegetation and climate changes during the Late Pleistocene to Holocene inferred from pollen record in Jinju area, South Korea. *Geosci. J.* 10:423–431.
- CHUNG, C.-H., H. S. LIM and H. J. LEE. 2010. Vegetation and climate history during the late Pleistocene and early Holocene inferred from pollen record in Gwangju area, South Korea. *Quatern. Int.* 227:61–67.
- CHUNG, M. Y., J. D. NASON, B.-Y. SUN, M.-O. MOON, J. M. CHUNG, C.-W. PARK and M. G. CHUNG. 2010. Extremely low levels of genetic variation in the critically endangered monotypic fern genus *Mankyua chejuense* (Ophioglossaceae) from Korea: Implications for conservation. *Biochem. Syst. Ecol.* 38:888–896.
- CLAYTON, J. W. and D. N. TRETIAK. 1972. Amine citrate buffers for pH control in starch gel electrophoresis. *J. Fish. Res. Board Canada* 29:1169–1172.
- CORNUET, J. M. and G. LUIKART. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144:2001–2014.
- DÖPP, W. 1950. Eine die Antheridienbildung bei farnen fördernde substanz in den prothallien von *Pteridium aquilinum* (L.) Kuhn. *Ber. Deutsch. Bot. Ges.* 63:139–147.
- DUMINIL, J., S. FINESCHI, A. HAMPE, P. JORDANO, D. SALVINI, G. G. VENDRAMIN and R. J. PETIT. 2007. Can population genetic structure be predicted from life-history traits? *Amer. Naturalist* 169:662–672.
- EDGINTON, J. A. 2007. Dynamics of long-distance dispersal: the spread of *Asplenium adiantum-nigrum* and *Asplenium trichomanes* (Aspleniaceae; Pteridophyta) on London walls. *Fern Gaz.* 18:31–38.
- FERNÁNDEZ-PALACIOS, J. M., L. DE NASCIMENTO, R. OTTO, J. D. DELGADO, E. GARCÍA-DEL-REY, J. R. ARÉVALO and R. J. WHITTAKER. 2011. A reconstruction of Palaeo-Macaronesia, with particular reference to the long-term biogeography of the Atlantic island laurel forests. *J. Biogeogr.* 38:226–246.
- FLINN, K. M. 2006. Reproductive biology of three fern species may contribute to differential colonization success in post-agricultural forests. *Amer. J. Bot.* 93:1289–1294.

- GODT, M. J. W. and J. L. HAMRICK. 2001. Genetic diversity in rare southeastern plants. *Nat. Areas J.* 21:61–70.
- GOTANDA, K. and Y. YASUDA. 2008. Spatial biome changes in southwestern Japan since the Last Glacial Maximum. *Quatern. Int.* 184:84–93.
- GOUDET, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. *J. Heredity* 86:485–486.
- GRAY, A. 1996. Genetic diversity and its conservation in natural populations of plants. *Biodivers. Lett.* 3:71–80.
- HAMRICK, J. L. and M. J. W. GODT. 1989. Allozyme diversity in plant species, Pp. 43–63, *In*: A. H. D. Brown, M. T. Clegg, A. L. Kahler and B. S. Weir, eds. *Plant population genetics, breeding and genetic resources*. Sinauer Associates, Sunderland.
- HAMRICK, J. L. and J. D. NASON. 1996. Consequences of dispersal in plants, Pp. 203–236, *In*: O. E. Rhodess Jr, R. K. Chesser and M. H. Smith, eds. *Population dynamics in ecological space and time*. University of Chicago Press, Chicago.
- HAMRICK, J. L., Y. B. LINHART and J. B. MITTON. 1979. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Annual Rev. Ecol. Syst.* 10:173–200.
- HARDY, O. J. and X. VEKEMANS. 2002. SPAGeDi: a versatile computer program to analyze spatial genetic structure at the individual or population levels. *Molec. Ecol. Notes* 2:618–620.
- HARRISON, S. P., G. YU, H. TAKAHARA and I. C. PRENTICE. 2001. Diversity of temperate plants in east Asia. *Nature* 413:129–130.
- HATTORI, T. 1985. Synecological study on the lucidophyllous forest of *Castanopsis-Persea* type in Japan proper. *Bull. Kobe. Geobot. Soc.* 1:1–98 (in Japanese with English abstract).
- HAUFLER, C. H. 1985. Enzyme variability and modes of evolution in *Bommeria* (Pteridaceae). *Syst. Bot.* 10:92–104.
- HAUFLER, C. H. and G. J. GASTONY. 1978. Antheridiogen and the breeding system in the fern genus *Bommeria*. *Canad. J. Bot.* 56:1594–1601.
- HAUFLER, C. H. and T. A. RANKER. 1985. Effects of differential antheridiogen response on evolutionary mechanisms in *Cystopteris*. *Amer. J. Bot.* 71:878–881.
- HAUFLER, C. H. and C. B. WELLING. 1994. Antheridiogen, dark spore germination, and outcrossing mechanisms in *Bommeria* (Adiantaceae). *Amer. J. Bot.* 81:616–621.
- HAUK, W. D. and C. H. HAUFLER. 1999. Isozyme variability among crytic species of *Botrychium* subgenus *Botrychium* (Ophioglossaceae). *Amer. J. Bot.* 86:614–633.
- HEWITT, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* 58:247–276.
- HEWITT, G. M. 1999. Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* 68:87–112.
- HEWITT, G. M. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405:907–913.
- HEWITT, G. M. 2011. Quaternary phylogeography: the roots of hybrid zones. *Genetica* 139:617–638.
- HOLDEREGGER, R. and J. J. SCHNELLER. 1994. Are small isolated populations of *Asplenium septentrionale* variable? *Biol. J. Linn. Soc.* 51:377–385.
- HOPE, G., A. P. KERSHAW, S. VAN DER KAARS, X. SUN, P.-M. LIEW, L. E. HEUSSER, H. TAKAHARA, M. MCGLONE, N. MIYOSHI and P. T. MOSS. 2004. History of vegetation and habitat change in the Austral-Asian region. *Quatern. Int.* 118–119:103–126.
- HU, F. S., A. HAMPE and R. J. PETIT. 2009. Paleoecology meets genetics: deciphering past vegetational dynamics. *Frontiers Ecol. Environ.* 7:371–379.
- HUTSEMÉKERS, V., P. SZÖVÉNYI, A. J. SHAW, J.-M. GONZÁLEZ-MANCEBO, J. MUÑOZ and A. VANDERPOORTEN. 2011. Oceanic islands are not sinks of biodiversity in spore-producing plants. *Proc. Natl. Acad. Sci. USA* 108:18989–18994.
- IWATSUKI, K. 1992. *Ferns and fern allies of Japan*. Heibonsha Ltd., Tokyo, Japan.
- JIMÉNEZ, A., L. G. QUINTANILLA, S. PAJARÓN and E. PANGUA. 2009. Genetic variation in the allotetraploid *Dryopteris corleyi* and its diploid parental species in the Iberian Peninsula. *Amer. J. Bot.* 96:1880–1886.
- JIMÉNEZ, A., R. HOLDEREGGER, D. CSENCICS and L. G. QUINTANILLA. 2010. Microsatellites reveal substantial among-population genetic differentiation and strong inbreeding in the relict fern *Dryopteris aemula*. *Ann. Bot.* 106:149–155.

- KLEKOWSKI, E. J. 1972. Genetical features of ferns as contrasted to seed plants. *Ann. Missouri Bot. Gard.* 59:138–151.
- KLEKOWSKI, E. J. 1973. Sexual and subsexual systems in the homosporous ferns: a new hypothesis. *Amer. J. Bot.* 60:535–544.
- KLEKOWSKI, E. J. and H. G. BAKER. 1966. Evolutionary significance of polyploidy in the Pteridophyta. *Science* 153:305–307.
- LEE, E., S. KIM and S. NAM. 2008. Paleo-Tsushima Water and its effect on surface water properties in the East Sea during the last glacial maximum: Revisited. *Quatern. Int.* 176–177:3–12.
- LI, J. and C. H. HAUFLE. 1999. Genetic variation, breeding systems, and patterns of diversification in Hawaiian *Polypodium* (Polypodiaceae). *Syst. Bot.* 24:339–355.
- LLOYD, R. M. 1974. Reproductive biology and evolution in the Pteridophyta. *Ann. Missouri Bot. Gard.* 61:318–331.
- LÓPEZ-PUJOL, J., F.-M. ZHANG, H.-Q. SUN, T.-S. YING and S. GE. 2011. Centres of plant endemism in China: places for survival or for speciation? *J. Biogeogr.* 38:1267–1280.
- LUIKART, G., F. W. ALLENDORF, J. M. CORNUET and W. B. SHERWIN. 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. *J. Heredity* 89:238–247.
- MANTEL, N. A. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27:209–220.
- MATSUOKA, K. and N. MIYOSHI. 1998. Chapter III-4, Pp. 224–236, *In: Y. Yasuda and N. Miyoshi, eds. The illustrated vegetation history of the Japanese Archipelago.* Asakura-shoten, Toyko, Japan (in Japanese).
- MCCAULEY, D. E., D. P. WHITTIER and L. M. REILLY. 1985. Inbreeding and the rate of self-fertilization in a grape fern, *Botrychium dissectum*. *Amer. J. Bot.* 72:1978–1981.
- MITTON, J. B., Y. B. LINHART, K. B. STURGEON and J. L. HAMRICK. 1979. Allozyme polymorphisms detected in mature needle tissue of ponderosa pine. *J. Heredity* 70:86–89.
- NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583–590.
- NEI, M., T. MARUYAMA and R. CHAKRABORTY. 1975. The bottleneck effect and genetic variability in populations. *Evolution* 29:1–10.
- NEVO, E., A. BEILES and R. BEN-SHLOMO. 1984. The evolutionary significance of genetic diversity: Ecological, demographic and life history correlates, Pp. 13–213, *In: G. S. Mani, ed. Evolutionary dynamics of genetic diversity – Lecture Notes in Biomathematics (vol. 53).* Springer-Verlag, Berlin.
- PAJARÓN, S., E. PANGUA and L. GARCÍA-ÁLVAREZ. 1999. Sexual expression and genetic diversity in populations of *Cryptogramma crispa* (Pteridaceae). *Amer. J. Bot.* 86:964–973.
- PARK, S.-C., D.-G. YOO, C.-W. LEE and E.-I. LEE. 2000. Last-glacial sea level changes and paleogeography of the Korea (Tsushima) Strait. *Geo-Mar. Lett.* 20:64–71.
- PECK, J. H., C. J. PECK and D. R. FARRAR. 1990. Influences of life history attributes on formation of local and distant fern populations. *Amer. Fern J.* 80:126–142.
- PETIT, R. J., I. AGUINAGALDE, J. L. DE BEAULIEU, C. BITTKAU, S. BREWER, R. CHEDDADI, R. ENNOS, S. FINESCHI, D. GRIVET, M. LASCoux, A. MOHANTY, G. MUELLER-STARCK, B. DEMESURE-MUSCH, A. PALME, J. P. MARTIN, S. RENDELL and G. G. VENDRAMIN. 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* 300:1563–1565.
- PIRY, S., G. LUIKART and J.-M. CORNUET. 1999. Bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. *J. Heredity* 90:502–503.
- PRENTICE, I. C., S. P. HARRISON and P. J. BARTLEIN. 2011. Global vegetation and terrestrial carbon cycle changes after the last ice age. *New Phytol.* 189:988–998.
- PRYOR, K. V., J. E. YOUNG, F. J. RUMSEY, K. J. EDWARDS, M. W. BRUFORD and H. J. ROGERS. 2001. Diversity, genetic structure and evidence of outcrossing in British populations of the rock fern *Adiantum capillus-veneris* using microsatellites. *Molec. Ecol.* 10:1881–1894.
- QIU, Y.-X., C.-X. FU and H. P. COMES. 2011. Plant molecular phylogeography in China and adjacent regions: Tracing the genetic imprints of Quaternary climate and environmental change in the world's most diverse temperate flora. *Molec. Phylogen. Evol.* 59:225–244.

