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# Additions to the Pteridophyte Flora of Kalimantan, Indonesian Borneo

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Abstract.—A list of 59 newly recorded Pteridophytes from the Bukit Baka- Bukit Raya National Park in Central Kalimantan is presented. Nine species are recorded for Indonesian Borneo (Kalimantan) for the first time: *Diplazium polycarpum*, *Pronephrium peltatum* var. *persetiferum*, *Pteris asperula*, *Selliguea enervis*, *Sphaerostephanos latebrosus*, *Sphaerostephanos reconditus*, *Syngramma quinata*, *Tectaria tricuspis*, and *Trichomanes humile*. Previously, *Sphaerostephanos reconditus*, and *Tectaria palmata* var. *dimorpha* were only known from their type localities.

Borneo lies in the center of the Southeast Asian archipelago and is divided politically between the Republic of Indonesia (Kalimantan), the Malaysian Federation (Sarawak and Sabah), and Brunei Darussalam. Bukit Baka-Bukit Raya National Park (181.090 ha) includes the highest regions of the Schwaner Mountains (Bukit Baka: 1617 m, Bukit Raya: 2278 m), and forms the borderland between West and Central Kalimantan. In the past the area was densely covered with lowland forest, dipterocarp hill forest, submontane forest, and montane forest, but since the end of the 20th century the area is suffering severe illegal logging. The temperature is relatively constant throughout the year and ranges between 25°C and 35°C in the lowlands. In Borneo there are very few months with rainfall below 200 mm, and most regions of the mountainous inland receive between 2000 and 4000 mm per year (MacKinnon *et al.*, 1996).

Malesia is a major hotspot of global biodiversity and harbors one of the greatest numbers of species of ferns and fern allies in the world. Next to New Guinea, Borneo holds the greatest species richness of Pteridophytes in this floristic region (Kato, 1990). However, surprisingly few studies are conducted on the ferns of Kalimantan (ca. 70% of Borneo), and West and Central Kalimantan are practically forgotten by botanists and scientists in general. In these provinces, the only significant collections of Pteridophytes were made in Bukit Baka- Bukit Raya National Park: Tumbang Riang and Bukit Raya (Nooteboom, 1987, material deposited in Leiden), and Kalaweit Research Station and surroundings (this study).

During an expedition in the summer of 2001, the first author collected 117 fertile specimens of Pteridophytes in the area around Kalaweit Research Station. This field station is situated in the southern part of the National Park

[1] Corresponding author.

(00°52′S; 112°29′E, alt. ca 350 m), in the drainage of the Sungai Bemban. We identified our collection using material deposited in Ghent (GENT) and Leiden (L), and arrived at 88 species: 59 representing new records for the National Park and Central Kalimantan, 9 of which also being new records for Indonesian Borneo (Kalimantan). Based on Nooteboom (1987) and Jarvie *et al.* (1998) we had counted 76 pteridophytes for Bukit Baka- Bukit Raya National Park, our 59 new records thus raise the total number of species for the National park to 135.

## Material and Methods

Our list, which only includes the new records, is based on the herbarium specimens collected by the first author during her fieldwork from August to October 2001 in the Bukit Baka-Bukit Raya National Park at the Kalaweit Research Station. All specimens are deposited in the herbarium of Ghent University (GENT). The list is ordered alphabetically by family, genus and species. Circumscriptions of families and genera follow Kramer and Green (1990); for easier reference to Holttum's 2nd edition of Flora of Malaya (1968) and to other treatments in Flora Malesiana, we include a limited number of synonyms. Bibliographic references to Bornean material, based on material primarily deposited in BM, BO, E, K, KYO, L, MICH, MSC, SAR, SING, and US, are added for each species. It should be noted that only the herbarium material deposited in GENT and L was studied for the present publication.

## Aspleniaceae

***Asplenium macrophyllum*** Sw.

**Indonesia:** Central Kalimantan, *Bellefroid EB34* (new for Central Kalimantan); East Kalimantan, Sebulu (Iwatsuki and Kato, 1980b); South Kalimantan, Sungai Doesson; Banjarmasin (Miquel, 1869). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: widespread in the Paleotropics: from Madagascar and the Mascarene Islands to Polynesia, north to Nepal, southern China, and the Philippines.

***Asplenium nitidum*** Sw. [= *Asplenium glaucophyllum* Alderw.]

**Brunei:** Seria Distr., Kampong Mendaram (Tagawa, 1965). **Indonesia:** Central Kalimantan, *Bellefroid EB37, EB96, EB107* (new for Central Kalimantan); South Kalimantan, Sungai Doesson and Mount Pamatton (Miquel, 1869). **Malaysia:** Sarawak, Bintulu Distr., Ulu Sungai Sinonok; Sungai Latai (Tagawa, 1965).

*General distribution*: western Malesia (Borneo, Java, Malay Peninsula, Sumatra), north to Indochina, very rare in South India, Sri Lanka and the Himalayas (Nepal to Assam), east to Samoa.

***Asplenium phyllitidis*** D.Don. subsp. ***malesicum*** Holttum

**Brunei:** Tutong Distr., Lake Merimbun (Tagawa, 1965); Temburong Distr., Bukit Bangar; Sungai Tongkat; Sungai Lacquan (Tagawa, 1965). **Indonesia:** Central Kalimantan, *Bellefroid EB13* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Gunung Batukenye (Iwatsuki & Kato, 1980b). **Malaysia:** Sabah, Sipitang; Penampang; Telupid: Bukit Tawai; Tawau (Tagawa, 1976); Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Bintulu Distr., Sungai Kakus (Tagawa, 1965); Mardi Distr., Gunung Mulu (Tagawa, 1965, Parris *et al.*, 1984).

*General distribution*: throughout Malesia, north to Thailand and Vietnam.

***Asplenium squamulatum*** Blume

**Indonesia:** Central Kalimantan, *Bellefroid EB7*, *EB38*, *EB67* (new for Central Kalimantan); East Kalimantan, Sekatak; Tabang; Jelini; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sarawak, Bintulu Distr., Sungai Bejangang; Bukit Kana (Tagawa, 1965); Mardi Distr., Gunung Mulu (Tagawa, 1965).

*General distribution*: endemic to Malesia.

***Asplenium subaquatile*** Ces.

**Indonesia:** Central Kalimantan, *Bellefroid EB61*, *EB66* (new for Central Kalimantan); East Kalimantan, Jelini (Iwatsuki and Kato, 1980b); West Kalimantan, along Sungai Melaku (Van Steenis, 1981). **Malaysia:** Sarawak, Bintulu Distr. (Van Steenis, 1981); Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo.

***Asplenium tenerum*** G.Forst. var. ***retusum*** C. Chr.

**Brunei:** Temburong Distr., Sungai Tongkat (Tagawa, 1965). **Indonesia:** Central Kalimantan, *Bellefroid EB76* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Jelini; Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Bintulu Distr., Ulu Sungai Minah; Sungai Keyan (Tagawa, 1965).

*General distribution*: *A. tenerum* is found from Sri Lanka to Polynesia, and north to China; var. *retusum* is reported for the Malay Peninsula and Borneo.

## Blechnaceae

***Blechnum finlaysonianum*** Hook. *et* Grev.

**Indonesia:** Central Kalimantan, *Bellefroid EB52*, *EB95* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Gunung Batukenye;

Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980b); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980b). **MALAYSIA:** SABAH, Telupid; Bukit Tawai (Tagawa, 1976); Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Bukit Kana; Sungai Minah; Ulu Sungai Minah; Bukit Keyan (Tagawa, 1967); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Malesia (Holttum, 1968).

## CYATHEACEAE

***Cyathea contaminans*** (Wall. *ex* Hook.) Copel.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB43* (within Malesia this is the most widespread species of *Cyathea* [Holttum, 1963], surprisingly it appears to be new for Central Kalimantan); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Penampang (Tagawa, 1974); Mount Kinabalu (Tagawa, 1974; Parris *et al.*, 1992).

*General distribution*: from India to Malesia, north to Myanmar (Holttum, 1963).

## DAVALLIACEAE

***Davallia denticulata*** (Burm.f.) Mett. *ex* Kuhn var. ***denticulata*** [=*Davallia chaerophylloides* (Poir.) Desv. (*fide* Nooteboom, 1998a)].

**BRUNEI:** TEMBURONG DISTR., Bukit Bangar (Tagawa, 1965). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB19*, *EB98* (new for Central Kalimantan); EAST KALIMANTAN, Samarinda; Sebulu; Tabang; Gunung Beratus (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Bukit Kana (Tagawa, 1965).

*General distribution*: Paleotropics: from southern and tropical Africa, and Madagascar, to Southeast Asia, China (Hainan), Malesia, Queensland, and the Pacific (Samoa, Tahiti) (Nooteboom, 1998a).

## DENNSTAEDTIACEAE

***Lindsaea napaea*** Alderw.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB103* (new for Central Kalimantan); EAST KALIMANTAN, Gunung Buduk Kelawak; Gunung Long Api; Gunung Kongbotak; Gunung Buduk Rakik; Tadur Bangar-Pa Panik; Gunung Batu Linanit; Gunung Leputung; Sinar Baru-Ruan Ruwan (Iwatsuki and Kato, 1983a).

*General distribution*: endemic to southern Peninsular Thailand and western Malesia (Kramer, 1971; Iwatsuki and Kato, 1983a).

***Lindsaea oblanceolata*** Alderw.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB53* (new for Central Kalimantan); EAST KALIMANTAN, Tarakan (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Southeast Asia (Annam, Thailand) and western Malesia (Kramer, 1971).

***Lindsaea obtusa*** J.Sm. *in* Hook.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB88* (new for Central Kalimantan); EAST KALIMANTAN, Sekatak; north of Sebulu; Tabang; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Malesia, north to Taiwan, south to Queensland, and east to the Solomon Islands (Kramer, 1971).

***Lindsaea parallelogramma*** Alderw.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB105* (according to Kramer [1971] this species is "apparently quite common" on Borneo; on his map showing the distribution of this species Bornean localities are concentrated in southwestern Sarawak, Brunei, Sabah, and eastern Kalimantan. Noting that Kramer [1971] could not locate several localities for this island, this taxon appears to be new for Central Kalimantan); EAST KALIMANTAN, Tarakan; Sekatak; north of Sebulu; Tabang; Jelini; Gunung Batukenye; Gunung Mendam (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992), Gunung Magdalena (Kramer, 1971); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic in western Malesia and peninsular Thailand (Kramer 1971).

***Lindsaea parasitica*** (Roxb. *ex* Griff.) Hieron.

**BRUNEI:** SERIA DISTR., Bukit Teraja (Tagawa and Iwatsuki, 1966). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB55* (new for Central Kalimantan); EAST KALIMANTAN, Sekatak; Gunung Batukenye (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Sungai Minah (Tagawa and Iwatsuki, 1966).

*General distribution*: endemic in western Malesia and peninsular Thailand (Kramer, 1971).

***Microlepia speluncae*** (L.) Moore

**Brunei:** Temburong Distr., Bukit Peradayan (Tagawa, 1965); Seria Distr.: Kampong Mendaram (Tagawa, 1965). **Indonesia:** Central Kalimantan, *Bellefroid EB112* (new for Central Kalimantan); East Kalimantan, Sekatak; Samarinda; Sebulu; Tabang; Gunung Mendam (Iwatsuki and Kato, 1980a); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980a). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992); Tenom (Tagawa, 1974); Tawau (Tagawa, 1974); Sarawak, Mardi Distr., Gunung Mulu (Tagawa, 1965; Parris *et al.*, 1984).