- RANKER, T. A. and J. M. O. GEIGER. 2008. Population genetics, Pp. 175–198, *In*: R. A. Ranker and C. H. Haufler, eds. *Biology and evolution of ferns and lycophytes*. Cambridge University Press, Cambridge.
- RANKER, T. A., C. E. C. GEMMILL, P. G. TRAPP, A. HAMBELTON and K. HA. 1996. Population genetics and reproductive biology of lava-flow colonising species of Hawaiian *Sadleria* (Blechnaceae), Pp. 581–598, *In*: J. M. Camus, M. Gibby and R. J. Johns, eds. *Pteridophytes in perspective*. Royal Botanic Gardens, Kew.
- ROUSSET, F. 1997. Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics* 145:1219–1228.
- ROUX, J. P. 2011. The genus *Cyrtomium* (Pteridophyta: Dryopteridaceae) in Africa and Madagascar. *Bot. J. Linn. Soc.* 167:449–465.
- SCHNELLER, J. J. and R. HOLDEREGGER. 1996. Colonisation events and genetic variability within populations of *Asplenium ruta-muraria* L., Pp. 571–580, *In*: J. M. Camus, M. Gibby and R. J. Johns, eds. *Pteridophytes in perspective*. Royal Botanic Gardens, Kew.
- SHI, Y. 2002. Characteristics of late Quaternary monsoonal glaciation on the Tibetan Plateau and in East Asia. *Quatern. Int.* 97–98:79–91.
- SOLTIS, D. E. and P. S. SOLTIS. 1986. Electrophoretic evidence for inbreeding in the fern *Botrychium virginianum* (Ophioglossaceae). *Amer. J. Bot.* 73:588–592.
- SOLTIS, D. E. and P. S. SOLTIS. 1989. Polyploidy, breeding systems, and genetic differentiation in homosporous Pteridophytes. Pp. 241–258, *In*: D. E. Soltis and P. S. Soltis, eds. *Isozymes in plant biology*. Dioscorides Press, Portland.
- SOLTIS, D. E. and P. S. SOLTIS. 1992. The distribution of selfing rates in homosporous ferns. *Amer. J. Bot.* 79:97–100.
- SOLTIS, D. E., C. H. HAUFLE, D. C. DARROW and G. J. GASTONY. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *Amer. Fern J.* 73:9–27.
- SOLTIS, D. E., A. B. MORRIS, J. S. MCLACHLAN and P. S. MANOS. 2006. Comparative phylogeography of unglaciated eastern North America. *Molec. Ecol.* 15:4261–4293.
- SOLTIS, P. S., D. E. SOLTIS and B. D. NESS. 1989. Population genetic structure in *Cheilanthes gracillima*. *Amer. J. Bot.* 76:1114–1118.
- SUN, M. and K. C. WONG. 2001. Genetic structure of three orchid species with contrasting breeding systems using RAPD and allozyme markers. *Amer. J. Bot.* 88:2180–2188.
- SUTER, M., J. J. SCHNELLER and J. C. VOGEL. 2000. Investigations into the genetic variation, population structure and breeding system of the fern *Asplenium trichomanes* subsp. *quadrivalens*. *Int. J. Pl. Sci.* 161:233–244.
- TRYON, R. 1970. Development and evolution of fern floras of oceanic islands. *Biotropica* 2:76–84.
- TRYON, R. 1972. Endemic areas and geographic speciation in tropical American ferns. *Biotropica* 4:121–131.
- TSUKADA, M. 1984. A vegetation map in the Japanese Archipelago approximately 20,000 years B.P. *Jap. J. Ecol.* 34:203–208 (in Japanese with English abstract).
- VAN DER BANK, H., M. VAN DER BANK and B.-E. VAN WYK. 2001. A review of the use of allozyme electrophoresis in plant systematics. *Biochem. Syst. Ecol.* 29:469–483.
- VOGEL, J. C., F. J. RUMSEY, J. J. SCHNELLER, J. A. BARRETT and M. GIBBY. 1999a. Where are the glacial refugia in Europe? Evidence from pteridophytes. *Biol. J. Linn. Soc.* 66:23–37.
- VOGEL, J. C., F. J. RUMSEY, S. J. RUSSELL, C. J. COX, J. S. HOLMES, W. BUJNOCH, C. STARK, J. A. BARRETT and M. GIBBY. 1999b. Genetic structure, reproductive biology and ecology of isolated populations of *Asplenium csikii* (Aspleniaceae, Pteridophyta). *Heredity* 83:604–612.
- WATANO, Y. and N. SAHASHI. 1992. Predominant inbreeding and its genetic consequences in a homosporous fern genus, *Sceptridium* (Ophioglossaceae). *Syst. Bot.* 17:486–502.
- WEEDEN, N. F. and J. F. WENDEL. 1989. Genetics of plant isozymes. Pp. 46–72, *In*: D. E. Soltis and P. S. Soltis, eds. *Isozymes in plant biology*. Dioscorides Press, Portland.
- WEIR, B. S. and C. C. COCKERHAM. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38:1358–1370.
- WEISS, S. and N. FERRAND, (eds.) 2007. *Phylogeography of southern European refugia – Evolutionary perspectives on the origins and conservation of European biodiversity*. Springer, Dordrecht.

- WIDMER, A. and C. LEXER. 2001. Glacial refugia: sanctuaries for allelic richness, but not for gene diversity. *Trends Ecol. Evol.* 16:267–269.
- WRIGHT, S. 1965. The interpretation of population structure by *F*-statistics with special regard to systems of mating. *Evolution* 19:395–420.
- WUBS, E. R., G. A. DE GROOT, H. J. DURING, J. C. VOGEL, M. GRUNDMANN, P. BREMER and H. SCHNEIDER. 2010. Mixed mating system in the fern *Asplenium scolopendrium*: implications for colonization potential. *Ann. Bot.* 106:583–590.
- XU, D., H. LU, N. WU and Z. LIU. 2010. 30 000-Year vegetation and climate change around the East China Sea shelf inferred from a high-resolution pollen record. *Quatern. Int.* 227:53–60.
- YEH, F. C., YANG, R.-C. and T. B. J. BOYLE. 1999. POPGENE version 1.31-Microsoft Windows-based freeware for population genetic analysis. Quick users' guide. University of Alberta, Edmonton.
- YI, S. 2011. Holocene vegetation responses to East Asian monsoonal changes in South Korea. Pp. 157–178, *In*: J. Blanco and H. Kheradmand, eds. *Climate change–geophysical foundations and ecological effects*. In Tech, Rijeka (Croatia).
- YI, S. and S.-J. KIM. 2010. Vegetation changes in western central region of Korean Peninsula during the last glacial (ca. 21.1–26.1 cal kyr BP). *Geosci. J.* 14:1–10.

SEM Studies on Tracheids of Lycopodiaceae; Observations on Adaptations in *Phylloglossum*

SHERWIN CARLQUIST

Santa Barbara Botanic Garden, 1212 Mission Canyon Road, Santa Barbara, CA 93105, USA,
email: s.carlquist@verizon.net

EDWARD L. SCHNEIDER

Department of Horticultural Sciences, 258 Alderman Hall, University of Minnesota, Twin Cities
Campus, St. Paul, MN 55108, and University of Minnesota Landscape Arboretum, 3675 Arboretum
Drive, Chaska, MN 55318, USA, email: edschnei@umn.edu

KEVIN F. KENNEALLY

School of Earth and Geographical Sciences, Faculty of Natural and Agricultural Sciences,
The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia,
and Western Australian Herbarium (PERTH), Department of Environment and Conservation,
Kensington, WA 6152, Australia, email: kevin.kenneally@uwa.edu.au

ABSTRACT.—Scanning electron microscope (SEM) studies of stem and strobilus longisections of *Huperzia*, *Lycopodium*, and *Phylloglossum* were undertaken to explore ultrastructure of pit membranes in tracheids. The membranes do not characteristically have pores and may often lack evidence of cellulosic fibrils. Some pit membranes in *Lycopodium* did show cellulosic fibrils. Porose membranes were seen in some tracheids, an appearance probably related to scraping away of layers in pit membranes by the sectioning process, or in other cases, artifact formation. Metaxylem tracheids have wide borders on pits. Truly “reticulate” metaxylem tracheids are few. Protoxylem tracheids have helices with borders in *Huperzia* and *Lycopodium*, but in *Phylloglossum* protoxylem annuli and helices are non-bordered. *Phylloglossum*, which appears nested in *Huperzia*, lacks metaxylem and has numerous other adaptations to the distinctive ephemeral vernal bogs of Australia and New Zealand, similar to those in Droseraceae and Orchidaceae.

KEY WORDS.—adaptation to fire, *Huperzia*, *Lycopodium*, *Phylloglossum*, pit membranes, tracheid ultrastructure, vernal bogs, xylem

Tracheids of *Lycopodium* were studied by Bierhorst (1960, 1971) and by Wilder (1970) by means of light microscopy. Although the total number of species studied was small, the differences among species were not great, so a broad-based survey of tracheids in the family has not been undertaken by those authors or by us. Cook and Friedman (1998) and Friedman and Cook (2000) offered some fascinating new data on ultrastructure of *Huperzia* (formerly a section of *Lycopodium*) tracheids. They demonstrated that the secondary wall of *Huperzia* tracheids is composed of a “template layer” on which is superimposed a “resistant layer.” The template layer is lignin-poor, whereas the resistant layer is rich in lignin.

Cook and Friedman (1998) and Friedman and Cook (2000) as well as Kenrick and Crane (1991, 1997) and Edwards (1993) have sought to integrate information on tracheid ultrastructure of early vascular plants with facts on the ultrastructure of tracheary elements in extant groups of vascular plants.

Lycopodiaceae, along with *Equisetum*, play an important role in these considerations because they may retain some characteristics of tracheary element ultrastructure from early times (e.g., Devonian).

Our goals in the present study complement the abovementioned studies by offering scanning electron microscope (SEM) studies of primary walls in three of the four genera currently recognized in Lycopodiaceae. We investigated primary wall microstructure with respect to protoxylem versus metaxylem. Also, we have included the first studies on ultrastructure of *Phylloglossum* tracheids. We have focused on the possible existence of thin areas or porosities in tracheid primary walls, and whether or not cellulosic fibrils are visible with SEM in primary walls of tracheids. Such structures have been evident in tracheids and/ or vessel elements of ferns (Carlquist and Schneider, 2007), *Equisetum* (Carlquist and Schneider, 2011a), cycads (Schneider *et al.*, 2007), and various families of monocotyledons (Carlquist and Schneider, 2010a, 2010b; Carlquist, 2012). Presence of pores and abundance of cellulosic fibrils vary considerably among these groups. For example, the end wall of a grass vessel contains a single large pit membrane that dissolves as the vessel matures; we were unable to demonstrate cellulosic fibrils in those pit membranes (Carlquist and Schneider, 2011b). By contrast, the cellulosic fibrils in perforations of *Canna* vessel elements are striking and persist in the mature vessel elements (Carlquist and Schneider, 2010b). The presence of a reticulum of cellulosic fibrils or of pores in an intact pit membrane appears to mediate the balance between conductive safety (restricting air bubbles to a single tracheary element) and conductive efficiency (porousness permitting passage of greater volumes of water per unit time than non-porous pit membranes). Where the tracheids of Lycopodiaceae fall in this gamut is the point of interest in our investigation.

Study of three genera of Lycopodiaceae permits a preliminary examination of diversity of tracheid ultrastructure for the family. One of these genera, *Phylloglossum*, is of especial interest because of the distinctive habit, which in turn is related to a special ecological niche. *Phylloglossum* plants consist of a so-called tuber (coexisting with the production of a single new tuber), a root, several leaves, and a strobilus. There is no stem in any accepted sense, merely a junction among these organs. Each spring, as moisture permits, the tuber produces leaves, a root, and a strobilus, and a geotropic new tuber. The plant oversummers and survives fire by means of the tuber. The vascular system of *Phylloglossum* consists of a plexus of tracheids interconnecting root, leaves, and strobilus. No vascular tissue enters the tuber, and thus vascular tissue is not intercontinuous from one year's plant body to the next year's. The distinctive habitat occupied by *Phylloglossum* can be characterized as ephemeral bogs, shallow depressions of acid sand, underlain by a hardpan, that accumulate rainwater during the winter months but evaporate as spring progresses into summer. The interrelationships between this distinctive habitat and the unique morphology of *Phylloglossum* form an obvious reason for special attention to *Phylloglossum* xylem. The habits of remaining Lycopodiaceae, which consist of horizontal and/or upright or pendant stems,

is relatively uniform, but no less interesting in terms of mechanical and physiological aspects.

MATERIALS AND METHODS

Sources of material are as follows. *Huperzia lucidula* (Michx.) Trevis: supplied by Carolina Biological Supply Company. *Lycopodium annotinum* L., *L. dichotomum* L., and *L. complanatum* L.: collected by E. L. Schneider at the moose-viewing platform along the Gunflint Trail, Minnesota, on September 12, 2011. *Phylloglossum drummondii* Kunze: specimen used for paraffin sectioning: collected between 120 and 121 mile post on highway between Brookton and Mt. Barker, Western Australia, on flat with grasses and sedges and annual *Stylidium* species; 9 October 1974, by Sherwin Carlquist (RSA). *Phylloglossum drummondii* used for SEM work: collected at Forrestdale Lake Nature Reserve, Western Australia, in seasonally waterlogged sandy clay flat (palusplain) with *Drosera* spp., *Utricularia multifida* and *Philydrella pygmaea*, 6 September 2007 by C. Tauss 1640 (PERTH). Recognition of genera in this paper follows that of Wikström and Kenrick (1997). The system for the family by Wagner and Beitel (1992) preceded molecular investigations and some genera recognized by them have not been followed in subsequent treatments.