*General distribution*: throughout the Tropics, mainly Paleotropical and apparently rare in the Neotropics.

## Dicksoniaceae

***Cystodium sorbifolium*** (Sm.) J.Sm.

**Indonesia:** Central Kalimantan, *Bellefroid EB51* (new for Central Kalimantan); East Kalimantan, Gunung Kongkat-Gunung Kongbotak; Gunung Kongkat-Tuun Alut Salah (Iwatsuki *et al.*, 1983a). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992), Sarawak, Mardi Distr.: Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: eastern Malesia, and from northern Borneo to the Louisiade Archipelago (Holttum, 1963).

## Dryopteridaceae

***Diplazium polycarpum*** (Copel.) C.Chr.

**Indonesia:** Central Kalimantan, *Bellefroid EB2*, *EB113* (new for Indonesian Borneo). **Malaysia:** Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Christensen, 1913; van Alderwerelt van Rosenburgh, 1917).

***Diplazium riparium*** Holttum [= *Athyrium riparium* (Holttum) Holttum]

**Indonesia:** Central Kalimantan, *Bellefroid EB39* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Jelini (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic in western Malesia (Malay Peninsula, east Sumatra, Borneo).

***Diplazium tomentosum*** Blume [= *Athyrium tomentosum* (Blume) Milde]

**Indonesia:** Central Kalimantan, *Bellefroid EB99*, *EB106* (new for Central Kalimantan); East Kalimantan, Sebulu; Gunung Batukenye; Gunung Mendam

(Iwatsuki & Kato, 1980b); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980b). **MALAYSIA:** SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to peninsular Myanmar (Dickason, 1946) and western Malesia [Malay Peninsula (van Alderwerelt van Rosenburgh, 1909; Holttum, 1968), and Borneo].

***Ctenitis vilis*** (Kunze) Ching

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB89* (new for Central Kalimantan); EAST KALIMANTAN, Sebulu; Long Keluh (Iwatsuki and Kato, 1983b); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980b). **MALAYSIA:** SARAWAK (Holttum, 1991).

*General distribution*: endemic in western Malesia (Malay Peninsula, Sumatra to Ambon), and peninsular Thailand (Holttum, 1991).

***Pleocnemia irregularis*** (C.Presl) Holttum

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB5* (new for Central Kalimantan); EAST KALIMANTAN, Samarinda; Tabang; Sebulu; Jelini; Gunung Mendam (Iwatsuki and Kato, 1980b); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980b). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: throughout Malesia, via southern Myanmar and Thailand north to Vietnam, east to the Pacific (Caroline Is. to Fiji [Holttum, 1991]).

***Pleocnemia olivacea*** (Copel.) Holttum

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB83* (new for Central Kalimantan); EAST KALIMANTAN, Gunung Batukenye; Gunung Mendam (Iwatsuki and Kato, 1980b); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980b). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: western Malesia (Malay Peninsula, Sumatra, Borneo) (Holttum, 1991).

***Tectaria palmata*** (Mett.) Copel. var. ***dimorpha*** Holttum

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB109* (this variety was only known from its type locality in Tabang, East Kalimantan (Holttum, 1991), new for Central Kalimantan); EAST KALIMANTAN, Tabang (type locality) (Holttum, 1991).

*General distribution*: endemic to Borneo (Holttum, 1991).

***Tectaria tricuspis*** (Bedd.) Copel.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB100* (new for Indonesian Borneo). **MALAYSIA:** SARAWAK, LUNDU DISTR., Lundu (Holttum, 1991).

*General distribution*: endemic to the Malay Peninsula and Borneo (Holttum, 1991).

GLEICHENIACEAE

***Gleichenia truncata*** (Willd.) Spreng. var. ***truncata***

**BRUNEI:** BRUNEI DISTR.: east of Brunei Town (Iwatsuki, 1965a). **INDONESIA:** Central KALIMANTAN, *Bellefroid EB44, EB111* (new for Central Kalimantan); EAST KALIMANTAN, Samarinda; Sekatak; north of Sebulu; Gunung Batukenye (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Tagawa, 1974; Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Iwatsuki, 1965a; Parris *et al.*, 1984).

*General distribution*: endemic to Malesia (Holttum, 1959a).

HYMENOPHYLLACEAE

***Hymenophyllum denticulatum*** Sw. [= *Meringium denticulatum* (Sw.) Copel.]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB14* (new for Central Kalimantan); EAST KALIMANTAN, Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., valley of Ulu Sungai Bejangang (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Iwatsuki, 1965a, Parris *et al.*, 1984).

*General distribution*: from Sri Lanka, northeastern India and Bhutan, through Malesia to Fiji (Holttum, 1968).

***Hymenophyllum polyanthos*** Sw. [= *Mecodium polyanthos* (Sw.) Copel.]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB15, EB62, EB86* (new for Central Kalimantan); EAST KALIMANTAN, Tabang; Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Sungai Kakus and Bukit Kana (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Pantropical.

***Trichomanes bimarginatum*** Bosch [= *Microgonium bimarginatum* (Bosch) Bosch]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB11* (new for Central Kalimantan); EAST KALIMANTAN, Sekatak; Gunung Beratus (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992).

*General distribution*: from Sri Lanka to Samoa and Queensland (Holttum, 1968).

***Trichomanes bipunctatum*** Poir. *in* Lam. [= *Crepidomanes bipunctatum* (Poir. in Lam.) Copel.]

**BRUNEI:** TEMBURONG DISTR., along Sungai Lacquan (Iwatsuki, 1965a). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB9, EB23, EB36, EB63* (new for Central

Kalimantan); EAST KALIMANTAN, Sebulu; Tabang; Jelini; Gunung Batukenye; Gunung Beratus; Gunung Mendam (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Sungai Mah to Sungai Sinonok (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Iwatsuki, 1965a; Parris *et al.*, 1984).

*General distribution*: Paleotropics: from Madagascar to Tahiti, north to Thailand (Holttum, 1968; Tagawa and Iwatsuki, 1979).

***Trichomanes humile*** G.Forst. [= *Crepidopteris humilis* (G.Forst.) Copel.]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB6, EB87* (new for Indonesian Borneo). **MALAYSIA:** SARAWAK, BINTULU DISTR., Ulu Sungai Sinonok (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Malesia to Tahiti (Holttum, 1968).

***Trichomanes maximum*** Blume [= *Vandenboschia maxima* (Blume) Copel.]

**BRUNEI:** TEMBURONG DISTR., Sungai Tongkat (Iwatsuki, 1965a). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB101* (new for Central Kalimantan); EAST KALIMANTAN, Gunung Mendam (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Iwatsuki, 1965a; Parris *et al.*, 1984).

*General distribution*: Malesia to Tahiti, north to Thailand and Taiwan (Holttum, 1968).

***Trichomanes singaporeanum*** (Bosch) Alderw. [= *Cephalomanes singaporeanum* Bosch]

**BRUNEI:** TUTONG DISTR., Lake Merimbun (Iwatsuki, 1965a); TEMBURONG DISTR., Bukit Bangar (Iwatsuki, 1965a). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB28, EB93* (new for Central Kalimantan); EAST KALIMANTAN, Tarakan; Sekatak; Sebulu; Tabang (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar (Iwatsuki and Kato, 1980a). **MALAYSIA:** SARAWAK, BINTULU DISTR., Sungai Kakus; along Sungai Puteh; Ulu Sungai Minah (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to the Malay Peninsula and Borneo (Holttum, 1968).

## LOMARIOPSIDACEAE

***Bolbitis sinuata*** (C.Presl) Hennipman

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB80, EB115* (new for Central Kalimantan); EAST KALIMANTAN, Sekatak; Sebulu; Tabang; Gunung Mendam

(Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Sandakan; Gomanton Caves (Tagawa, 1976); Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Malesia, north to the Nicobar Islands and Thailand (Hennipman, 1977).

***Lomariopsis lineata*** (C.Presl) Holttum

**Indonesia:** Central Kalimantan, *Bellefroid EB108* (new for Central Kalimantan); East Kalimantan, Sebulu; Long Keluh; Sungai Menubar (Iwatsuki and Kato, 1983b). **Malaysia:** Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: throughout Malesia (except east New-Guinea), north to southern Myanmar, South Thailand, and South Vietnam (Holttum, 1978).

***Teratophyllum aculeatum*** (Blume) Mett. *ex* Kuhn var. ***aculeatum***

**Indonesia:** Central Kalimantan, *Bellefroid EB16*, *EB65* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Jelini; Gunung Batukenye (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Tawau (Tagawa, 1976); Sarawak, Mardi Distr.: Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: throughout Malesia, north to southern Myanmar (Holttum, 1978).

## Lycopodiaceae

***Lycopodium aellenii*** (Herter) Tagawa [= *Lycopodium horizontale* Alderw. (1912), *non* C.Presl (1825)]

**Indonesia:** Central Kalimantan, *Bellefroid RV9045* (new for Central Kalimantan); East Kalimantan, Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1981). **Malaysia:** Sabah, Mount Kinabalu (Tagawa, 1974); Tenom, Bukit Malatut (Tagawa, 1974).

*General distribution*: southwestern Malesia (Borneo, Java, Sumatra, Timor) (Nessel, 1939).

## Nephrolepidaceae

***Nephrolepis biserrata*** (Sw.) J.Scott

**Indonesia:** Central Kalimantan, *Bellefroid EB60* (new for Central Kalimantan); East Kalimantan, Sekatak; Samarinda; Sebulu (Iwatsuki and Kato, 1980a). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992).

*General distribution*: Pantropical (Holttum, 1968).

POLYPODIACEAE

***Drynaria sparsisora*** (Desv.) T.Moore

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB77* (new for Central Kalimantan); EAST KALIMANTAN, Sebulu; Sekatak; Samarinda; Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1981). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., Sungai Kakus (Tagawa, 1967); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Southeast Asia to Australia (Hovenkamp and Roos, 1998).

***Goniophlebium percussum*** (Cav.) W.H.Wagner & Grether [= *Polypodium verrucosum* Mett.]

**BRUNEI:** TEMBURONG DISTR., Bukit Paradayan (Tagawa, 1967). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB48* (new for Central Kalimantan); EAST KALIMANTAN, Sinar Baru-Ruan Ruwan; Ruan Ruwan-Pa Poon; Gunung Seribu; Gunung Batu Harun (Iwatsuki and Kato, 1984), Sekatak; Sebulu; Samarinda (Iwatsuki and Kato, 1981, as *Polypodium cyatheoides* SW.). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: from Thailand to Australia (Queensland) (Hovenkamp and Rödl-Linder 1998).

***Goniophlebium persicifolium*** (Desv.) Bedd. [= *Polypodium persicifolium* Desv.]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB82* (new for Central Kalimantan); EAST KALIMANTAN, Gunung Beratus (Iwatsuki and Kato, 1981). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992).

*General distribution*: from the Himalayas to the Pacific (Hovenkamp and Rödl-Linder 1998).

***Leptochilus macrophyllus*** (Blume) Noot. var. ***fluviatilis*** (Lauterb.) Noot. [= *Colysis fluviatilis* Ching]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB32* (new for Central Kalimantan); EAST KALIMANTAN, Jelini; Gunung Batukenye (Iwatsuki and Kato, 1981, under *Colysis fluviatilis* Ching).