All collections were preserved in 50% aqueous ethanol. The 1974 collection of *Phylloglossum* was embedded in paraffin according to the usual techniques; sections were stained with a safranin–fast green combination. Collections of all other Lycopodiaceae were sectioned by hand with a single-edged razor blade. The sections were subjected to three changes of distilled water, dried (with pressure applied) on a warming table, mounted on aluminum stubs, sputter-coated with gold, and examined with a Hitachi S2600N SEM. These methods have been described in more detail by Carlquist and Schneider (2007).

RESULTS

Huperzia lucidula (Fig. 1A–D). SEM micrographs of *H. lucidula* in our preparations show a range of conditions. The metaxylem pit membranes in Fig. 1A show numerous holes of various sizes, but no convincing evidence of cellulosic fibrils. The holes may represent thin areas in the pit membrane, revealed only when portions of the pit membrane are shaved away by the sectioning process. No holes are evident in the pit membrane shown in Fig. 1B, which has not been affected by the sectioning process. Likewise, the metaxylem tracheids in Fig. 1C show no evidence of pores in the pit membranes (tears represent obvious artifacts).

In protoxylem of *H. lucidula* (Fig. 1D), primary walls (equivalent to pit membranes in metaxylem) without pores were observed. This appearance accords with the illustration for this species by Friedman and Cook (2000). Also in agreement with their illustration, we found a relatively abrupt shift from protoxylem to metaxylem wall patterns, with few reticulate tracheids.

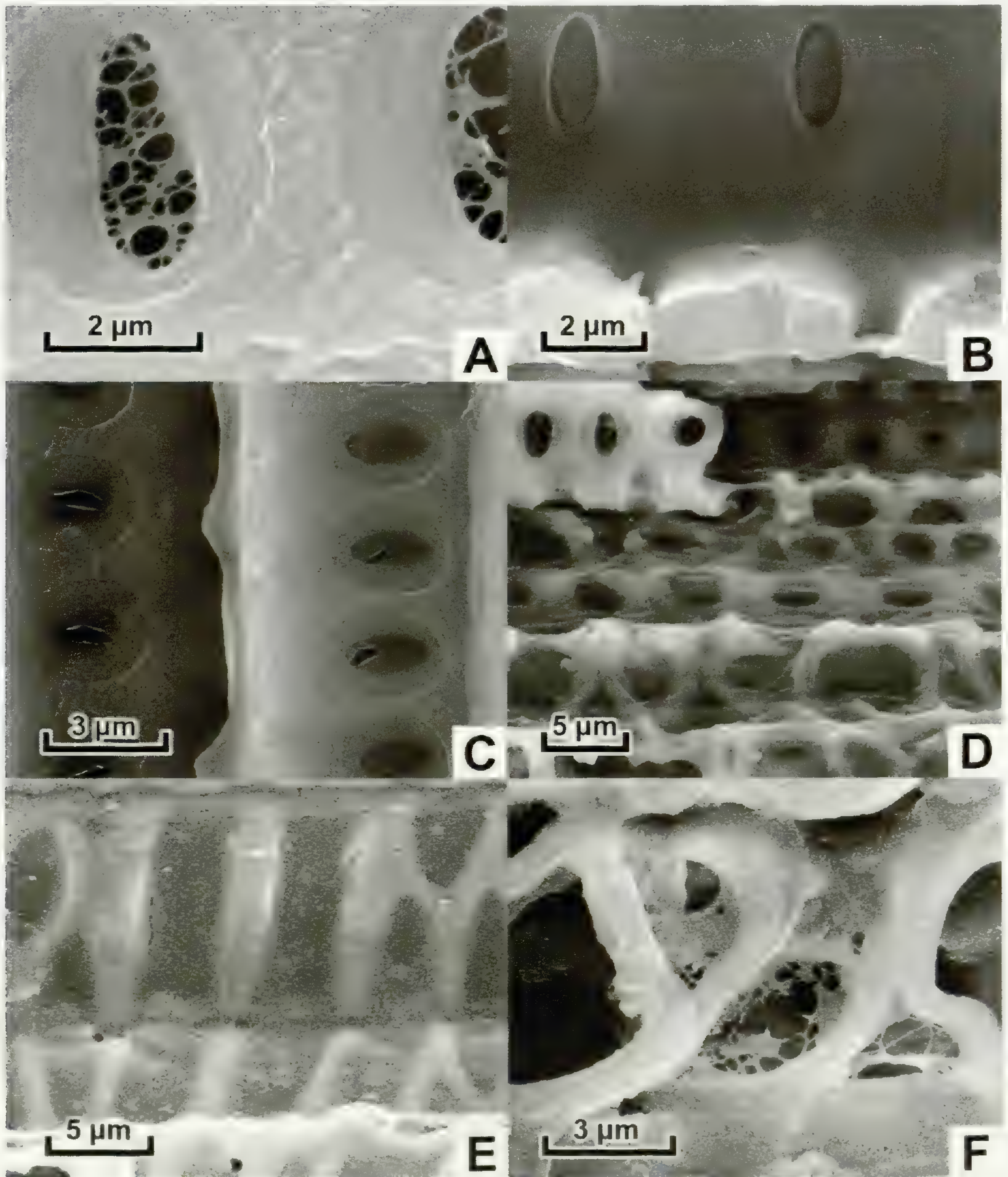


FIG. 1. SEM micrographs of tracheids from longisections of stems of Lycopodiaceae. A–D. *Huperzia lucidula*. A. Metaxylem tracheid outer surface, showing porous appearance of the pit membrane. B. Metaxylem tracheid in sectional view, showing pit membranes in face view (above) and in sectional view (lower right); no porosities evident. C. Outer surfaces of two metaxylem tracheids; no pores evidence in pit membranes (which do show some tears). D. Protoxylem (below) and metaxylem (above). E–F. *Lycopodium complanatum*. E. Protoxylem; some interconnections between the secondary wall helices occur in the tracheid above; no pores evident in primary wall. F. Porous primary wall in protoxylem, probably related to the sectioning process.

Lycopodium complanatum (Fig. 1E–F).

Protoxylem tracheids of *L. complanatum* in Fig. 1E show thin non-porose pit membranes on helical (below) and transitional (above) tracheids. Pores may be found on primary wall areas scraped away by sectioning (Fig. 1F), although the primary walls are otherwise non-porose.

L. annotinum (Fig. 2A–D).

In metaxylem, pit membranes of *L. annotinum* tracheids are typically non-porose (Fig. 2A). Tracheid pits that presumably have not been affected by sectioning (because they are viewed from the tracheid inside and seem intact), some pit membranes have a few small holes (Fig. 2B–C). These porosities may be irregularly distributed (Fig. 2B), and may represent some degree of artifact formation. In pits that have experienced scraping from the knife, a fibrillar structure is evident (Fig. 2D).

L. dichotomum (Fig. 2E–H).

Scattered small pores were observed in an apparently intact metaxylem tracheid (Fig. 2E). Protoxylem tracheids show pores to the degree that portions of the primary wall are shaved away by the sectioning process (Fig. 2F–G). The pit membrane portion shown in Fig. 2H is suggestive of presence of a fibrillar reticulate background. In the other instances, there is no indication of a fibrillar background because the porosities are circular rather than angular.

Phylloglossum drummondii (Fig. 3A–F).

The xylem studied in this species is derived from study of the vascular plexus, which interconnects strobilus, leaves, and root (see Introduction for a description of the plant body). *Phylloglossum* has no metaxylem. The bands of secondary wall in protoxylem tracheids are annular in a few places, but mostly helical (Fig. 3A). There are very few interconnections (indicating an approach to reticulate wall pattern) between the helices. The secondary wall annuli and helices are not bordered (Fig. 3B).

Primary walls of *Phylloglossum* tracheids are thin and homogeneous (Fig. 3C, E). Some areas show faint dark spots as recorded by the SEM (Fig. 3D). This rendering suggests that they are depressions. Areas of primary wall that have experienced some shaving from the sectioning process show pores (Fig. 3F) that correspond to the depression seen in Fig. 3D.

DISCUSSION AND CONCLUSIONS

Ultrastructure of the primary walls of tracheids.—Pits in metaxylem tracheids are oval in shape and prominently bordered in all Lycopodiaceae, as shown in the illustrations of Bierhorst (1960, 1971), Wilder (1970), Cook and Friedman (1998), and Friedman and Cook (2000). The appearances of pit

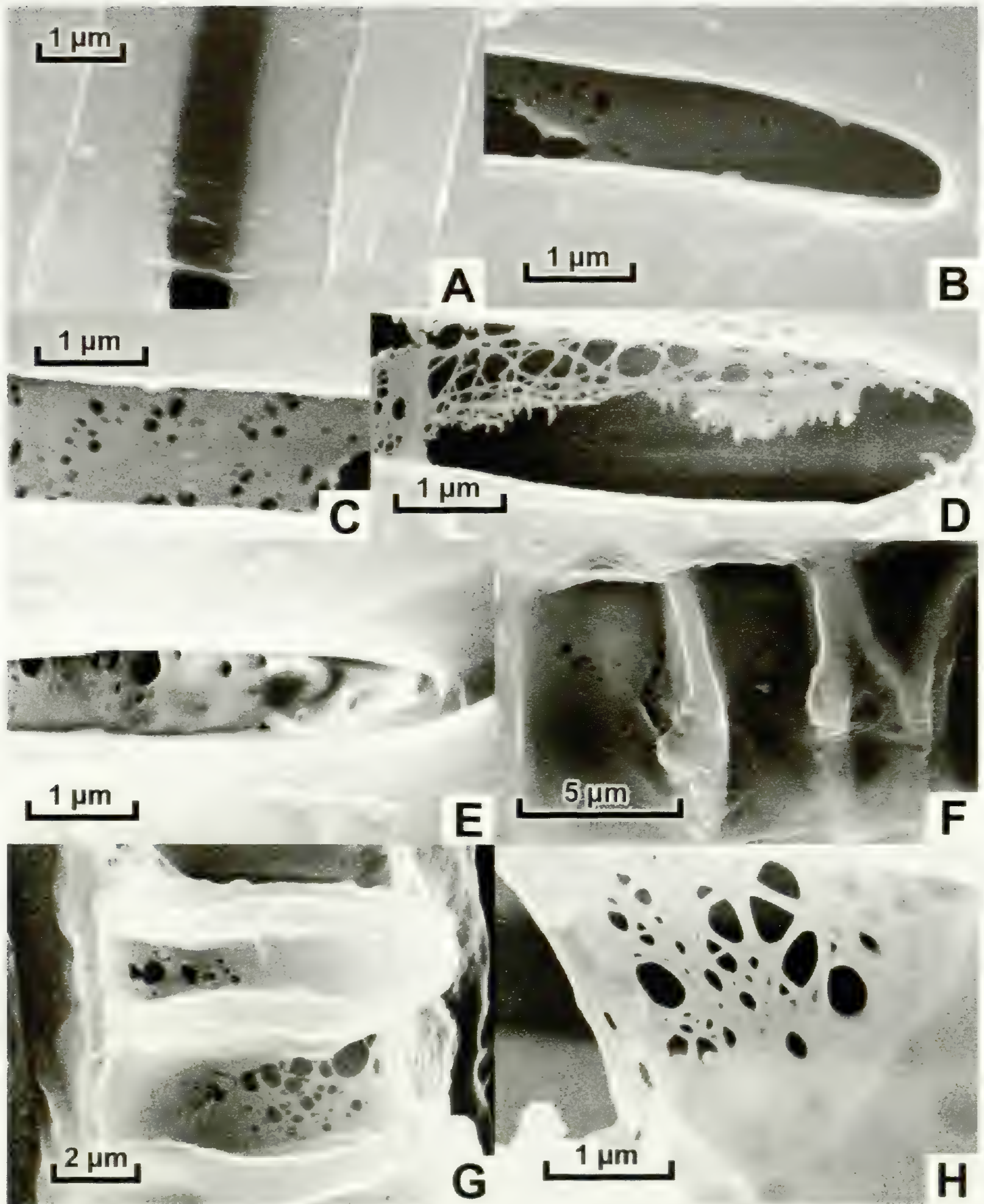


FIG. 2. SEM micrographs of tracheids from longisections of *Lycopodium*. A–D. *L. annotinum*. A. Outer surface of metaxylem tracheid, showing non-porous pit membrane extending across the pit border. B, C. Views of metaxylem pit membranes that have not been affected by sectioning, seen from inside the tracheid. B. A few small pores adjacent to a torn area (left.). C. Porosities scattered within pit membrane. D. Metaxylem pit membrane that has been partially scraped away by the sectioning process, revealing a reticulum with strands. E–H. *L. dichotomum*. E. Pit membrane seen from inside a metaxylem tracheid, showing some holes of various sizes. F–H. Portions of protoxylem tracheids seen from their outer surfaces. F. Primary wall non-porous, except at left; secondary wall thickenings are bordered. G. Porose appearance in primary wall of helical tracheid. H. A portion of a primary wall from a helical tracheid, showing a reticulate appearance suggestive of a fibrillar background.

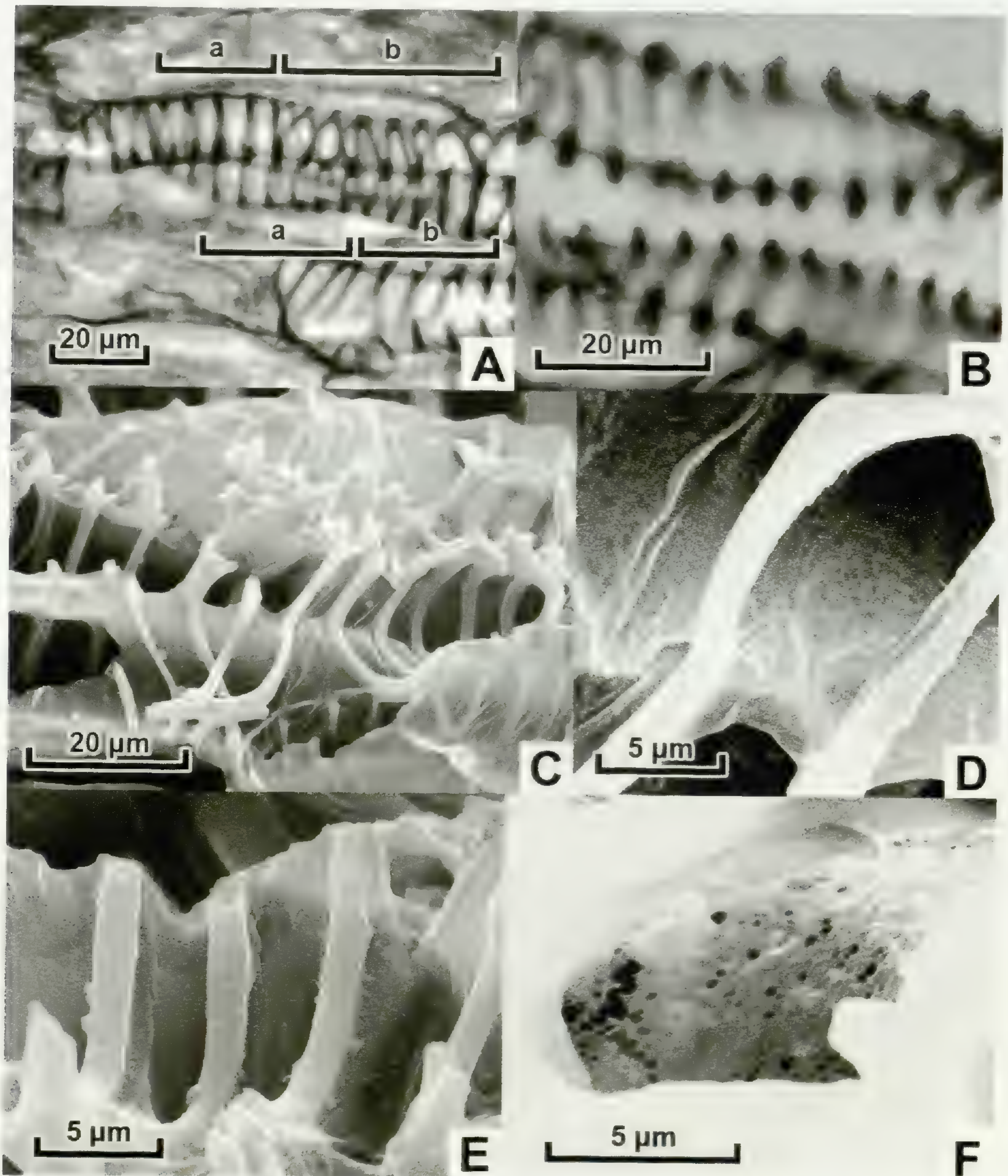


FIG. 3. Longisections of tracheids from the vascular plexus in plants of *Phylloglossum drummondii*. A, B. Light micrographs. A. Portions of tracheids to show annular (a) and helical (b) secondary wall bands. B. Optical sections of secondary wall bands (center), showing non-bordered condition. C–F. SEM micrographs. C. Portions of several adjacent tracheids, showing inner surfaces; primary wall portions non-porous. D. Portion of primary wall and two secondary wall helices from inner surface of tracheid; faint spots on the primary wall suggest presence of depressions. E. Inner surface of tracheid; secondary wall helices are borderless; primary wall is homogeneous in appearance. F. Shaved away portion of primary wall; minute pores are illustrated.

membranes that have experienced some degree of shaving away of wall material during the sectioning process suggest the presence of cellulosic fibrils, but this is not evident in the intact pit membranes we viewed. The circular shape and distribution of pores we observed in pit membranes that have not been affected by sectioning suggests the possibility that such holes may be artifacts caused by drying. However, porose appearances that are related to shaving away of wall material could indicate genuine thin areas in primary walls.

The abovementioned appearances are also found in primary walls of protoxylem tracheids. Homogeneous non-porose wall surfaces predominate in our material. This finding is in accord with the illustration of Friedman and Cook (2000) for *Huperzia lucidula*, which is based upon an SEM image of rotary microtome sections.

The paucity of cellulosic fibrils in primary walls of lycopodiaceous tracheids contrasts with appearances found in ferns (Carlquist and Schneider, 2007), cycads (Schneider *et al.*, 2007), and monocots (Carlquist and Schneider, 2010a, 2011b). In the monocots, fibrillar appearances are common in end walls of tracheary elements of families with more numerous plesiomorphic features (Carlquist, 2012). Cellulosic fibrils are, by contrast, few in the pit membranes of the end walls, prior to lysis, of vessel elements of grasses (Carlquist and Schneider, 2011b), which have simple perforation plates. More observations on a diversity of pit membranes, preferably with transmission electron microscopy (TEM) is desirable. The electron microscope data of Friedman and Cook (2000) were focused on the secondary walls of *Huperzia* tracheids.

In monocots (Carlquist, 2012) and in some genera of ferns, (e.g., *Blechnum*, Carlquist and Schneider, 2007), there is differentiation between end walls and lateral walls of tracheary elements with respect to size of pits (or perforations), pit membrane porousness, and presence of evident cellulosic webs. This may be related to incipient tendencies toward acquisition of some characteristics of vessels. Such differentiation was not evident to any appreciable extent in our studies on cycad tracheids (Schneider *et al.*, 2007). There is no differentiation between end walls and lateral walls in tracheids of Lycopodiaceae. This suggests that fascicles of tracheids, as in vascular cryptogam steles, serve as conductive units conjunctively, whereas individual vessels or tracheids with differentiation of end walls are the conductive units in monocots.

Unique adaptations of Phylloglossum.—Wikström and Kenrick (1997) found that *Huperzia* is paraphyletic, because *Phylloglossum* is nested within it (*Lycopodium* + *Lycopodiella* forms the other clade of the family). If we regard *Phylloglossum* as an extreme adaptation of the *Huperzia* clade, a number of adaptations become evident. The meristem that gives rise to each new geotropic tuber is exogenous, formed from surface tissue where the leaves and strobili join the old tuber. As the tuber elongates geotropically, a negatively geotropic meristem, which will give rise to the next year's leaves and stems, forms within the new tuber at its upper end (see Bierhorst, p. 25). This is a unique structure within vascular plants. At this stage when the parent plant has produced leaves and a strobilus, the new tuber has no vascular tissue. The

vascular plexus that interconnects root with juncture between leaves and strobilus does not even take the form of any discernable stem or stelar configuration. As the current year's plant body dries with the onset of the warm and dry season, the only surviving portion is the maturing new tuber, which contains no vascular tissue. Vascular tissue is initiated from the negatively geotropic meristem of the new tuber, apparently in response to leaf and strobilus initiation.

The vascular plexus, as we have seen, consists wholly of protoxylem. This fact is compatible with changing turgor in the plant, facilitated by the annular and helical patterns of the secondary wall within the tracheids. This correlates with changes in water availability in the wet vernal flats—ephemeral bogs of a sort—in which *Phylloglossum* grows.

Friedman and Cook (2000) illustrated borders on secondary wall in protoxylem tracheids of *Huperzia*. This corresponds with our observations on *Huperzia* and *Lycopodium* tracheids, although the sections of Friedman and Cook (2000) are clear in this respect because they observed rotary microtome sections with SEM. *Phylloglossum*, by contrast, lacks borders on the secondary wall annuli and helices of tracheids. The borderless condition confers more flexibility, because the investment in cellulosic wall material is less.

There are several interesting implications of the anatomy of *Phylloglossum* and its xylem. One can regard the plant body of *Phylloglossum* as paedomorphic, as Wikström and Kenrick (1997) do, in that it produces so few leaves and only one root, and yet produces a strobilus with this minimal vegetative apparatus. The tuber does not represent a juvenilization of a stem, but a new kind of appendage. This innovation within Lycopodiaceae is truly remarkable, because it runs counter to the intuitive idea that an ancient group of vascular plants is less likely to produce a vegetative structure *sui generis*. The gemmae of *Huperzia* spp. are rather easily categorized, in contrast, as products of shoot dimorphism.

The xylem of *Phylloglossum* may certainly be regarded as paedomorphic, because it consists of protoxylem only, whereas all other Lycopodiaceae have metaxylem as well as protoxylem. The wall strength of metaxylem is sufficient to promote a self-supporting stem structure. Although sclerenchyma develops in the cortex of some *Lycopodium* species, it develops later than metaxylem (original data). The central issue at hand is whether or not one invokes the term “paedomorphic,” a functional correlation exists between the mechanical strength provided by cellulose deposition and the mechanical strength (or lack of it) in tracheids of *Phylloglossum* as compared to those of the remaining Lycopodiaceae. This relative lack of tracheids with reticulate wall thickenings in tracheids of Lycopodiaceae other than *Phylloglossum* suggests that elongation (congruent with annular and helical wall thickenings of protoxylem) abruptly yields to self-support (a characteristic of pitted tracheary elements with appreciable wall thickness).

Interestingly, *Phylloglossum* shares its habitat with other vascular plants that produce tuber-like structures with various modes of origin, notably

Droseraceae, certain Stylidiaceae, and *Utricularia menziesii* R. Br. (Utriculariaceae). Studies on the comparative physiology and timing of photosynthate storage and retrieval of these plant assemblages together with xylem characteristics would be of interest. One should point out, as do Wikström and Kenrick (1997) with respect to *Phylloglossum*, that tuber formation is also a strategy of fire avoidance. Data on soil temperature gradients with respect to the species with tuber-like perennation structures in these areas would be of special interest, because most of these structures are relatively close to the ground surface in the “vernal bog” habitats. More deeply buried tuber-like structures are formed in various Orchidaceae native to sand areas of Australia (especially Western Australia), a fact which correlates with the fact that those Orchidaceae tend to co-exist with more shrubs, which would provide greater heat when burned.

LITERATURE CITED

- BIERHORST, D. W. 1960. Observations on tracheary elements. *Phytomorphology* 10:249–305.
- BIERHORST, D. W. 1971. Morphology of vascular plants. The Macmillan Company, New York.
- CARLQUIST, S. 2012. Monocot xylem revisited: new information, new paradigms. *Bot. Rev.* 78:87–153.
- CARLQUIST, S. and E. L. SCHNEIDER. 2007. Tracheary elements in ferns: new techniques, observations, and concepts. *Amer. Fern J.* 97:25–37.
- CARLQUIST, S. and E. L. SCHNEIDER. 2010a. Origin and nature of vessels in monocotyledons. 12. Pit membrane microstructure diversity in tracheary elements of *Astelia*. *Pac. Sci.* 64:607–618.
- CARLQUIST, S. and E. L. SCHNEIDER. 2010b. Origin and nature of vessels in monocotyledons. 11. Primary xylem microstructure, with examples from Zingiberales. *Int. J. Pl. Sci.* 171:258–266.
- CARLQUIST, S. and E. L. SCHNEIDER. 2011a. *Equisetum* xylem: SEM studies and their implications. *Amer. Fern J.* 101:133–141.
- CARLQUIST, S. and E. L. SCHNEIDER. 2011b. Origin and nature of vessels in monocotyledons. 13. Scanning electron microscope studies on xylem in large grasses. *Int. J. Pl. Sci.* 172:345–351.
- COOK, M. E. and W. E. FRIEDMAN. 1998. Tracheid structure in a primitive extant plant provides an evolutionary link to earliest fossil tracheids. *Int. J. Pl. Sci.* 159(6):881–890.
- EDWARDS, D. 1993. Cells and tissues in the vegetative sporophytes of early land plants. *New Phytol.* 125:225–247.
- FRIEDMAN, W. E. and M. E. COOK. 2000. The origin and early evolution of tracheids in vascular plants: integration of palaeobotanical and neobotanical data. *Phil. Trans. R. Soc. London B* 355:857–868.
- KENRICK, P. and P. R. CRANE. 1991. Water-conducting cells in early fossil land plants: implications for the early evolution of tracheophytes. *Bot. Gaz.* 152:335–356.
- KENRICK, P. and P. R. CRANE. 1997. The origin and early diversification of land plants: a cladistic study. Washington and London, Smithsonian Institution Press.
- SCHNEIDER, E. L., S. CARLQUIST and J. G. CHEMNICK. 2007. Scanning electron microscope studies of cycad tracheids. *S. African J. Bot.* 73:512–517.
- WAGNER, W. H. and M. J. BEITEL. 1992. Generic classification of modern North American Lycopodiaceae. *Ann. Mo. Bot. Gard.* 79:676–686.
- WIKSTROM, N. and P. KENRICK. 1997. Phylogeny of Lycopodiaceae (Lycopsidea) and the relationships of *Phylloglossum drummondii* Kunze based on *rbcL* sequences. *Int. J. Pl. Sci.* 158:862–871.
- WILDER, G. J. 1970. Structure of tracheids in three species of *Lycopodium*. *Amer. J. Bot.* 57:1093–1107.