*General distribution*: endemic to Borneo and the Philippines (Nooteboom, 1998b).

***Selliguea enervis*** (Cav.) Ching [= *Crypsinus enervis* (Cav.) Copel.]

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB73* (new for Indonesian Borneo). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Indochina and Malesia (Hovenkamp, 1998).

***Selliguea lateritia*** (Baker) Hovenkamp [= *Crypsinus taeniophyllus* (Copel.) Copel.] [= *Selliguea heterocarpa auct. non* Blume]

**Indonesia:** Central Kalimantan, *Bellefroid EB94* (apparently very common in Borneo with many recent records [Hovenkamp, 1998], but apparently not previously recorded for Central Kalimantan); East Kalimantan, Gunung Batu Harun; Gunung Paris; Gunung Leputung; Gunung Long Api; Ruan Ruwan; Gunung Batu Linanit; Pa Panik-Pa Pelinitan; Long Pa Riman-Gunung Tapa Sia; Long Pa Binuang; Gunung Kongkat (Iwatsuki and Kato, 1984, under *Crypsinus taeniophyllus*). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992).

*General distribution*: Indochina, peninsular Thailand and Malesia (Borneo, Malay Peninsula, Philippines, Sulawesi, Sumatra, and New-Guinea) (Hovenkamp, 1998).

## Pteridaceae

***Pteris asperula*** J.Sm.

**Indonesia:** Central Kalimantan, *Bellefroid EB42* (new for Indonesian Borneo). **Malaysia:** Sarawak, Bintulu Distr., Bukit Kana (Tagawa, 1965); Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic in Malesia (Holttum, 1968).

***Pteris furcans*** Baker

**Indonesia:** Central Kalimantan, *Bellefroid EB84* (new for Central Kalimantan); East Kalimantan, Tabang; Tanjung Lapang; Sebulu (Iwatsuki and Kato, 1983a). **Malaysia:** Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo and Sumatra (van Alderwerelt van Rosenburgh, 1909, 1917).

***Syngramma alismifolia*** (C.Presl) J.Sm.

**Brunei:** Seria Distr.: Bukit Puan (Tagawa and Iwatsuki, 1966); Tutong Distr.: Lake Merimbun (Tagawa and Iwatsuki, 1966); Temburong Distr.: Sungai Lacquan (Tagawa and Iwatsuki, 1966). **Indonesia:** Central Kalimantan, *Bellefroid EB49*, *EB70* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Gunung Batukenye; Gunung Mendam (Iwatsuki and Kato, 1980a). **Malaysia:** Sabah, Telupid; Bukit Tawai; Tawau (Tagawa, 1975); Mount Kinabalu (Tagawa, 1975; Parris *et al.*, 1992); Sarawak, Bintulu Distr., Sungai Kakus; Ulu Sungai Minah; Sungai Keyan; Bukit Kana (Tagawa and Iwatsuki, 1966); Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Malesia (Holttum, 1968).

***Syngramma quinata*** (Hook.) Carruth.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB49*, *EB70* (new for Indonesian Borneo). **MALAYSIA:** SARAWAK, MARDI DISTR., Gunung Mulu (Tagawa and Iwatsuki, 1966).

*General distribution*: Malesia to Fiji (not known from Java and probably absent from southeast Malesia) (Holttum, 1968).

SCHIZAEACEAE

***Lygodium circinnatum*** (Burm.f.) Sw.

**BRUNEI:** BRUNEI DISTR., east of Brunei town (Iwatsuki, 1965a); TEMBURONG DISTR.: south of Labu (Iwatsuki, 1965a). **INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB91* (new for Central Kalimantan); EAST KALIMANTAN, Samarinda; Sebulu (Iwatsuki and Kato, 1980a); SOUTH KALIMANTAN, Gunung Besar; south of Banjarmasin (Iwatsuki and Kato, 1980a). **MALAYSIA:** SABAH, Telupid; Bukit Tawai; Bukit Doji (Tagawa, 1974), Mount Kinabalu (Tagawa, 1974; Parris *et al.*, 1992); SARAWAK, BINTULU DISTR., along Sungai Bejangang (Iwatsuki, 1965a); MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: Sri Lanka, northeast India to southern China, Thailand, and from the Nicobar Islands throughout Malesia to Micronesia, the New Hebrides, and the Solomon Islands (Holttum, 1959b).

SELAGINELLACEAE

***Selaginella boschai*** Hieron.

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB1a* (*Selaginella boschai* was previously only known from its type locality near Sambas in West Kalimantan [Hieronymus, 1911], and from Gunung Mulu, Sarawak [Parris *et al.*, 1984]. New for central Kalimantan.); WEST KALIMANTAN, Sambas (Hieronymus, 1911). **MALAYSIA:** SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Reed, 1965–1966).

***Selaginella lobbii*** H.J.Veitch *ex* A. Braun

**INDONESIA:** CENTRAL KALIMANTAN, *Bellefroid EB4* (new for Central Kalimantan); EAST KALIMANTAN, Sekatak; Jelini; Sebulu; Samarinda; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1981). **MALAYSIA:** SABAH, Mount Kinabalu (Parris *et al.*, 1992); SARAWAK, MARDI DISTR., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Reed, 1965–1966).

***Selaginella paxii*** Hieron.

**Indonesia:** Central Kalimantan, *Bellefroid EB1b* (new for Central Kalimantan); East Kalimantan, Sekatak; Jelini; Tabang; Sebulu; Gunung Mendam (Iwatsuki and Kato, 1981); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1981). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1991); Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Reed, 1965–1966).

## Thelypteridaceae

***Mesophlebion motleyanum*** (Hook.) Holttum [= *Thelypteris motleyana* (Hook.) Holttum]

**Brunei:** Seria Distr., from Kampong Mendram to Bukit Teraja; Bukit Teraja (Iwatsuki, 1965b); Temburong Distr., Bangar to Sungai Betia (Iwatsuki, 1965b). **Indonesia:** Central Kalimantan, *Bellefroid EB58* (new for Central Kalimantan); East Kalimantan, Sekatak; Tabang; Sebulu; Gunung Mendam (Iwatsuki and Kato, 1980b). **Malaysia:** Sarawak, Bintulu Distr., Bukit Kana; Sungai Mah to Sungai Sinonok; Sungai Bejangang (Iwatsuki, 1965b); Mardi Distr., Gunung Mulu (Iwatsuki, 1965b; Parris *et al.*, 1984).

*General distribution*: Peninsular Thailand and western Malesia (Borneo, Malay Peninsula, Sumatra) (Holttum, 1981).

***Pneumatopteris truncata*** (Poir.) Holttum [= *Cyclosorus truncatus* (Poir.) Farwell]

**Brunei:** Temburong Distr.: Bukit Paradayan (Iwatsuki, 1965b). **Indonesia:** Central Kalimantan, *Bellefroid EB41, EB110* (new for Central Kalimantan); East Kalimantan, Jelini; Sebulu (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Mount Kinabalu (Holttum, 1981; Parris *et al.*, 1992); Sarawak, Bintulu Distr., Sungai Bejangang (Iwatsuki, 1965b); Mardi Distr., Gunung Mulu (Iwatsuki, 1965b, Holttum, 1981; Parris *et al.*, 1984).

*General distribution*: Sri Lanka and southern India, northeast India to south China, west Malesia (Holttum, 1981).

***Pronephrium peltatum*** (Alderw.) Holttum var. ***persetiferum*** Holttum

**Indonesia:** Central Kalimantan, *Bellefroid EB46* (new for Indonesian Borneo). **Malaysia:** Sabah, Mount Kinabalu (type locality) (Parris *et al.*, 1992); Sarawak, Mardi Distr., Gunung Mulu (Holttum, 1981; Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Holttum, 1981).

***Sphaerostephanos heterocarpus*** (Blume) Holttum [= *Cyclosorus heterocarpus* (Blume) Ching var. *heterocarpus*]

**Brunei:** Temburong Distr., Sungai Tongkat (Iwatsuki, 1965b). **Indonesia:** Central Kalimantan, *Bellefroid EB85*, *EB116* (new for Central Kalimantan); East Kalimantan, Sekatak; Jelini; Sebulu; Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Bintulu Distr., Sungai Minah; Bukit Kana; Ulu Sungai Sinonok (Iwatsuki, 1965b); Mardi Distr., Gunung Mulu (Iwatsuki, 1965b; Holttum, 1981; Parris *et al.*, 1984).

*General distribution*: from peninsular Thailand and south China (Hainan, Guangdong), through Malesia to northern Queensland, east to the Solomon Islands, New Hebrides, Fiji, and Samoa (Holttum, 1981).

***Sphaerostephanos latebrosus*** (Kunze *ex* Mett.) Holttum [= *Cyclosorus heterocarpus* (Blume) Ching var. *glaucostipes* (Bedd.) Holttum]

**Indonesia:** Central Kalimantan, *Bellefroid EB102* (new for Indonesian Borneo). **Malaysia:** Sabah, Mount Kinabalu (Holttum, 1981; Parris *et al.*, 1992); Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: endemic in western Malesia and the Philippines (Holttum, 1981).

***Sphaerostephanos reconditus*** Holttum

**Indonesia:** Central Kalimantan, *Bellefroid EB75* (This taxon was previously only known from its type locality on Gunung Mulu [Holttum, 1981]. Our record shows that it may be present in other areas in and around the central mountain range of Borneo. New for Indonesian Borneo.). **Malaysia:** Sarawak, Mardi Distr., Gunung Mulu (Holttum, 1981; Parris *et al.*, 1984).

*General distribution*: endemic to Borneo (Holttum, 1981).

## Vittariaceae

***Antrophyum callifolium*** Blume

**Indonesia:** Central Kalimantan, *Bellefroid EB3* (new for Central Kalimantan); East Kalimantan, Sekatak; Sebulu; Tabang; Jelini; Gunung Batukenye; Gunung Mendam; Gunung Beratus (Iwatsuki and Kato, 1980b); South Kalimantan, Gunung Besar (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Mount Kinabalu (Parris *et al.*, 1992); Sarawak, Mardi Distr., Gunung Mulu (Parris *et al.*, 1984).

*General distribution*: tropical Southeast Asia (Holttum, 1968).

***Monogramma trichoidea*** J.Sm.

**Indonesia:** Central Kalimantan, *Bellefroid EB81* (new for Central Kalimantan); East Kalimantan, Tabang; Gunung Batukenye (Iwatsuki and Kato, 1980b). **Malaysia:** Sabah, Tenom (Tagawa, 1975); Mount Kinabalu (Parris *et al.*, 1992).

*General distribution*: endemic to west Malesia (Borneo, Malay Peninsula) and the Philippines (Holttum, 1968).

## Discussion

Because large parts of the forests of Indonesian Borneo have been degraded, without prior inventories, to farmlands, production forests, and areas of desertification, it is difficult to obtain a general biogeographical picture of the flora of Kalimantan. About 10% of the pteridophytes found in the area studied are endemic to Borneo. More than half (54%) have a wide distribution (pantropical or panmalesian), 23% are west Malesian, 9% are east and west Malesian, and 3% are west and south Malesian. The local species composition shows significant resemblance to those of Gunung Mulu and Mount Kinabalu: for Borneo 20% of our species only occur on Gunung Mulu, 14% only on Mount Kinabalu, and 48% are restricted to our area and both Gunung Mulu and Mount Kinabalu. Only 18% grow neither on Gunung Mulu nor on Mount Kinabalu.

Malaysian Borneo (Sabah and Sarawak) is better known pteridologically than Kalimantan because fern research was conducted mainly on Mount Kinabalu (4101 m) in Sabah (Parris *et al.*, 1992), and on Gunung Mulu (2377 m) in Sarawak (Parris *et al.*, 1984). Parris *et al.* (1992) reported 609 pteridophytes for Mount Kinabalu, and gave an estimate of about 446 species for Gunung Mulu (Parris *et al.*, 1984, 1992).

Because the lowland and hilly regions of Gunung Mulu, and especially Mount Kinabalu, had practically disappeared before significant inventories could take place, a comparison with our area situated between 300 and 600 m is not evident. For the more comparable Danum Valley (150–550 m) in eastern Sabah, Parris and Edwards (unpublished; in Parris *et al.*, 1992), reported 152 taxa. Because the list of Danum Valley taxa is not available, an exact comparison with our area is impossible. However, if we decrease our provisional list of 135 Bukit Baka-Bukit Raya National Park species with the 25 taxa Nooteboom (1987) collected on Bukit Raya (2278 m), this leaves us with 110 species for the lower areas of the National Park. Accepting a diversity similar to that in the Danum Valley (152 taxa) would mean that about 73% of the lowland species are known to date. This seems a reasonable estimate considering that we did not collect sterile plants or high growing epiphytes.

Because of the similar altitudinal range of Bukit Raya (2278 m) and Gunung Mulu (2377 m), the expected number of species for the entire Bukit Baka- Bukit Raya National Park may be comparable to that of Gunung Mulu, or to about 446 taxa. Our provisional number of 135 species would then represent only 30% of this total. Considering that about 73% of the lowland flora is known this clearly demonstrates the need of more and thorough inventories at higher elevations of Bukit Raya.

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# Herbivore Damage to Ferns Caused by a Chrysomelid Beetle from Lower Gangetic Plains of West Bengal, India

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Abstract.—This paper records the occurrence of a polyphagous beetle, *Schenklingia bhaumiki* (Coleoptera: Chrysomelidae), feeding on ten fern species growing in the Lower Gangetic Plains of West Bengal, India viz., *Christella dentata*, *Ampelopteris prolifera*, *Cyclosorus sp.*, *Pteris vittata*, *Nephrolepis cordifolia*, *N. exaltata*, *Adiantum philippense*, *Drynaria propinqua*, *Pyrrosia adnascens* and *Phymatosorus scolopendria* for the first time. The adult beetles are leaf surface scraper and skeletonize the lamina. The larvae are leaf miners and produce linear–blotch mines between the epidermal layers with continuous spiral black frass. Young leaves of all ten species of ferns are significantly less damaged than mature ones indicating that both the adults and the larvae attack leaves of all ages. Herbivore damage of the beetle infested ferns ranged from 1.94% to 25.47% and 2.68% to 54.86% for scraping feeding and mining feeding respectively. Among the host ferns, the members of Thelypteridaceae viz., *Christella* (Scraping feeding 25.47%; mining feeding 54.86%), *Ampelopteris* (Scraping feeding 24.10%; mining feeding 53.60%) and *Cyclosorus* (Scraping feeding 16.06%, mining feeding 27.12%) suffered maximum herbivore damage. Interspecific variation of plant size and biogeographic range of the fern species are not related to herbivore damage. Insects may perhaps attack fewer ferns than angiosperms, but there is no evidence that ferns are generally less damaged than angiosperms.

Pteridophytes, especially ferns in a broad sense, are among the most primitive land plants and are generally considered to be difficult plants for herbivores to exploit (Eastop, 1973; Hendrix, 1977, 1980; Cooper-Driver, 1978; Soo Hoo and Fraenkel, 1964; Kaplains *et al.*, 1967). This underutilization of ferns by herbivores has been attributed to host resistance factors such as texture (Soo Hoo and Fraenkel, 1964), toxins (Muenscher, 1939), amino acid deficiency (Smith and Agiza, 1951), poor nutritional composition (Moon and Pal, 1949), and the presence of cyanogens (Lawton, 1976) and thiaminase (Somogyi, 1949). Although it has also been suggested by Auerback and Hendrix (1980), Balick *et al.* (1978) and Gerson (1979) that this assumption may not be well founded and may be due to less documentation of herbivory on ferns. Fossilized ferns showing damage attributed to herbivores are known from the Carboniferous (Smart and Hughes, 1973) to the Upper Triassic (Ash, 1999, 2000). Recent work on fern herbivory, however has revealed that a fairly large number of insects of different groups efficiently utilize fern hosts for their growth and development (Mound, 1967; Room *et al.*, 1981; Ottosson and Anderson, 1983; Lawton and MacGarvin, 1985; Mohan-Daniel and Chandrasekar, 1986; Misra *et al.*, 1986; Kraus *et al.*, 1993; Bera *et al.*, 1994; Bera and Ghorai, 1995a, b, 1997a, b, 1999a, b; Gilman and Cooper-Driver, 1998; Pemberton, 1998; Patra and Bera, 2002; Bera *et al.*, 2003; Mehltreter and Tolome, 2003, Barker *et al.*, 2005). The objective of this study was to contribute new data to the field of insect-fern interactions and to demonstrate that

although insects may attack fewer ferns than angiosperms, ferns are not totally free from insect attack. This paper presents a first report of interactions between a chrysomelid beetle and ferns in India.

## Materials and Methods

Beetle-fern interactions were recorded from ten different sites in the lower Gangetic Plains of West Bengal (21°25′–26°50′ N, 86°30′–89°85′ E), India during May 1999–December 2000. Field photographs were taken while the adult beetles and their larvae were feeding on the ferns, at which time they were caught alive, kept in small vials, and then sent to the Coleoptera Department, Zoological Survey of India, Calcutta, for identification. Infested host ferns were preserved on herbarium sheets numbered (CU/ IFI 1–11) and deposited in the pteridophyte repository, Department of Botany, Calcutta University, Calcutta.

Feeding habit, occurrence and abundance of the beetle on different fern hosts were studied directly in the field. Abundance and numbers of beetles per leaf were estimated by counting the number of adult beetles present on each leaf of the host ferns once a week during each month of the study period. Mean values of beetles/leaf/month were calculated for each species. Monthly average temperature and rainfall for the study area and mean number of beetle/leaf/month were graphically represented to establish the influence of environmental parameters on the seasonal abundance of the beetle. Herbivore damage in terms of leaf area lost and and total leaf area were estimated by placing individual leaflets on millimeter graph paper, tracing the outline of both the leaflets as well as their damaged areas and then counting the included squares. Finally, mean herbivore damage as a percentage of leaf area was calculated. In each case a minimum of 50 plants were studied. Herbivore damage in young and mature leaves were compared using student's t- test. Correlation coefficients were used to establish the relationship between leaf age and herbivore damage. Measurement of the longest leaf, which we considered as a measure of the plant size, was also taken. Leaves of less than 3 months were considered to be young whereas those of more than 3 months were considered mature. In order to test whether the beetle and its larvae fed only on ferns as opposed to angiosperms, a feeding trial was conducted. Adult beetles and their larvae were exposed to randomly selected terrestrial and aquatic ferns and angiosperms growing in the study area. These feeding trials were conducted on three occasions for a minimum of seven days in nylon gauze cages enclosed in perforated polythene sheets. Control sets with beetles and their recorded host ferns were also maintained.

For scanning electron microscopic studies the beetles were dried, coated with gold and scanned using a Leica Leo S-440 electron microscope and photographed with a High Resolution Record Unit (HRRU) using 35 mm automatic camera. Protein, total amino acids, total sugar, and total phenol of young and mature leaves were estimated following the methods employed by Lowry *et al.* (1951), Lee and Takahasi (1966), Somogyi (1945), and Malik and

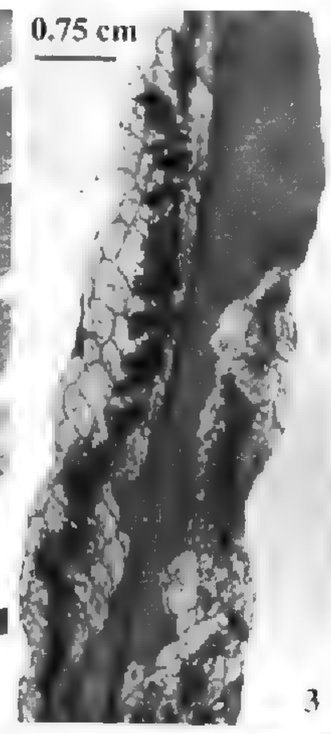
Singh (1980) respectively. Fecal pellets, frass, of feeding insects, were dissolved in a small amount of water to form a suspension. Drops of this suspension were mounted in glycerin jelly and examined under the microscope for residual undigested plant parts.

## Results

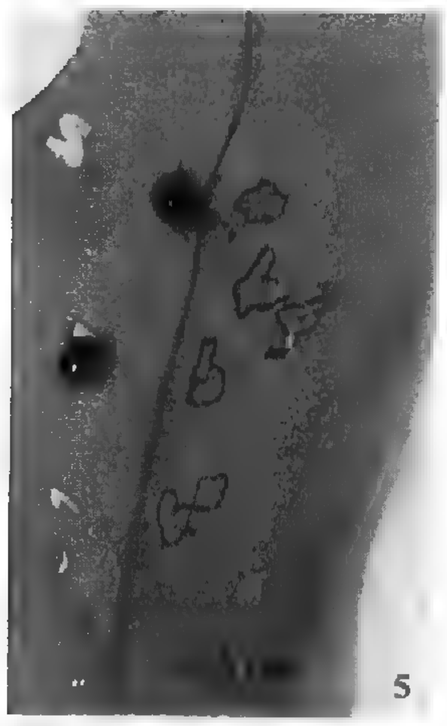
The adult beetles, and larvae found feeding on different species of ferns of Lower Gangetic plains of West Bengal were identified as *Schenklingia bhaumiki* Basu and Sengupta.(C. R. Basu collection 18.iv. 1976, ZSI, Calcutta). Csiki and Heikertinger (1940) proposed the name *Schenklingia* for *Eucycla*. The genus has 25 species distributed in India, Sri Lanka, Taiwan, Indonesia, New Guinea, Solomon Island and Micronesia (Basu and Sengupta, 1981). The Indian species of the genus include *Schenklingia bhaumiki, S. heteropunctata* and *S. himalayensis* (Basu and Sengupta, 1981). The adult beetles are small (2.25 mm in length, 1.90 mm in width), somewhat rounded, strongly convex, shiny, and reddish brown with 5 pairs of black spots on the elytra (Basu and Sengupta, 1981). Earlier, Basu and Sengupta (1981) collected adults of the *Schenklingia bhaumiki* for their taxonomic studies from ferns in Darjeeling (West Bengal) and Sikkim Himalayas. There are three larval instars but the external morphologies of the larvae are more or less the same across all instars. Full grown larvae are 7 × 1.5 mm, considerably flattened and show characteristics of leaf miners. They have a dark head, and strong, well developed mandibles, each with two sharp chitinous blades. The body is yellow with transverse furrows and ridges. The larval period is 42 days and the third instar larva is pupated on leaflets and lasts up to 14 days.