Phlegmariurus changii (Huperziaceae), a New Hanging Firmoss from Taiwan

TUNG-YU HSIEH

School of Forestry and Resource Conservation, National Taiwan University, No. 1, Section 4, Roosevelt Road, Taipei, 10617, Taiwan

KENT A. HATCH

Biology Department, C.W. Post Campus of Long Island University, 720 Northern Boulevard, Brookville, NY 11548, USA

YUAN-MOU CHANG*

Department of Ecoscience and Ecotechnology, National University of Tainan, 33 Su-Lin Street, Section 2, Tainan 700, Taiwan

ABSTRACT.—We describe and illustrate a new firmoss, *Phlegmariurus changii* (Huperziaceae), which is endemic to eastern Taiwan. This new species is most similar to *Phlegmariurus carinatus* (Desv. ex Poiret) Ching; however, it differs by leaves that are flat abaxially. In addition, the sporophylls and trophophylls are conspicuously dimorphic for *Phlegmariurus changii*, but essentially monomorphic in *Phlegmariurus carinatus*. The ecology, conservation status, and morphology of *P. changii* is compared to that of species in three other sections (Sect. *Phlegmariurus*, L. B. Zhang, Sect. *Huperzioides* H. S. Kung et L. B. Zhang, and Sect. *Carinaturus* (Herter) H. S. Kung et L. B. Zhang) of *Phlegmariurus* in East Asia.

KEY WORDS.—*Phlegmariurus*, flora of Taiwan, taxonomy, ornamental fern, tassel fern, extinct in the wild

In the spring of 2006, we collected a unique hanging firmoss at Hsilin Village, Wanrong Township, Hualien County in eastern Taiwan. After consulting the relevant literature (Ching, 1981b; Ching, 1981a; Ching and Zhang, 1983; Ching, 1982; Holub, 1991; Kuo, 1985; Li *et al.*, 1975; Ma, 1990; Medeiros *et al.*, 1996; Wagner *et al.*, 1999; Wagner *et al.*, 1995; Wagner, 1993; Yang, 1984; Zhang, 2004; Zhang and Kung, 1999; Zhang and Kung, 2000; Fernández Prieto *et al.*, 2008; Rothmaler, 1944; Knapp, 2011; Kuo, 1997; Huang, 1994) and abundant herbarium specimens, we found that this species is new and we describe it herein.

This species belongs to the Lycopodiales. The Lycopodiales were historically considered a single family, the Lycopodiaceae, which contained two genera, *Phylloglossum* Kunze and *Lycopodium* L. *Phylloglossum* is a genus containing only one species and endemic to Australia and New Zealand, whereas *Lycopodium sensu* Linnaeus are widely distributed in temperate and tropical regions. *Lycopodium* L. is a complex group and has undergone many changes in taxonomy and nomenclature (Holub, 1991; Wagner and Beitel,

*Corresponding author; email: changyuanmou@gmail.com

1992; Wagner, 1993; Øllgaard, 1987). *Lycopodium* has been separated into two families, the Urostachyaceae Rothm. (=Huperziaceae Rothm.) and the Lycopodiaceae (Rothmaler, 1944), and several genera. There are two genera—*Phlegmariurus* and *Huperzia* Bernh.—in Huperziaceae. In this article, we follow Wagner's classification and include this species in *Phlegmariurus* (Wagner and Beitel, 1992).

Many species of *Phlegmariurus* (Herter) Holub are important traditional medicines and popular ornamental ferns in the flower markets, such as *Phlegmariurus squarrosus* (Forst.) L'ève et L'ève, *P. carinatus* (Desv. ex Poiret) Ching, and *P. cunninghamioides* (Hayata) Ching. Consequently, over-collection of plants from the wild for medicinal and horticultural purposes threatens many species (Yumkham and Singh, 2011). Of the ten species of *Phlegmariurus* that occur in Taiwan, all but *P. fordii* (Baker) Ching are threatened (Moore, 2000; Moore, 2001; Kuo, 1997). This makes the finding of a new species in Taiwan important.

Phlegmariurus changii T. Y. Hsieh, *sp. nov.* TYPE.—TAIWAN. Hualien County: Wanrong Township, Hsilin Village. 6 April 2006, *Tung-Yu Hsieh 516* (holotype: TAI 281321). **Figs. 1, 2.**

This new species is similar to *P. carinatus* (Desv. ex Poiret) Ching, but differs by having leaves that are flat, vs. carinate or raised abaxially in *P. carinatus*.

Epiphytes, pendant firmoss; stems 0.6–0.9 m long, 3–5 mm in diameter, dichotomously branching 5–8 times. Leaves sessile, leathery, lanceolate, entire, with tapering apex, 7–9 mm long, 3–4 mm wide, imbricate, pointing towards the apex of the shoot, appressed, decreasing in size towards apex and gradually changing into sporophylls. Fertile spikes terminal, 0.15–0.2 m long, 2 mm thick. Sporangia reniform, borne in the axil of the sporophyll, green turning to yellow when mature, ca. 1.3 × 1.2 mm. Spores trilete, radially symmetrical, foveolate, tetrahedral from the polar view, having a laesura with three radiating branches near to the equator, ca. 34 × 32 μm.

ADDITIONAL SPECIMENS EXAMINED.—TAIWAN. Hualien County: Wanrong Township, Hsilin Village, ca. 200 m alt., 6 Apr 2006, *T. C. Hsu 461* (TAIF); same loc., *s.d.*, *Liang-Ru Chang s.n.* (TAI).

ETYMOLOGY.—The specific epithetic commemorates the original discoverer of this species, Liang-Ru Chang. He is an active amateur fern and orchid lover (Lin *et al.*, 2006).

NOTES.—Liang-Ru Chang first found this hanging firmoss in the spring of 2006, from the type locality at Hsilin Village, Wanrong Township, Hualien County. The holotype was collected from the trunk of an old *Schefflera* tree (*Schefflera octophylla* (Lour.) Harms) on a cliff beside a valley, at about 200m altitude, growing with many plants of *Vittaria zosterifolia* Willd. (Fig. 2A).

After the initial discovery, the first author conducted a long-term, regular and exhaustive field investigation for *P. changii* thorough the island during the field work for his PhD study in 2006–2011 (Hsieh *et al.*, 2007; Hsieh *et al.*,

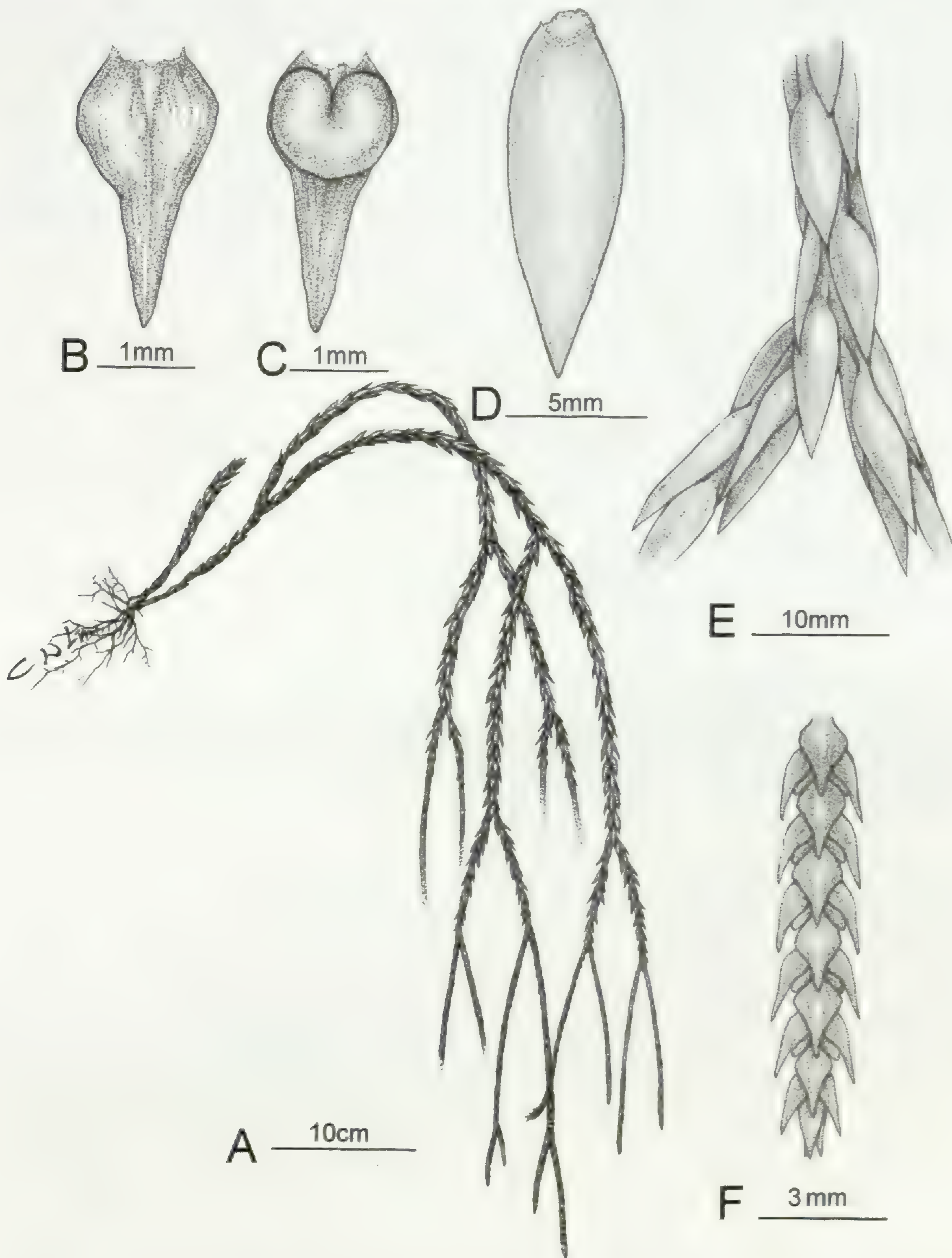


FIG. 1. *Phlegmariurus changii* T. Y. Hsieh. A. Whole plant; B. Abaxial side of sporophyll; C. Adaxial side of sporophyll and sporangium; D. Trophophyll; E. Middle part of stem; F. Fertile spike.

2011; Hsieh, 2011). During this period, only three habitats were found. All three were found at separate locations in lowland of Taitung and Hualien County, in eastern Taiwan. Unfortunately, all known wild individuals have since been removed by other collectors. There are two cultivated individuals

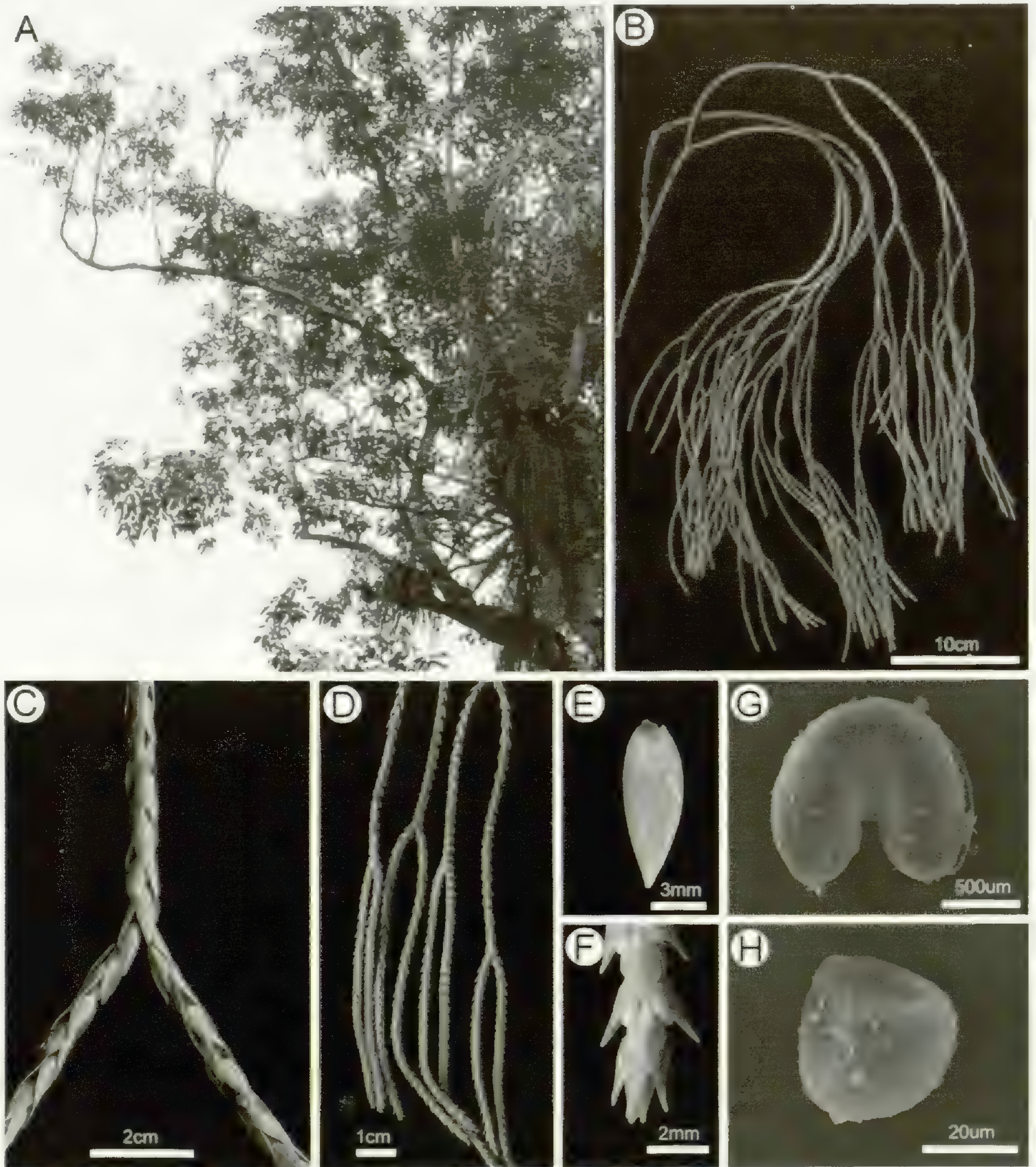


FIG. 2. *Phlegmariurus changii* T. Y. Hsieh. A. Habitat; B. Whole plant; C. Middle part of stem; D. Fertile spikes; E. Trophophyll; F. Sporophylls; G. Sporangium, SEM microphotograph; H. Spore, SEM microphotograph.

as far as we know. Given this situation, according to the IUCN (The International Union for Conservation and Natural Resources) ranking system (IUCN, 2008), this species should be considered extinct in the wild (EW) temporarily. We will continue our field investigation for this species and hope that we can find the individuals of this species in the wild again in the future. By doing so, we can re-evaluate the conservation status of this species.

Phlegmariurus carinatus is the only species that is morphologically similar to *P. changii*. *Phlegmariurus changii* can be distinguished from species in the

three sections of *Phlegmariurus* in East Asia., Sect. *Phlegmariurus*, L. B. Zhang, Sect. *Huperzioides* H. S. Kung et L. B. Zhang, and Sect. *Carinaturus* (Herter) H. S. Kung et L. B. Zhang. *Phlegmariurus changii* can be distinguished from species in both Sect. *Phlegmariurus* and Sect. *Huperzioides* by having all leaves appressed on the stem, imbricate, and pointing towards the apex of the shoot. This is not the case in the later two groups (Zhang and Kung, 1999; Zhang and Kung, 2000). Compared to species of Sect. *Carinaturus*, the leaves of *P. changii* are relatively large and flat. By comparison, the leaves of Sect. *Carinaturus* are carinate or raised on the abaxial side, whereas they are but flat in *P. changii*. Sporophylls and trophophylls are homomorphic for species of Sect. *Carinaturus* (Zhang and Kung, 2000), but dimorphic for *P. changii* (Figs. 1B, 1D, 2E, 2F).

ACKNOWLEDGMENTS

We greatly appreciate Jian-Hong Chen for help with fieldwork; Che-Wei Lin for preparing the line drawings; Liang-Ru Chang and Tian-Chuan Hsu for the useful habitat information; and Kuang-Ming Hsu for taking SEM photos. We also thank Professor Tsung-Hsin Hsieh and two anonymous reviewers for commenting on an earlier draft of this manuscript. This study was supported in part by grants from the Research Center for Biodiversity, Academia Sinica (Taipei, Taiwan) to Ching-I Peng (HAST) and the Division of Silviculture, Taiwan Forestry Research Institute (Taipei, Taiwan) to Ching-Te Chien.

LITERATURE CITED

- CHING, R. C. 1981a. The taxonomy of Chinese Lycopodiaceae (sen. lat.) II. Acta Botan. Yunnan. 3:291–305.
- CHING, R. C. 1981b. The taxonomy of Chinese Lycopodiaceae (sen. lat.) I. Acta Botan. Yunnan. 3:1–9.
- CHING, R. C. 1982. The taxonomy of Chinese Lycopodiaceae (sen. lat.) III–IV. Acta Botan. Yunnan. 4:119–128.
- CHING, R. C. and C. F. ZHANG. 1983. New ferns of Zhejiang Province. Bull. Bot. Res., Harbin 3:1–55.
- FERNÁNDEZ PRIETO, J. A., C. AGUIAR, E. DIAS, M. D. L. A. F. CASADO and J. HOMET. 2008. The genus *Huperzia* (Lycopodiaceae) in the Azores and Madeira. Bot. J. Linn. Soc. 158:522–533.
- HOLUB, J. 1991. Some taxonomic changes within Lycopodiales. Folia Geobot. Phytotax. 26:81–94.
- HSIEH, T. Y. 2011. Taxonomy and distribution of indigenous *Actinidia* in Taiwan. PhD, National Chung-Hsing University, Taichung, Taiwan.
- HSIEH, T. Y., T. C. HSU, Y. KONO, S. M. KU and C. I. PENG. 2007. *Gentiana bambuseti* (Gentianaceae), a new species from Taiwan. Botanical Studies 48:349–355.
- HSIEH, T. Y., S. M. KU, C. T. CHIEN and Y. T. LIOU. 2011. Classifier modeling and numerical taxonomy of *Actinidia* (Actinidiaceae) in Taiwan. Botanical Studies 52:337–357.
- HUANG, T. C. E. A. (ed.) 1994. Flora of Taiwan, 2nd edition., Vol. 1., Taipei, Editorial Committee of the Flora of Taiwan.
- IUCN. 2008. IUCN Red list categories and criteria: version 7, IUCN—The World Conservation Union, Gland, Switzerland and Cambridge, United Kingdom.
- KNAPP, R. 2011. Ferns and Fern Allies of Taiwan, KBCC Press.
- KUO, C. M. 1985. Taxonomy and phytogeography of Taiwanese pteridophytes. Taiwaniana 30:5–100.
- KUO, C. M. 1997. *Lycopodium*. Rare and Endangered Plants in Taiwan (II), Council of Agriculture, Taipei.
- LI, H. L., T. S. LIU, T. P. HUANG, T. KOYAMA and E. CHARLES. 1975. Flora of Taiwan (1st edn.), Vol. 1, Pteridophyta and Gymnospermae. Epoch Publishing Company.

- LIN, W. M., L. L. KUO HUANG and T. LIN. 2006. Newly discovered native orchids of Taiwan. *Taiwania* 51:162.
- MA, C. Y. 1990. New Taxa of genus *Phlegmariurus* from China. *Bull. Bot. Res.*, Harbin 10:57–169.
- MEDEIROS, A., W. WAGNER JR and R. HOBODY. 1996. A new Hawaiian hanging firmoss (Lycopodiaceae: *Phlegmariurus*) from the eastern Hawaiian Islands. *Amer. Fern J.* 86:89–97.
- MOORE, S. J. 2000. *Lycopodium*. Rare and Endangered Plants in Taiwan (V), Council of Agriculture, Taipei.
- MOORE, S. J. 2001. *Lycopodium*. Rare and Endangered Plants in Taiwan (VI), Council of Agriculture, Taipei.
- ØLLGAARD, B. 1987. A revised classification of Lycopodiaceae s. lat. *Opera Bot.* 92:153–178.
- ROTHMALER, W. 1944. Pteridophyten-Studien I. Repertorium novarum specierum regni vegetabilis 54:55–82.
- WAGNER, W., F. S. WAGNER and T. FLYNN. 1995. Taxonomic notes on pteridophytes of Hawaii. *Contr. Univ. Michigan Herb.* 20:241–260.
- WAGNER, W. H. 1993. A New Combination for a North American Lycopod. *Novon* 3:305.
- WAGNER, W. H. and J. M. BEITEL. 1992. Generic classification of modern North American Lycopodiaceae. *Ann. Missouri Bot. Gard.* 79:676–686.
- WAGNER, W. H., D. D. PALMER and R. W. HOBODY. 1999. Taxonomic notes on the pteridophytes of Hawaii: II. *Contr. Univ. Michigan Herb.* 22:135–187.
- YANG, C. Y. 1984. A new species of the genus *Phlegmariurus* from Guangxi, China. *Acta Phytotax. Sin.* 22:87–88.
- YUMKHAM, S. D. and P. K. SINGH. 2011. *Huperzia squarrosa* (G. Forst.) Trev. (Lycopodiaceae) in Manipur: Taxonomy and Biological Aspects. *Taiwania* 56:157–164.
- ZHANG, L. B. 2004. *Phlegmariurus*. *Flora Reipublicae Popularis Sinicae* 6:31–54.
- ZHANG, L. B. and H. S. KUNG. 1999. On the taxonomy of *Phlegmariurus* (Herter) Holub sect. *Huperzioides* H. S. Kung et L. B. Zhang (sect. nov.) with notes on the infrageneric classification of the genus *Phlegmariurus* in China. *Acta Phytotax. Sin.* 37:40–53.
- ZHANG, L. B. and H. S. KUNG. 2000. Two sections of *Phlegmariurus* (Herter) Holub. (Huperziaceae) from China. *Acta Phytotax. Sin.* 38:23–29.

Lectotypification of *Marsilea quadrifolia* L. (Marsileaceae)

DUILIO IAMONICO

Laboratory of Phytogeography and Applied Geobotany, Department DATA, Section Environment and Landscape, University of Rome Sapienza, 00185 Rome, Italy,
e-mail: d.iamonico@yahoo.it

ABSTRACT.—The typification of the binomium *Marsilea quadrifolia* L. (Marsileaceae) is discussed. To fix the application of the species name an iconography by de Jussieu is designated as the lectotype.

KEY WORDS.—*Marsilea*, Linnaean names, nomenclature, typification

Marsilea L. (Marsileaceae Mirb.: Salviniiales Bartl.) is a genus of approximately 45–50 species and has a cosmopolitan distribution, although it is infrequent in cool-temperate regions and oceanic islands (Kubitzki, 1990; Johnson, 1993; Nagalingum *et al.*, 2007).

Linnaeus published three names under *Marsilea* (*M. minuta*, *M. natans*, *M. quadrifolia*; Jarvis, 2007: 657), of which one (*M. natans*) is now placed in *Salvinia* Ség. (*Salviniaceae* T. Lestib.), as *S. natans* (L.) All. Of the other two names, only *M. quadrifolia* appears not to be typified. It is investigated here.

Typification

Linnaeus' protologue (Linnaeus, 1753: 1099) consists of a short diagnosis, with seven synonyms cited from de Jussieu (1740: 263), Guettard (1747: 62), Bauhin (1623: 362; 1651: 789), Mappus (1742: 166), Morison (1699: 619), and Matthioli *et al.* (1586: 853). All these authors (except Guettard, 1747) provided illustrations that are thus original materials.

Bobrov (1984: 20) indicated the sheet No. 1254.2 at LINN as type. Although this plant agrees with the diagnosis, the sheet lacks the relevant *Species Plantarum* number ("2" in the case of *M. quadrifolia*) including only the Linnaean script "*Marsilea quadrifolia*." So, it is to be considered a post-1753 addition to the collection and therefore not original material for the name (see Jarvis, 2007). According to Art. 9.2 of the ICNB (McNeill *et al.*, 2012) a lectotype is "... a specimen ... designated from the original material ..." and, as reported in the Art. 9.3 "... original material comprises: (a) those specimens and illustration (... published either prior to or together with the protologue) upon which it can be shown that the description or diagnosis validating the name was based ...". So, the choice by Bobrov (1984) is not correct. Johnson (1986: 35) proposed a de Jussieu collection (No. 1599-A at P-JU) as lectotype, but this would not have been studied or examined by Linnaeus (see Jarvis, 2007). In fact, although Stearn (1957: 106) reported that Linnaeus received



FIG. 1. Lectotype of *Marsilea quadrifolia* L. (from de Jussieu, 1740, pl. 15).