The adult beetles are host surface scrapers and the damage they cause is very distinctive. They are confined primarily to the central portions of the fronds and tissues between the veins are selectively eaten, partially skeletonizing the lamina, leaving it riddled with holes (Figs. 1, 2, 5 & 10). The upper and lower leaflet surfaces of the host ferns are indiscriminately scraped by the adult beetles (Fig. 14) leaving the veins intact. Subsequent to the removal of some of the laminar tissue, much of the remaining tissue becomes necrotic and brown, giving the infested region a distinctive pattern (Figs. 1, 2, 5, 7 & 10). The beetle larvae are leaf miners, and eat tissue between the upper and lower epidermis. The damage appears as linear-blotch mines with continuous spiral black frass trails (Figs. 2, 3, 4 & 6). Initially the mines are about 1.2 mm–1.5 mm broad, but as the larvae grow, the trails gradually become broader (10 mm–15 mm or more). A mine constructed by one larva is generally confined to one side of the midrib (Figs. 3 & 4) but occasionally may cross it. Inside the mine, the larvae are capable of moving rapidly backward and forward and when removed from the mine, they are capable of re-entering the leaflet. They are leaf defoliators and may consume the whole laminar tissue of the frond leaving only a light-colored, wrinkled upper and lower epidermis behind (Figs. 3, 4, 6, 8 & 9).

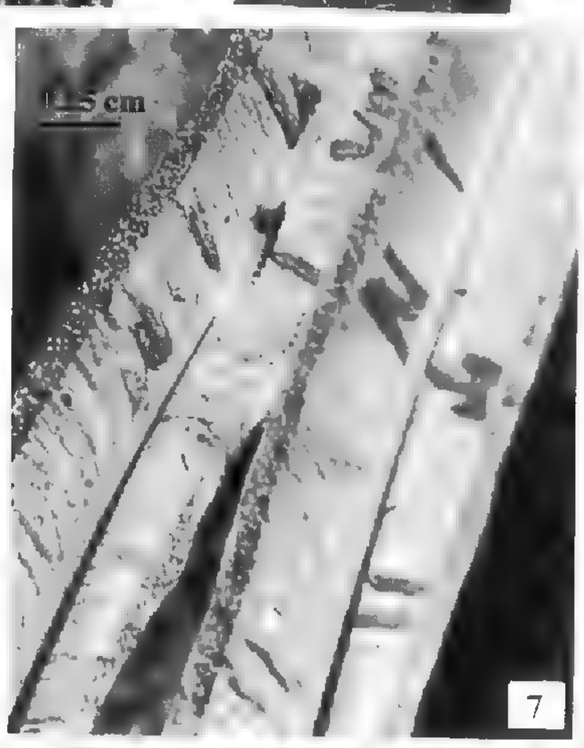


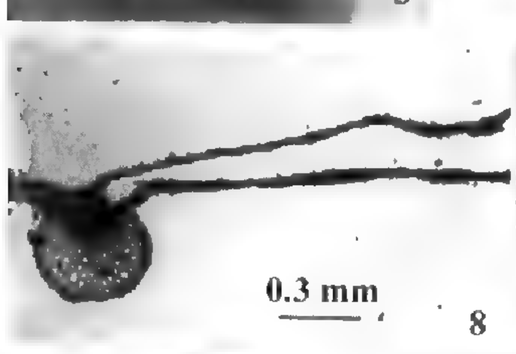


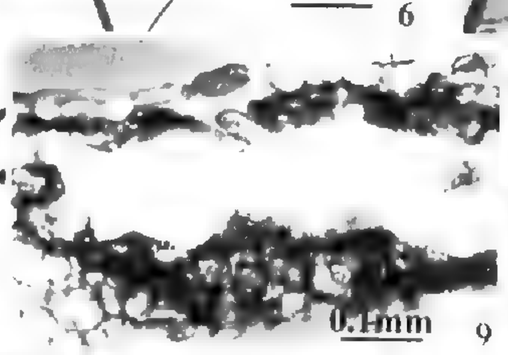


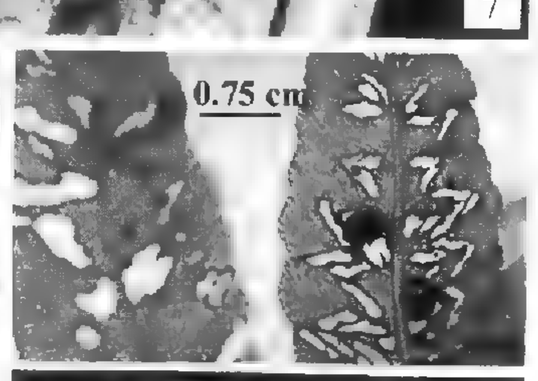




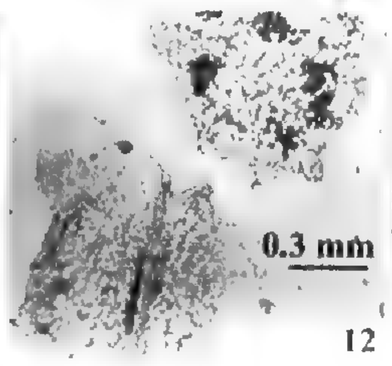


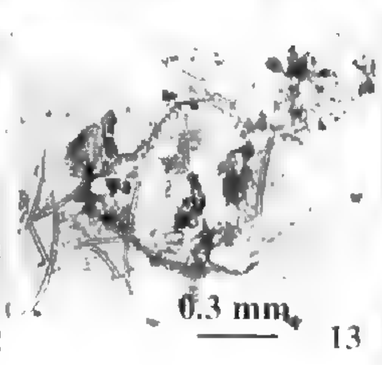



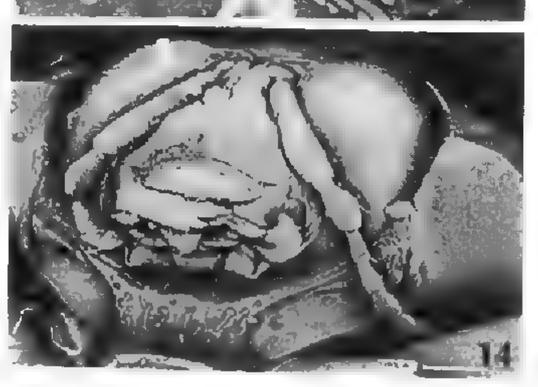








FIGS. 1, 5, 10. Beetle infestation on *Christella dentata*, *Drynaria propinqua* and *Ampleopteris prolifera*. Figs. 2, 3, 4, 6. Linear blotch mines showing continuous spiral black frass on the leaflets of *Christella dentata* (fig.2), *Drynaria propinqua*(fig.3), *Ampelopteris prolifera*(fig.4) and *Adiantum philippense*(fig.6) caused by beetle larvae. Fig. 7. Feeding scars on the abaxial leaflet surface of *Pteris vittata*; note larva on the costa. Figs. 8, 9. Sections of mined leaflets from *Pteris vittata* and *Drynaria propinqua* showing loss of mesophyll tissue. Fig. 11. Mined leaflet of. *Drynaria propinqua* with continuous spiral black frass deposited by larvae. Figs. 12, 13. Fecal matter from the mining larvae and adult beetles showing undigested remains of mesophyll tissue and acicular trichomes with fragments of laminar tissue respectively. Fig. 14. SEM *Schenklingia bhaumiki* showing mouthparts of adult beetle.

Young leaves of the ten fern species were significantly less damaged than mature leaves in both scraping and mining feeding ($t > 3.44$, $P < 0.05$; $t > 3.83$, $P < 0.05$; respectively) (Table 1). The occurrence and abundance of the beetle was purely seasonal. They started appearing in June, became very abundant

TABLE 1. Herbivore damage (%) of young and mature leaves of ten Indian fern species of Lower Gangetic Plains of West Bengal fed by the adult beetle *Schenklingia bhaumiki* (scraping feeding) and its larvae (mining feeding), expressed as percent leaf area loss. Data are mean ± standard errors, N = 50.

| Species | Scraping feeding | | Mining feeding | |
|---|---|---|---|---|
| | Young | Mature | Young | Mature |
| 1.*Christella dentata* | 2.69 ± 0.2 | 25.47 ± 1.66 | 2.95 ± 0.2 | 54.86 ± 0.7 |
| 2.*Ampelopteris prolifera* | 1.35 ± 0.1 | 24.10 ± 1.7 | 2.12 ± 0.3 | 53.60 ± 0.2 |
| 3.*Cyclosorus sp.* | 0.36 ± .02 | 16.06 ± 1.2 | 0.69 ± 0.03 | 27.12 ± 0.6 |
| 4.*Adiantum philippense* | Not found | 13.22 ± 1.2 | Not found | 23.19 ± 0.1 |
| 5.*Pteris vittata* | 0.53 ± .05 | 7.07 ± 0.7 | 1.21 ± 0.1 | 19.37 ± 0.9 |
| 6.*Nephrolepis cordifolia* | 0.36 ± .01 | 8.25 ± 1 | 0.53 ± .02 | 14.72 ± 0.1 |
| 7.*Nephrolepis exaltata* | 0.35 ± .01 | 6.15 ± 1.2 | 0.35 ± 0.04 | 9.25 ± 0.2 |
| 8.*Pyrrosia adnascens* | 0.27 ± .01 | 3.5 ± 1 | Not found | Not found |
| 9.*Drynaria propinqua* | 0.29 + .04 | 2.79 ± 1 | 0.50 ± .05 | 22.55 ± 0.1 |
| 10.*Phymatosorus scolopendria* | Not found | 1.94 ± 0.3 | Not found | 2.68 + 0.2 |

from July to mid August, decreased in numbers from the end of August, and completely disappeared by December. This seasonal behavior of the beetle was found to be correlated with atmospheric temperature and precipitation (Fig. 15). The number of adult beetles per leaf was high in July–August when monthly average temperature (mean maximum) and precipitation were 25°C–31°C and 14.1 mm respectively. With the decrease of atmospheric temperature and precipitation in the subsequent months the number of beetles per leaf