“many” specimens from de Jussieu, it is very difficult to know which specimens may have come from the author as there are no explicit annotation that might indicate this. The collection No. 1599-A at P-JU is therefore not original material for the name, and thus Johnson’s (1986) lectotypification is incorrect, too. We have been unable to trace original material in any of the other Linnaean and Linnaeus-linked herbaria.

All original material (the images cited by Linnaeus from de Jussieu, Bauhin, Mappus, Morison and Matthioli *et al.*) clearly show leaves whose blades (cruciform, consisting of two pair of opposite and sessile leaflets) are the only feature that marks the Linnaean concept of the species (“*MARSILEA foliis quaternatis*”). de Jussieu’s illustration (1740: pl. 15; see Fig. 1) is the most complete, showing a large part of a plant (letter “A” in the de Jussieu plate) including details of two leaves (letters “n” and “o”) and a series of 11 drawings (some magnified) of the sporocarps (both entire and in longitudinal or transversal sections), sori, and spores (letters “B” – “m”). This iconography also agrees with the current application of this name (e.g., Akeroyd, 1993; Johnson, 1993). Thus, it is here designated as the lectotype for the name *Marsilea quadrifolia*.

***Marsilea quadrifolia* L., Sp. Pl. 2: 1099. 1753. Lectotype (designated here):**
Lemma, pl. 15 in de Jussieu (1740: Histoire de Lemma). Fig. 1

LITERATURE CITED

- AKERROYD, J. R. 1993. *Marsilea* L. Vol. 1, Pp. 31–32, In: T. G. Tutin, V. H. Heywood, D. M. Moore, D. H. Valentine, S. M. Walters and D. A. Webb, eds. *Flora Europaea*, ed. 2. Cambridge University Press, Cambridge.
- BAUHIN, C. 1623. *Pinax theatri botanici*. Ludovici Regis, Basileae.
- BAUHIN, C. 1651. *Historia Plantarum Universalis* 3. Ebroduni.
- BOBROV, A. E. 1984. Conspectus filicarum Asiae Mediae et Kazakhstaniae. *Novosti Syst. Vyssh. Rast.* 21:5–21.
- GUETTARD, M. 1747. *Observation sur les plantes* 1. Durand, Paris.
- JOHNSON, D. M. 1986. Systematics of the New World species of *Marsilea* (Marsileaceae). *Syst. Bot. Monogr.* 11:1–87.
- JOHNSON, D. M. 1993. Marsileaceae Mirb. Vol. 2, Pp. 331–335, In *Flora of North America* Editorial Committee, eds. *Flora of North America North of Mexico*. Oxford University Press, New York and Oxford.
- JUSSIEU, B. DE. 1740. Histoire du Lemma. *Mém. Acad. Roy. Sci.* 1740:263–275.
- KUBITZKI, K. 1990. *Pteridophytes and gymnosperms*, Vol. 1, Pp. 1–404, In K. U. Kramer and P. S. Green, eds. *The families and genera of vascular plants*. Springer-Verlag, Berlin.
- MAPPUS, M. 1742. *Historia Plantarum Alsaticarum*. Petrum Mortier, Amstelodami.
- MATTHIOLI, P. A., J. CAMERARIO and F. CALCEOLARIO. 1586. *De plantis epitome utilissima*. Francoforti ad Moenum.
- MCNEILL, J., BARRIE, F. R., BUCK, W. R., DEMOULIN, V., GREUTER, D. L., HAWKSWORTH, D. L., HERENDEEN, P. S., KNAPP, S., MARHOLD, PRADO, J., PROUD’HOMME VAN REINE, W. F., SMITH, J. F. and WIERSEMA, J. H., eds. 2012. *International Code of Nomenclature for algae, fungi and plants (Melbourne Code)*. *Regnum Vegetabile* 154. Gantner, Ruggell.
- MORISON, R. 1699. *Plantarum historia universalis oxoniensis* 3. Theatro Sheldoniano, Oxonii.

- NAGALINGUM, N. S., H. SCHNEIDER and K. M. PRYER. 2007. Molecular phylogenetic relationships and morphological evolution in the heterosporous fern genus *Marsilea*. *Syst. Bot.* 32(1):16–25.
- STEARNS, W. T. 1957. *An introduction to the Species Plantarum and cognate botanical works of Carl Linnaeus* 1. Ray Society, London.

Referees for 2012

All papers submitted to the journal are peer reviewed. Members of the editorial board and the American Fern Society, as well as additional scientists in cognate areas do these reviews on a voluntary basis. It is their work that contributes to the high quality of articles in the *American Fern Journal* and to its continued success. The American Fern Society and I extend our thanks to the following reviewers for the assistance, diligence, and patience in the year 2012 (My sincere apologies if I inadvertently omitted anyone from this list).

NAN CRYSTAL ARENS
CURTIS BJORK
MARIA REGINA T. BOEGER
PATRICK BROWNSEY
SHERWIN CARLQUIST
WEN-LIAN CHIOU
MARTHA COOK
JIM DUNN
ASHLEY FIELD
CHRISTOPHER FRASER-JENKINS
JOSE MARÍA GABRIEL Y GALAN
JENNIFER GEIGER
GARY GREER
G.A. DE GROOT
AMANDA GRUSZ

CHRISTOPHER H. HAUFLER
PAULO LABIAK
OLGA MARTINEZ
ANDY McCALL
KLAUS MEHLTRETER
JORDAN METZGAR
ROBBIN C. MORAN
NATHALIE NAGALINGUM
JOEL NITTA
JARMILA PITTERMANN
MÓNICA PONCE
JEFFERSON PRADO
KAREN RENZAGLIA
CARL ROTHFELS
GERMINAL ROUHAN

ED SCHNEIDER
JIM SEAGO
EMILY SESSA
JOANNE SHARPE
KIRK STOWE
MEI SUN
MICHAEL SUNDUE
WES TESTO
DORI THOMPSON
OLENA VASHEKA
MAGGIE R. WAGNER
JAMES E. WATKINS, JR.
DEAN WHITTIER
MICHAEL WINDHAM
LIBING ZHANG

Statement of Ownership, Management, and Circulation

Publication title and number: *American Fern Journal* (0002-8444). Date of filing: September 4, 2012. Frequency of issue: quarterly. Annual subscription price 2012: \$25.00. Office of Publisher: c/o Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299. Editor: Jennifer Geiger. The *American Fern Journal* is wholly owned by the American Fern Society, Inc., with no bond holders. Physical address of the Society: c/o Missouri Botanical Garden, 4344 Shaw Blvd., St. Louis, MO 63110. The purpose, function, and nonprofit status of the Society and its tax exempt status for Federal income tax purposes remains the same as in past years. The average press run for Volume 102 is 800, and was 800 for the issue appearing immediately prior to the filing date, for which 712 copies were mailed as paid circulation and 0 copies were mailed as free distribution, leaving 88 copies for office use and back-issues sales. I certify that these statements are correct and complete. GEORGE YATSKIEVYCH, Membership Secretary of AFS.

Table of Contents for Volume
(A list of articles arranged alphabetically by author)

AHAD, BURHAN, ZAFAR A. RESHI, AND AIJAZ H. GANAIE. <i>Azolla cristata</i> in the Kashmir Himalaya	224
ALENCAR DE MENEZES, I. R. (See T. M. DE SOUZA)	198
ANDRADE, J. C. (See M. F. B. MORAIS-BRAGA)	154
BANDYOPADHYAY, M. (See S. DAS)	47
BENNIAMIN DR. A. AND CHRIS FRASER-JENKINS. Obituary: The Rev. Father Dr. V. S. Manickam S. J. (1944–2012)	236
BERA, S. (See S. DAS)	47
BIRAL, LEONARDO AND JEFFERSON PRADO. First Record of <i>Pellaea ovata</i> (Pteridaceae) from Brazil	83
BOLIGON, A. A. (See T. M. DE SOUZA)	198
BRAGA, M. F. B. M. (See T. M. DE SOUZA)	198
BUENO, D. C. (See T. M. DE SOUZA)	198
CARLQUIST, SHERWIN, EDWARD L. SCHNEIDER, AND KEVIN F. KENNEALLY. SEM Studies on Tracheids of Lycopodiaceae; Observations on Adaptations in <i>Phylloglossum</i>	273
CARVALHO, FERNANDA ANTUNES, ALEXANDRE SALINO, AND CHARLES EUGENE ZARTMAN. New Country and Regional Records from Brazilian Side of Neblina Massif	228
CHANG, Y.-M. (See T.-Y. HSIEH)	283
CHENG, X. (See K. M. ZHANG)	208
CHUNG, J. M. (See M. Y. CHUNG)	256
CHUNG, MI YOON, JORDI LÓPEZ-PUJOL, JAE MIN CHUNG, KI-JOONG KIM, AND MYONG GI CHUNG. Low Within Population Genetic Variation and High Among Population Differentiation in <i>Cyrtomium falcatum</i> (L.f.) C. Presl (Dryopteridaceae) in Southern Korea: Inference of Population-Establishment History	256
COSTA, J. G. M. (See T. M. DE SOUZA)	198
COSTA, J. G. M. (See M. F. B. MORAIS-BRAGA)	154
COUTINHO, H. D. M. (See T. M. DE SOUZA)	198
COUTINHO, H. D. M. (See M. F. B. MORAIS-BRAGA)	154
DA ROCHA, J. B. T. (See T. M. DE SOUZA)	198
DAS, SAYANTANI, MAUMITA BANDYOPADHYAY, AND SUBIR BERA. Optimization of Protocol for Isolation of Genomic DNA from Leaves of <i>Selaginella</i> Species Suitable for RAPD Analysis and Study of their Genetic Variation	47

DE ARIAS, A. R. (See T. M. DE SOUZA)	198
DE SOUZA, TEOGENES, M., MARIA F. B. M. BRAGA, ROGÉRIO A. SARAIVA, PABLO A. NOGARA, DIONES C. BUENO, ALINE A. BOLIGON, MARGARETH L. A. FONE, JOÃO B. T. DA ROCHA, MIRIAM ROLON, CELESTE VEGA, ANTONIETA ROJAS DE ARIAS, JOSE G. M. COSTA, IRWIN R. ALENCAR DE MENEZES, HENRIQUE D. M. COUTINHO AND ANTÔNIO A. F. SARAIVA. Cytotoxic and Tripanocide Activities of <i>Pityrogramma calomelanos</i> (L.) Link.	198
DEVO, J. A. (See G. K. GREER)	32
DIAMOND, HOPE L., HEATHER R. JONES, AND LUCINDA J. SWATZELL. The Role of Aquaporins in Water Balance in <i>Cheilanthes lanosa</i> (Adiantaceae) Gametophytes	11
DIETRICH, M. A. (See G. K. GREER)	32
DONG, YUAN-HUO, QING-FENG WANG, AND ROBERT WAHITI GITURU. Effect of Habitat Modification on the Distribution of the Endangered Aquatic Fern <i>Ceratopteris pteridoides</i> (Parkeriaceae) in China	136
DUTTA, S. (See R. GHANTA)	216
FANG, Y. M. (See K. M. ZHANG)	208
FONE, M. L. A. (See T. M. DE SOUZA)	198
FRASER-JENKINS, C. (See DR. A. BENNIAMIN)	236
GABRIEL Y GALÁN, JOSE MARÍA and CARMEN PRADA. Farina Production by Gametophytes of <i>Argyroschisma nivea</i> (Poir.) Windham (Pteridaceae) and its Implications for Cheilanthoid Phylogeny	191
GANAIE, A. H. (See B. AHAD)	224
GHANTA, REBECA, SIKHA DUTTA, AND RADHANATH MUKHOPADHYAY. Occurrence of Dark Septate Endophytes in the Sporophytes of <i>Christella dentata</i>	216
GI CHUNG, M. (See M. Y. CHUNG)	256
GITURU, R. W. (See Y.-H. DONG)	136
GÓMEZ-DOMÍNGUEZ, H. (See M. A. PÉREZ-FARRERA)	233
GREER, GARY K., MARGARET A. DIETRICH, JOSEPH A. DEVO, AND APRIL REBERT. The Effects of Exogenous Cytokinin on the Morphology and Gender Expression of <i>Osmunda regalis</i> Gametophytes	32
GUEDES, G. M. M. (See M. F. B. MORAIS-BRAGA)	154
HATCH, K. A. (See T.-Y. HSIEH)	283
HIRAYAMA, Y. (See C. TSUTSUMI)	55
HOSHIZAKI, B. J. (See D. G. YANSURA)	69
HSIEH, TUNG-YU, KENT A. HATCH, YUAN-MOU CHANG. <i>Phlegmariurus changii</i> (Huperziaceae), a New Hanging Firmoss from Taiwan	283