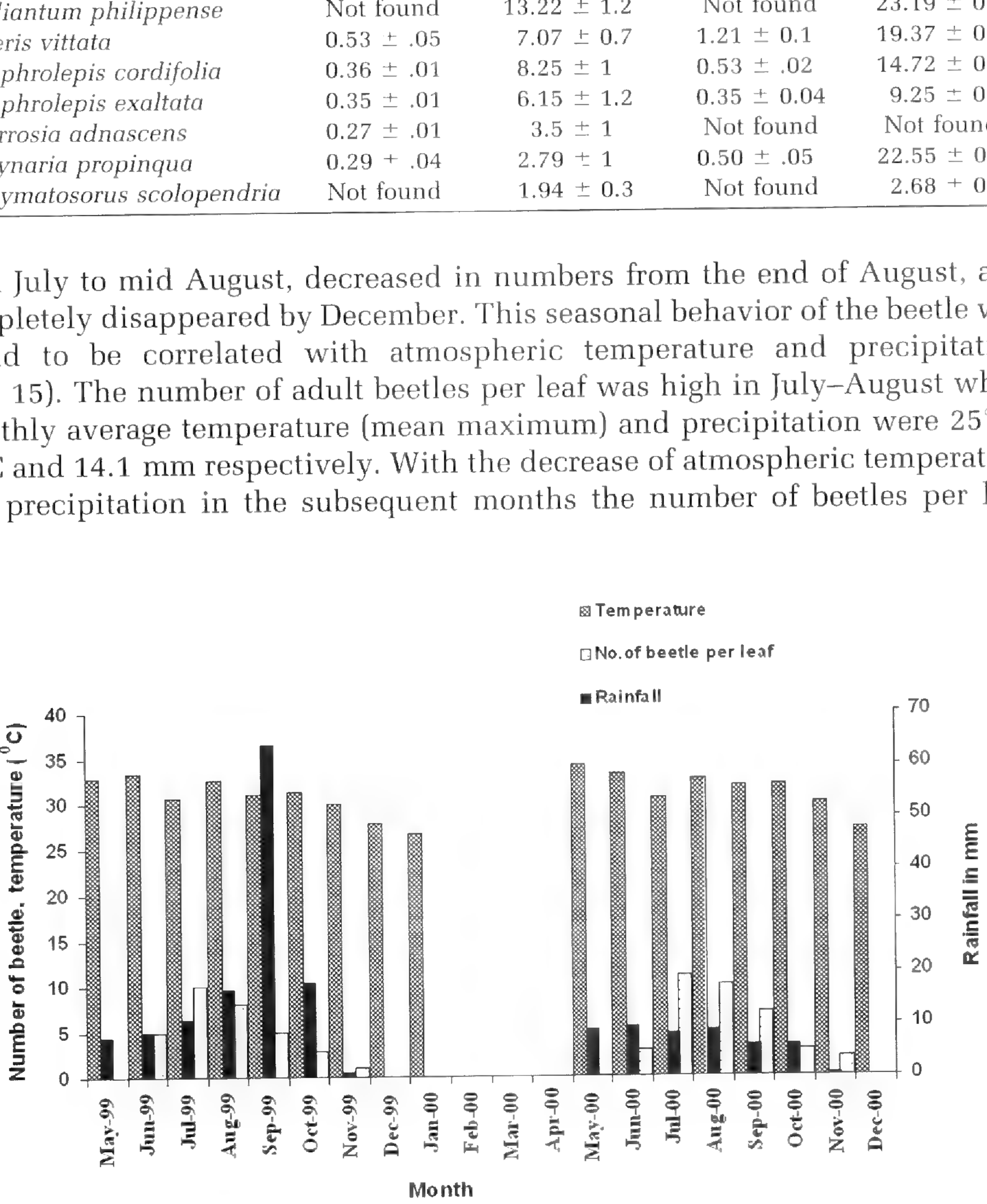



FIG. 15. Correlation between seasonal abundance of adults of *Schenklingia bhaumiki* on the most preferred host fern *Christella dentata* with mean maximum temperature and average monthly rainfall in Kolkata, India (1999–2000).

decreased and completely disappeared in December when atmospheric temperature felt sharply to 22 °C and precipitation became almost nil.

The interspecific variation of plant size was not correlated with herbivore damage to the host ferns (r = − 0.07) (Table 3). The beetle was specific to ferns and fed exclusively on selected species of ferns of Lower Gangetic Plains of West Bengal. No feeding occurred on aquatic ferns (*Marsilea sp.*, *Azolla sp.*, *Salvinia sp.*) or angiosperms (*Oryza sp.*, *Colocasia sp.*, *Solanum sp.*, *Lantana sp.*, and *Parthenium* sp.) growing in association with the host ferns, when tested in vitro where the beetles and larvae were provided with only single food sources.

Biochemistry of the fern frond varied quantitatively with its age. The results of the present investigation showed that young leaves of the most preferred (*Christella*, *Ampelopteris*, *Cyclosorus*) and the least preferred (*Phymatosorus*) host fern species have higher amounts of phenol, sugar, and protein than in mature leaves. Total amino acid levels ran in the reverse direction (Table 4). Analysis of fecal pellets of adult beetles showed the presence of undigested remains of cuticle, epidermal hairs, and mesophyll tissue (Fig. 13) whereas analyses of larval frass showed the remains of mesophyll tissue only (Fig. 12).

## Discussion

Strong and Levin (1979) hypothesized that ferns are subject to less herbivory than angiosperm herbs, shrubs and trees. If ferns are less frequently attacked by herbivores, it can be hypothesized that they should show less damage than angiosperms. The results of the present study do not support this hypothesis. In the present study, leaf damage in ten fern species ranged from 1.94% to 25.47% and from 2.68% to 54.86% for scraping and mining feeding activities respectively. These data are comparable to reported levels of leaf damage on angiosperms of 10.9% (Coley and Aide,1991) and on ferns of 5.8, 6.1, 11.1% (Mehltreter and Tolome, 2003), and 38% (Balick *et al.*, 1978). Although these variations in reported herbivore damage may be a consequence of differences among species in plant phenology, changes in herbivore pressure at different study sites, or diverse applied methodologies, the general hypothesis that ferns are generally less damaged by herbivores than angiosperms lacks evidence.

The herbivores caused more damage to mature fern fronds than young ones. Ottosson and Anderson (1983) and Lawton (1976) suggested that nutritional quality and levels of plant protection compounds are lower in mature fronds than in young fronds. When phenol is sufficiently synthesized it combines with sugars form tannins. These tannins combine irreversibly with available protein to form indigestible complexes, which result in nutritional deficiency of the host plants. This in turn affects the percentage of infestation and host preference (Raman and Ananthakrishnan, 1986). In the present study, a higher abundance of beetles and a higher herbivore damage were recorded on *Christella dentata* than on *Phymatosorus scolopendria* (Table 1 & 2). High concentrations of phenol and sugar, along with available protein, in *Phymatosorus* appear to act as repellent to beetle infestation. This interspecific

TABLE 2. Feeding behavior of the adult beetle on different species of ferns in West Bengal, India. Data are mean ± standard deviation.

| | Host | Feeding surface | Insect abundance/leaf |
|---|---|---|---|
| **Thelypteridaceae** | *Christella dentata* | Upper | 5.6 ± 3.2 |
| | *Ampelopteris prolifera* | Upper | 4.4 ± 2.6 |
| | *Cyclosorus sp.* | Lower | 3.9 ± 2.3 |
| **Adiantaceae** | *Adiantum philippense* | Upper | 2.8 ± 1.5 |
| **Pteridaceae** | *Pteris vittata* | Lower | 2 ± 1 |
| **Nephrolepidaceae** | *Nephrolepis cordifolia* | Upper | 1.7 ± 1.09 |
| | *Nephrolepis exaltata* | Upper | 1.3 ± 1.1 |
| **Polypodiaceae** | *Pyrrosia adnascens* | Upper | 1.5 ± 0.7 |
| | *Drynaria propinqua* | Lower | 1.08 ± 0.64 |
| | *Phymatosorus scolopendria* | Lower | 1 ± 0 |

variation in biochemistry makes *Christella* and *Phymatosorus* the most and the least preferred hosts, respectively. Similarly, the higher levels of herbivory in mature fern fronds may be attributed to differences in biochemical composition (protein, phenol and sugar) compared to young fronds (Table 4). Whereas, these data help explaine the differences in herbivory between *Christella* and *Phymatosorus*, they fail to explain interspecific variation in herbivory among the thelypterid ferns, *Christella*, *Ampelopteris* and *Cyclosorus*. Thus the involvement of certain morphological features of thelypterid ferns in the regulation of herbivory cannot be ruled out.

Two correlations between plant features and herbivore damage have been reported in literature. First, herbivore damage may increase with plant size (Marquis, 1992), perhaps because larger plants offer more resources and are easier to locate. Second, the biogeographic range of the plant species appears to affect the number of plant-animal interactions. Species with wider ranges should have more herbivores (Strong, 1979; Cooper-Driver, 1985) and consequently may suffer more damage. The present study however does not confirm these hypotheses. There is insufficient data to draw a final conclusion but comparisons of plant size, biogeographic range, and herbivore damages of the ferns in the present study show no such trends (Table 3).

Physical factors such as temperature, light, precipitation and humidity help regulate insect abundance because insects are cold-blooded animals and their growth, development, occurrence and abundance are largely dependent on these physical factors. In the present study, the number of beetles per host was higher during the rainy season, July to August, when the atmospheric conditions were amenable to their growth and development but insect numbers gradually declined at the end of rainy season. This study showed that herbivore damage is higher in thelypterid ferns (Table 1) than other host ferns of this study, perhaps due to lower phenol content, and thus may be considered to be the preferred host. From the present insect- fern interaction, it is clear that certain species of ferns are susceptible to the attack of the beetle *Schenklingia bhaumiki* and that aquatic ferns (*Azolla*, *Salvinia*, *Marsilea*) and certain angiosperms (*Oryza*, *Lantana*, *Colocasia*, *Parthenium*, *Solanum*) were

TABLE 3. Herbivore damage of mature leaflets, biogeographic range and maximum size of ferns from Lower Gangetic Plains of West Bengal, India.

| Host | Herbivore damage % ± s.d. | Biogeographic range | Maximum leaf size (cm) |
|---|---|---|---|
| *Christella dentata* | 25.47 ± 1.66 | Throughout the tropics and subtropics | 80 |
| *Ampelopteris prolifera* | 24.10 ± 1.75 | India, Nepal, Bhutan, Burma, Philippines, China, South Africa, Australia, New Caledonia | 68 |
| *Cyclosorus sp.* | 16.06 ± 1.2 | India, Nepal, Bhutan, Burma, Philippines, China, South Africa, Australia, New Caledonia | 90 |
| *Adiantum philippense* | 13.22 ± 1.2 | Throughout the tropics and subtropics | 40 |
| *Pteris vittata* | 7.07 ± 0.7 | Throughout the tropics and subtropics | 40 |
| *Nephrolepis cordifolia* | 8.25 ± 1 | Throughout the tropics and subtropics | 105 |
| *Nephrolepis exaltata* | 6.16 ± 1.2 | Throughout the tropics and subtropics | 132 |
| *Pyrrosia adnascens* | 3.5 ± 1 | India, China, Formosa, Malaysia to Polynesia | 13 |
| *Drynaria propinqua* | 2.79 ± 1 | India, Burma, China, Malay Peninsula, Malesian Islands | 65 |
| *Phymatosorus scolopendria* | 1.94 ± 0.3 | India, China, Formosa, Malaysia to Polynesia | 134 |

not susceptible to the beetle infestation. This may be due to nutritional inadequacy or to high levels of deterrent chemicals. The parameters responsible for such selective feeding of the beetle are, however, yet to be determined.

Included among the ferns studied are those with aesthetic (*Ampelopteris prolifera*, *Christella dentata*, *Adiantum philippense*, *Nephrolepis cordifolia*), food (*Ampelopteris prolifera*), and potential medicinal value (*Adiantum philippense*, *Nephrolepis cordifolia*, *Drynaria propinqua*) (Vasudeva, 1999). Young leaves of *Ampelopteris prolifera* and *Nephrolepis cordifolia* are cooked as leafy vegetables by local tribal people. The fronds of *Adiantum philippense* are used to fight fever, dysentery, asthma and bronchitis. Powdered rhizomes are used for dog bites and snakebite by local tribal people. Rhizomes of *Nephrolepis cordifolia* are mixed with water dropped from hair while bathing and administered to women orally once during their menstrual period for permanent sterility (Henry *et al.*, 1996). Thus herbivore damage to these

TABLE 4. Biochemical analysis of young and mature leaves of the most and least preferred host ferns of *Schenklingia bhaumiki*. Bracketed figures correspond to values for young leaves.

| Species | Amino acids (mg/g) | Phenol (mg/g) | Sugar (mg/g) | Protein (mg/g) |
|---|---|---|---|---|
| *Christella dentata* | 17.55 (13.56) | 0.123 (1.45) | 68.42 (76.11) | 3.10 (6.25) |
| *Ampelopteris prolifera* | 42.25 (32.5) | 0.29 (1.55) | 74.26 (91.55) | 3.94 (11.36) |
| *Cyclosorus* sp. | 38.11 (31.10) | 0.137 (1.73) | 113.5 (119.33) | 2.50 (3.75) |
| *Phymatosorus scolopendria* | 12.00 (18.30) | 32.5 (56.0) | 560 (725) | 1.20 (2.50) |

economically important ferns is of concern to the beneficiaries in Gangetic West Bengal, India and measures should be taken to insure their survival in the wild.

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# Gametophytic and Sporophytic Responses of *Pteris* spp. to Arsenic

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Abstract.—Few plant species have demonstrated the ability to hyperaccumulate heavy metals from contaminated soil. Recently, *Pteris vittata* L. has been identified as a hyperaccumulator of arsenic. Because gametophytic development is an essential stage in the fern life cycle, impacts of heavy metal hyperaccumulation on gametophytic and sporophytic tissue must be investigated if successional bioremediation efforts are to be implemented successfully. Our research showed that sporophytes as well as gametophytes of *P. vittata* are capable of As uptake and accumulation. Increased As (~ 2500 ppm) did not inhibit spore germination, and deleterious effects on gametophyte morphology were observed only after extended time periods on media with extremely high As concentrations (≥600 ppm). Six other *Pteris* species varied in ability to germinate on As-containing media. Sporophytes of *P. vittata* showed no adverse effects when exposed to the highest soil As levels (1650 ppm); in fact, root proliferation was observed in areas of increased As concentration (250 ppm). Foliar application of an arsenical herbicide (calcium acid methanearsonate) to sporophytes resulted in decreased chlorophyll and carotenoid concentrations. Phosphate additions inhibited As uptake by sporophytes, indicating As uptake involves the phosphate transport system.

Arsenic (As), a group 15 metalloid known for its toxicity, commonly occurs in earth's crustal rocks and soil but is minimally present in and often highly detrimental to biological organisms (Cullen and Reimer, 1989). While As concentrations in the environment are often low and non-problematic ($<10$ mg kg$^{-1}$ in soil and $<1$ mg kg$^{-1}$ in plants), anthropogenic activities, such as mining, industry, and agriculture as well as natural geophysical processes elevate As levels (Adriano, 1986; Meharg *et al.*, 1994; Brandstetter *et al.*, 2000; Schmöger *et al.*, 2000). Present techniques for decontamination of soils are costly and highly destructive to ecosystems by often requiring the physical removal of soil for chemical treatment or by rendering the substrate static (Lombi *et al.*, 2000; McGrath *et al.*, 2001).

Phytoremediation, using plants to ameliorate contaminated water or soil, is a much less disruptive procedure (McGrath, 1998; Salt *et al.*, 1998). One such method of phytoremediation, phytoextraction, involves sequestration of contaminants in the harvestable portions of a plant for later removal and subsequent disposal (Salt *et al.*, 1998; McGrath *et al.*, 2001). This method is

relatively more cost-effective and environmentally sound than engineering based clean-up processes. Often associated with phytoextraction are hyper-accumulators, plants that are tolerant of metals in plant tissues at levels higher than are found in the surroundings (McGrath *et al.*, 2001; Meharg, 2003).

Sporophytes of the Chinese ladder brake fern (*Pteris vittata* L.) have been discovered to hyperaccumulate As in concentrations up to 2.3% of dry biomass (Ma *et al.*, 2001), showing great potential as phytoremediators. Since this discovery, sporophytes of *Pteris cretica* var. *albolineata* Hooker, *Pteris cretica* '*Wimsetti*', *Pteris longifolia* L., *Pteris umbrosa* R. Br., and *Pityrogramma calomelanos* (L.) Link have also been found to hyperaccumulate As (Francesconi *et al.*, 2002; Visoottiviseth *et al.*, 2002; Zhao *et al.*, 2002).

In this study, the germination of spores as well as growth and morphology of gametophytes of *P. vittata* were assessed after exposure to and growth on As-containing media. Spore germination of six other *Pteris* spp. was examined in response to As. Root uptake and As accumulation by sporophytes of *P. vittata* were studied after growth on soils spiked with As. Competition between arsenic and phosphate for uptake by sporophytes was also studied. Root proliferation was observed directly in split soil rhizotrons with half arsenic-laden soil and half arsenic-free soil. Lastly, survival and plant health, based on chlorophyll and carotenoid levels, were evaluated in response to application of As-containing herbicide.

## Materials and Methods

*Spore collection and growth culturing conditions.*—Mature fertile fronds of naturalized *P. vittata* sporophytes were collected for spore generation from the Ocklawaha River floodplain (near Gainesville, FL). Upon desiccation of fertile fronds for 48 h at room temperature on sheets of paper, the spores released from sporangia were collected. Additionally, sporangia were scraped with a dissecting needle to collect any remaining spores. Once collected, spores were stored in 15 ml centrifuge tubes at 4°C until time for experimentation. Vouchered herbarium specimens of *Pteris vittata* were reposited in the Paul Hollister herbarium at Tennessee Tech University (Cookeville, TN.)

Sterilization and spore plating procedures were performed under a laminar-flow hood (ENVIRCO TT4830 [#10564]). Spores were first surface sterilized in a solution of 25% Chlorox and 0.1% Triton-X in sterile 15 ml centrifuge tubes for 10 min, then rinsed five times (10 min per rinse) with sterile distilled deionized (ddi) water. Spores were aseptically transferred using a 0.4% low melting agarose solution to Petri dishes containing MS salts, 3% sucrose, and 1% agar medium, with pH adjusted to 5.7 using 1 M NaOH (Murashige and Skoog, 1962). Plates were then stored in darkness for 24 h at 4°C and subsequently placed in a growth chamber with a 16/8 h light-dark cycle and 100 $\mu M \cdot m^{-2} \cdot sec^{-1}$ photon flux at 27°C ± 3°C. Upon spore germination, gametophytes were used in subsequent gametophyte experiments. For sporophyte material, gametophytes were grown on the aforementioned growth

media until fertilization occurred. Resultant sporophytes were transferred for growth on appropriate soil or culture media until ready for experimentation.

*Pteris vittata spore germination and gametophyte experiments.*—Surface sterilized spores of *P. vittata* were placed on nutrient agar containing As using the previously described method. Concentrations of As in the media were as follows: 0, 200, 625, 1250, and 2500 ppm (dry mass) in the form of potassium arsenate ($KH_2AsO_4$; Sigma-Aldrich [#A-6631]). All plates were kept in a growth chamber as described above for weekly observation.

Equal masses of ten-week-old gametophytes of *P. vittata* were transferred from As-free media to media containing As (0, 200, 625, 1250, and 2500 ppm As). Transfers were done under a laminar-flow hood using flame-sterilized utensils. After 10 weeks of growth, gametophytes were removed from each plate and fresh weights were obtained. The gametophytes were then desiccated in an oven at 70 C for 24 h to obtain bulked dry weights. Care was taken upon gametophyte removal from the plates that agar media was not included.

*Spore germination experiments of various pteris spp. on as media.*—Spores of *Pteris cretica* var. *albolineata*, *Pteris cretica* 'Major', *Pteris cretica* 'Parkeri,' *Pteris ensiformis* Burm. var. *victoriae* Baker, *Pteris gallinopes* Ching, and *Pteris pacifica* Hieron. were obtained through the American Fern Society spore exchange program. The spores were plated on nutrient agar containing the following As concentrations (ppm): 0, 250, 500, 1025, 1900, and 2500. Additionally spores were plated on media containing 250 ppm As + 500 ppm phosphate. Phosphate was added as potassium phosphate ($K_2HPO_4$; J. T. Baker Chemical Co. [#3252-1]). The plates were maintained in growth chamber conditions as previously described and checked for presence/absence of germination only.

*Pteris vittata rhizotron experiments.*—Arsenic-spiked soil was prepared by amending commercial soil media (Earth-Gro, Marysville, OH, USA; pH 5.7) with 250 ppm (± 30 ppm) arsenic (as analyzed-See ICP Spectrometry Methods), added in the form of potassium arsenate ($KH_2AsO_4$). The slightly moistened, amended soil was unused for 24 h to allow adsorption of As to soil particles. As-spiked soil and control (unaltered) soil were placed into rhizotrons with internal dimensions of 38 × 23 × 8 cm. Dividers, which were removed before experimentation to allow root proliferation in both soil types, ensured approximate division of the media to one half of the rhizotron.

Three-month-old *P. vittata* sporophytes were transferred to rhizotrons, centering the plant over the soil dividing point. All rhizotrons were maintained in a greenhouse at 27°C ± 3°C with a light/dark cycle of 16/8 h and 700 $\mu M \cdot s^{-1} \cdot m^{-2}$ photon flux for 15 weeks. Shoots were harvested and analyzed for dry weight, fresh weight and As content. Soil from each side of the rhizotron was sieved using "8" Newark #2 standard test sieves to separate soil from roots. Roots were rinsed with ddi water to remove residual soil prior to drying in an oven at 70°C for 24 h. Upon drying, roots were weighed to assess growth as a function of dry weight.

*Sporophytic arsenic accumulation and $PO_4$ interaction.*—Three-month-old *P. vittata* sporophytes were transplanted to 19 × 19 × 6 cm flats containing

1 kg of moist soil (pH 5.7) artificially spiked with 0, 550, 1100, and 1650 ppm (± 30 ppm) As. An additional treatment included soil spiked with 550 ppm As and 500 ppm (± 50 ppm) phosphate as potassium phosphate ($KH_2PO_4$; Sigma-Aldrich [#P 5379]). Fronds were sampled initially and then at two-week intervals for 10 weeks. Samples from each arsenic treatment were dried and homogenized prior to analysis for arsenic accumulations.

*Inductively coupled plasma spectrometry.*—Arsenic concentrations were determined by inductively coupled plasma (ICP) spectrometry (Perkin Elmer Emission Spectrometer Plasma 400) at the TTU Center for the Management, Utilization and Protection of Water Resources (Cookeville, TN). Dried samples were ground to a fine powder in liquid nitrogen. Each sample was wet-ashed by the addition of 4 ml 1+1 (35.4%) $HNO_3$ and 10 ml 1+4 (7.5%) HCl to 1 g (DW) of plant tissue following the method of Jones and Case (1990). Beakers containing the samples were covered and placed in a water bath for 30 min at 95°C. Samples were then washed twice with 15 ml of ddi water, vacuum filtered (0.45 µm), and completed to 50 ml with ddi water. An As-free control was prepared by completing the combined 4 ml 1+1 $HNO_3$ and 10 ml 1+4 HCl to 50 ml with ddi water. The ICP data were stoichiometrically evaluated to acquire ppm As values as a function of plant biomass (DW).