IAMONICO, DUILIO. Lectotypification of <i>Marselia quadrifolia</i> L. (Marseliaceae)	289
JACONO, C. C. (See W. M. WHITTEN)	114
JONES, H. R. (See H. L. DIAMOND)	11
KAMACHI, HIROYUKI AND MUNENORI NOGUCHI. Negative Gravitropism in Dark-Grown Gametophytes of the Fern <i>Ceratopteris richardii</i>	147
KATO, M. (See C. TSUTSUMI)	55
KENNEALLY, K. F. (See S. CARLQUIST)	273
KHOLIA, B. S. <i>Botrychium simplex</i> E. Hitchcock: a New Moonwort for the Indian Himalayan Mountains	86
KIELING-RUBIO, MARIA, A., AND PAULO G. WINDISCH. <i>Elaphoglossum montanum</i> , a New Species from Southern Brazil	78
KIM, K.-J. (See M. Y. CHUNG)	256
LABIAK, P. H. (See J. B. PEREIRA)	174
LABIAK, PAULO H., A New Species and a New Hybrid in the Grammitid Fern Genus <i>Stenogrammitis</i> (Polypodiaceae)	161
LIU, J. H. (See K. M. ZHANG)	208
LÓPEZ-MOLINA, MA. E. (See M. A. PÉREZ-FARRERA)	233
LÓPEZ-PUJOL, J. (See M. Y. CHUNG)	256
LORSCHBITTER, M. L. (See J. B. PEREIRA)	174
MAGRINI, SARA AND ANNA SCOPPOLA. Agravitropic Growth of the Early Leaves of Apogamous Sporophytes of <i>Dryopteris tyrrhena</i>	181
MARTÍNEZ, OLGA, G., AND IGNACIO VILTE. The Structure of Petioles in <i>Pteris</i> (Pteridaceae)	1
MARTÍNEZ-MELÉNDEZ, N. (See M. A. PÉREZ-FARRERA)	233
MATOS, F. B. (See C. M. MYNSSEN)	167
MENEZES, I. R. A. (See M. F. B. MORAIS-BRAGA)	154
MORAIS-BRAGA, M. F. B., T. M. SOUZA, K. K. A. SANTOS, J. C. ANDRADE, G. M. M. GUEDES, S. R. TINTINO, C. E. SOBRAL-SOUZA, J. G. M. COSTA, I. R. A. MENEZES, A. A. F. SARAIVA, AND H. D. M. COUTINHO. Antimicrobial and Modulatory Activity of Ethanol Extract of the Leaves from <i>Lygodium venustum</i> SW.	154
MORAN, ROBBIN C., The Life of Barbara Joe Hoshizaki (1928–2012)	252
MUKHOPADHYAY, R. (See R. GHANTA)	216
MYNSSEN, CLAUDINE M., AND FERNANDO B. MATOS. <i>Diplazium fimbriatum</i> (Athyraceae), a New Species from Brazil	167
NAGALINGUM, N. S. (See W. M. WHITTEN)	114

NOGARA, P. A. (See T. M. DE SOUZA)	198
NOGUCHI, M. (See H. KAMACHI)	147
PEREIRA, JOVANI B., PAULO G. WINDISCH, MARIA L. LORSCHAITTER, AND PAULO H. LABIAK. <i>Isoëtes mourabaptistae</i> , a New Species from Southern Brazil	174
PÉREZ-FARRERA, MIGUEL A., MA. EVANGELINA LÓPEZ-MOLINA, NAYELY MARTÍNEZ- MELÉNDEZ, AND HÉCTOR GÓMEZ-DOMÍNGUEZ. New Records Of Ferns From Chiapas, Mexico	228
PRADA, C. (See J. M. GABRIEL Y GALÁN)	191
PRADO, J. (See L. BIRAL)	83
PRYER, KATHLEEN M., The Distinguished Legacy of DMB: Donald MacPhail Britton (1923–2012)	241
REBERT, A. (See G. K. GREER)	32
RESHI, Z. A. (See B. AHAD)	224
ROLON, M. (See T. M. DE SOUZA)	198
ROTHFELS, C. J., E. M. SIGEL, AND M. D. WINDHAM. <i>Cheilanthes feei</i> T. Moore (Pteridaceae) and <i>Dryopteris erythrosora</i> (D.C. Eaton) Kunze (Dryopter- idaceae) New for the Flora of North Carolina	184
SALINO, A. (See F. A. CARVALHO)	228
SANTOS, K. K. A. (See M. F. B. MORAIS-BRAGA)	154
SARAIVA, A. A. F. (See T. M. DE SOUZA)	198
SARAIVA, A. A. F. (See M. F. B. MORAIS-BRAGA)	154
SARAIVA, R. A. (See T. M. DE SOUZA)	198
SCHNEIDER, E. L. (See S. CARLQUIST)	273
SCOPPOLA, A. (See S. MAGRINI)	181
SIGEL, E. M. (See C. J. ROTHFELS)	184
SOBRAL-SOUZA, C. E. (See M. F. B. MORAIS-BRAGA)	154
SOUZA, T. M. (See M. F. B. MORAIS-BRAGA)	154
SWATZELL, L. J. (See H. L. DIAMOND)	11
TINTINO, S. R. (See M. F. B. MORAIS-BRAGA)	154
TSUTSUMI, C., Y. HIRAYAMA, M. KATO, Y. YATABE-KAKUGAWA, AND S.-Z. ZHANG. Molecular Evidence on the Origin of <i>Osmunda × mildei</i> (Osmundaceae)	55
TURNER, M. D. (See R. A. WHITE)	198
VEGA, C. (See T. M. DE SOUZA)	91
VILTE, I. (See O MARTÍNEZ)	1

WANG, Q.-F. (See Y.-H. DONG)	136
WHITE, RICHARD A., AND MELVIN D. TURNER. The Anatomy and Occurrence of Foliar Nectaries in <i>Cyathea</i> (Cyatheaceae)	91
WHITTEN, W. MARK, COLETTE C. JACONO, AND NATHALIE S. NAGALINGUM. An Expanded Plastid Phylogeny of <i>Marsilea</i> with Emphasis on North American Species	114
WINDHAM, M. D. (See C. J. ROTHFELS)	184
WINDISCH, P. G. (See M. A. KIELING-RUBIO)	78
WINDISCH, P. G. (See J. B. PEREIRA)	174
YANSURA, DANIEL G., AND BARBARA J. HOSHIZAKI. The Tree Fern Highland Lace is a Cultivar of <i>Sphaeropteris cooperi</i>	69
YATABE-KAKUGAWA, Y. (See C. TSUTSUMI)	55
ZARTMAN, C. E. (See F. A. CARVALHO)	228
ZHANG, G. F. (See K. M. ZHANG)	208
ZHANG, H. J. (See K. M. ZHANG)	208
ZHANG, S.-Z. (See C. TSUTSUMI)	55
ZHANG, K. M., J. H. LIU, X. CHENG, G. F. ZHANG, Y. M. FANG, AND H. J. ZHANG. Effects of <i>Ageratina adenophora</i> on Spore Germination and Gametophyte Development of <i>Neochheiropteris palmatopedata</i>	208

Volume 102, number 1, January–March, pages 1–90, issued 21 June 2012
Volume 102, number 2, April–June, pages 91–190, issued 17 July 2012
Volume 102, number 3, July–September, pages 191–240, issued 15 October 2012
Volume 102, number 4, October–December, pages 241–298, issued 4 April 2013

INFORMATION FOR AUTHORS

Authors are encouraged to submit manuscripts pertinent to pteridology for publication in the *American Fern Journal*. Manuscripts should be sent to the editor at hauk@denison.edu. Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication.

Authors should adhere to the following guidelines; manuscripts not so prepared may be returned for revision prior to review. **Electronic submission is strongly encouraged**; however if it is necessary to submit hard copy, please submit one copy of the manuscript and include a review copy of illustrations and originals of illustrations. After acceptance, please submit final versions of manuscripts via FTP (contact the managing editor for instructions), email, or on diskette or CD ROM (see below for figure formatting). If submitting hard copies, use standard 8.5 by 11 inch paper of good quality, not "erasable" paper. **Double-space manuscripts throughout**, including title, author's names and full addresses, a short, informative abstract, key words, text (including heads and keys), literature cited, tables (separate from text), and figure captions (grouped as consecutive paragraphs separate from figures). Arrange parts of manuscript in order just given. Include author's name and page number in the upper right corner of every sheet, and provide an abbreviated running title. Provide margins of at least one inch (25 mm) all around on typed pages. Do not submit right-justified text, avoid footnotes, and do not break words at end of lines. Make table headings and figure captions self-explanatory. Use S.I. (metric) units for all measures (e.g., distance, elevation, weight) unless quoted or cited from another source (e.g., specimen citations). For nomenclatural matters (i.e., synonymy and typification), use one paragraph per basionym (see *Regnum Veg.* 58:39–40, 1968). Abbreviate titles according to *Botanico-Periodicum-Huntianum* (Lawrence *et al.*, 1968, Hunt Botanical Library, Pittsburgh) and its supplement (1991). References cited only as part of nomenclatural matter are not included in literature cited. For shorter notes and reviews, omit the abstract and put all references parenthetically in text. Use *Index Herbariorum* (*Regnum Veg.* 120:1–693, 1990; or <http://websun.nybg.org/bsci/ih/>) for designations of herbaria. For more detailed instructions on manuscript preparation, see <http://amerfernsoc.org/>.

Illustrations should be proportioned to fit page width (5 inches or 12.5 cm) with caption ultimately to be included on the same page. Halftone and color images should be scanned at a minimum of 300 pixels per inch (ppi). Line art should be scanned at 1200 ppi whenever possible. Please note that nearly all images that are downloaded from the Internet or that are in JPEG or GIF format will be 72 dpi and not acceptable for the printing process. Indicate the file format of the graphics. Please submit image files in TIFF (preferable) or EPS format. Provide margins of at least 25 mm on all illustrations. For continuous-tone illustrations, design originals for reproduction without reduction or by uniform amount. In composite blocks, abut edges of adjacent photographs. Avoid combining continuous-tone and line-copy in single illustrations or blocks. Coordinate sequence and numbering of figures (and tables), with order of citation in text. Explain scales and symbols in figures themselves, not in captions. Include a scale and reference to latitude and longitude in each map.

Instructions for downloading electronic proofs and reprint order forms are sent to authors by the printer. Authors should send proof corrections of corrected proofs to the editor and reprint orders to the printer. Authors will be assessed charges for extensive alterations made after type has been set.

For other matters of form or style, consult recent issues of the *American Fern Journal* and *The Chicago Manual of Style*, 14th ed. (1993, Univ. of Chicago Press, Chicago). Occasionally, departure from these guidelines may be justified. Authors are encouraged to consult the editor for assistance with any aspect of manuscript preparation.

Papers of longer than 32 printed pages may be sent to the Editor of *Pteridologia* (memoir Editor, see journal cover page 2).

PTERIDOLOGIA ISSUES IN PRINT

The following issues of *Pteridologia*, the memoir series of the American Fern Society, are available for purchase:

1. Wagner, David H. 1979. Systematics of *Polystichum* in Western North America North of Mexico. 64 pp. \$10.00 plus postage and handling.

2A. Lellinger, David B. 1989. The Ferns and Fern-allies of Costa Rica, Panama, and the Chocó (Part 1: Psilotaceae through Dicksoniaceae). 364 pp. \$32.00 plus postage and handling.

3. Lellinger, David B. 2002. A Modern Multilingual Glossary for Taxonomic Pteridology. 263 pp. \$28.00 plus postage and handling.

14. Hirai, Regina Y., and Jefferson Prado. 2012. Monograph of Moranopteris (Polypodiaceae). 113 pp. \$28.00 plus postage and handling.

For orders and more information, please contact our authorized agent for sales at: Missouri Botanical Garden Press, P.O. Box 299, St. Louis, MO 63166-0299, tel. 314-577-9534 or 877-271-1930 (toll free). For online orders, visit: <http://www.mbgpress.org>.

FIDDLEHEAD FORUM

The editor of the Bulletin of the American Fern Society welcomes contributions from members and non-members, including miscellaneous notes, offers to exchange or purchase materials, personalia, horticultural notes, and reviews of non-technical books on ferns.

SPORE EXCHANGE

Mr. Brian S. Aikin, 3523 Federal Ave, Everett, WA 98201-4647 (spores.afs@comcast.net), is Director. Spores exchanged and lists of available spores sent on request. <http://amerfernsoc.org/sporexy.html>

GIFTS AND BEQUESTS

Gifts and bequests to the Society enable it to expand its services to members and to others interested in ferns. Back issues of the Journal and cash or other gifts are always welcomed and are tax-deductible. Inquiries should be addressed to the Membership Secretary.

**VISIT THE AMERICAN FERN SOCIETY'S
WORLD WIDE WEB HOMEPAGE:**

<http://amerfernsoc.org/>