*Response of P. vittata to foliar as application.*—Arsenic-containing herbicide was applied to fully mature, spore-producing sporophytes of *P. vittata* in the form of calcium acid methanearsonate (CAMA) (commercially available in concentrated or ready-to-use forms as Ortho Weed-B-Gon Crabgrass Killer for Lawns). CAMA treatments contained 0.25%, 0.50%, and 1.0% elemental As. Each of the three treatment groups received 5 ml CAMA solution (to the saturation or dripping point), with the control group sprayed with ddi water only. All treatment groups and the control group were greenhouse-grown for 15 days. One week after initial application, solutions were reapplied in the same amount and concentrations after the Day 7 sampling. Day 0 was considered the first day of the experiment for the randomly selected, uniformly aged plants.

Approximately 100 mg of pinnae samples from each herbicide treatment were procured and placed in capped, glass vials containing 5 ml of *N,N*-Dimethylformamide (DMF; Sigma-Aldrich #154814). Samples were then stored in the dark at 4°C for 36 h. DMF solutions were analyzed spectrophotometrically for total chlorophyll and carotenoid contents following the methods of Inskeep and Bloom (1985) and Doong *et al.* (1993). A Spectronic 601 spectrophotometer (Milton Roy Co. Rochester, NY) was used to measure absorbance of each sample at 470 nm, 647 nm, and 664.5 nm. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Carot) concentrations (mg $L^{-1}$) in pinnae were calculated from the obtained values. Total chlorophyll ($Chl_{TOT}$) was calculated by summation of the Chl *a* and Chl *b* values.

*Data analysis.*—Three subsamples and three replicates of each experiment were used for statistical analysis. Statistical analysis followed a randomized complete block design for replicated experiments to ensure differences were due to treatments rather than block variation (Steel and Torrie, 1980). The

blocks represent the replicate series of each experiment. Mean separation for treatments with significant F values ($P \leq 0.05$) following analysis of variance was based on least significant difference (LSD) tests (Steel and Torrie, 1980).

## Results

*Pteris spp. spore germination.*—Germination, defined here as the emergence of gametophytic tissue from spores, of seven *Pteris* spp. was monitored on control and As-containing media for presence/absence of spore germintaion only. Spore germination of *P. cretica* var. *albolineata*, *P. cretica* 'Major', *P. gallinopes*, and *P. vittata* was observed on all As treatments tested (0–2500 ppm As). *Pteris cretica* 'Parkeri' spores germinated on all treatments except for the two highest As concentrations (1900 and 2500 ppm As) but did not germinate on As + phosphate treatment plates. *Pteris pacifica* spores germinated only on control, 100 ppm As and As + phosphate treatment plates. Germination of *P. ensiformis* var. *victoriae* was not observed at any As treatment; however, germination did occur on control plates.

*Long term P. vittata gametophyte growth and uptake of arsenic.*—After 10 weeks on As media, abnormal rhizoid growth was observed on gametophytic tissues at the 200 and 625 ppm As treatments. Figures 1 A and B show gametophytes grown on control media and 625 ppm As, respectively. Despite the obvious morphological changes, fertilization events, evidenced by sporophyte development, were still documented on the 200 ppm As medium. Additionally, the gametophytic fresh weights were greater when grown on the 200 and 625 ppm As treatments than the control media (Table 1; Fig. 2A). However, the dry weights of the control, 200 ppm As, and 625 ppm As treatments were not significantly different from one another (Fig. 2B). Compared to the control, the 1250 ppm As media gametophytic fresh weights were reduced 29% and dry weights were reduced 34% with dense rhizoids no longer being observed on the surface of the gametophytic tissue from this and greater As treatments. Additionally, these effects on plant growth became more pronounced with increasing As concentrations. Malformed gametophytes and sporadic gametophyte mortality were observed at treatments ≥1250 ppm As.

Accumulation of As by *P. vittata* gametophytes was elevated and significantly different from controls and between treatments as As increased in the growth media (Table 1). No significant difference in As accumulation was observed between the 1250 and 2500 ppm As level. In our study, arsenic accumulation was greatest on the media containing 1250 ppm As (Fig. 3).

*Rhizotron experiments.*—Rhizotrons with the As-spiked soil on one half of the rhizotron had significantly greater (1.40 g ± 0.06) dry weight root mass in

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FIG. 1. Growth of *P. vittata* gametophytes on control medium (A) and the presence of dense rhizoids on 625 ppm As medium (B). Note the many emerging sporophytes on control medium (A) and the absence of sporophytes on 625 ppm As medium (B).


A
B

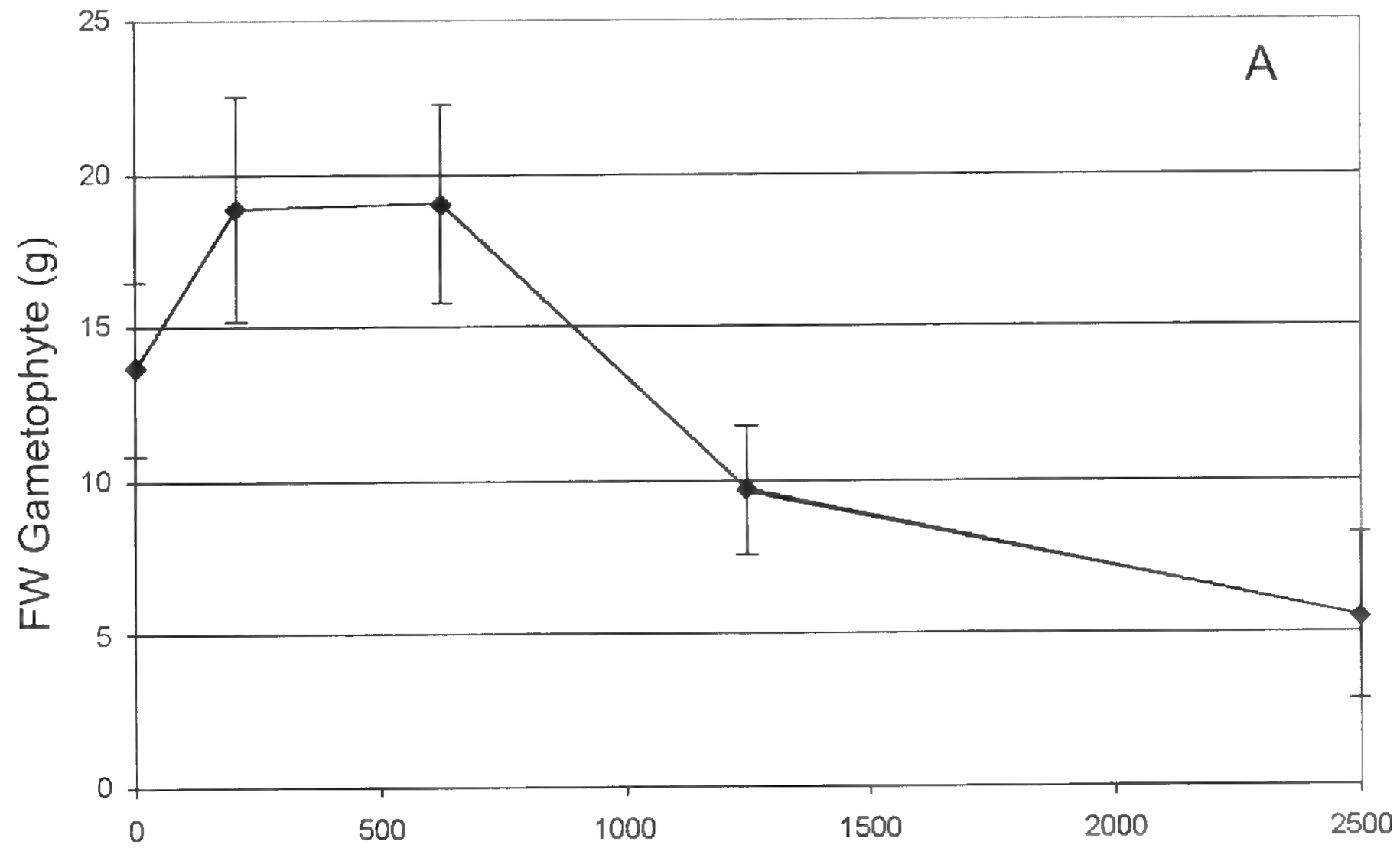
TABLE 1. Gametophyte fresh weights, dry weights, and As accumulation after 10 weeks of exposure to 0 ppm As (control), 200 ppm As, 625 ppm As, 1250 ppm As, and 2500 ppm As. Arsenic accumulation is given on a dry weight (DW) basis. Least significant difference (LSD) test ($P = 0.05$) was used for separation of means. Shared letters within a column represent no significant difference between treatments.

| As concentration (ppm) | Gametophytic weights (g) | | As accumulation (ppm DW) |
|---|---|---|---|
| | Fresh weight | Dry weight | |
| **Control** | 13.7 B | 1.31 A | 5 D |
| **200** | 18.8 A | 1.41 A | 4110 C |
| **625** | 19.0 A | 1.32 A | 8960 B |
| **1250** | 9.7 B | 0.87 B | 9700 A |
| **2500** | 5.5 C | 0.44 C | 9450 A |

the soil containing 250 ppm As than in the control soil side (0.89 g ± 0.06) ($n = 15$ for each set of rhizotron data analysis.) The root masses within control rhizotrons (which had arsenic-free soil on both sides) did not differ significantly in dry weight root mass on either side (data not shown). Roots of the As-free and As-spiked sides of the treatment rhizotrons were analyzed for As content. Arsenic concentrations in the roots of the control rhizotrons (20 ppm As) and As-free roots of experimental rhizotrons (40 ppm As) did not differ significantly. The majority of the As accumulated by sporophytes was translocated to fronds. Arsenic concentrations in sporophytes exposed to 250 ppm As were nearly ten times higher (3000 ppm ± 460 vs. 330 ppm ± 38 As) in fronds than in roots. In contrast, fronds of the ferns grown in control rhizotrons accumulated approximately 19 ppm As. Lastly, analysis did show some movement of arsenic via leaching over the course of this experiment. However, the level was minimal and resulted in less than 10 ppm As being found in the unspiked arsenic soil.

*Sporophytic arsenic accumulation.*—After two weeks of exposure, As concentrations were greatest in fronds of sporophytes grown on 1100 ppm As soil and remained as such through the tenth week, at which time As accumulation averaged 6500 ppm (Table 2). Arsenic accumulation at week 10, in order of decreasing concentration, was as follows: 1100 ppm As; 1650 ppm As; 550 ppm As; 550 ppm As + 500 ppm phosphate; 0 ppm As (Table 2). Addition of 500 ppm phosphate to 550 ppm As soil caused a 37% decrease in As uptake by sporophytes (2600 ppm vs. 4150 ppm As).

*Chlorophyll and carotenoid analysis post herbicide application.*—One week after application of CAMA, toxicity in sporophytes was observed. Chlorosis and downward curling of pinnae were noted for all treatments, with the most pronounced effects being observed in those exposed to the 0.50% and 1.0% As herbicide treatments. Chlorophyll and carotenoid concentrations decreased significantly in pinnae exposed to 0.50% and 1.0% arsenic as well, while changes were not significantly different between control and 0.25% As treatments (Tables 3 and 4). Total chlorophyll content in pinnae of all CAMA-treated sporophytes was significantly lower at day seven than the control.



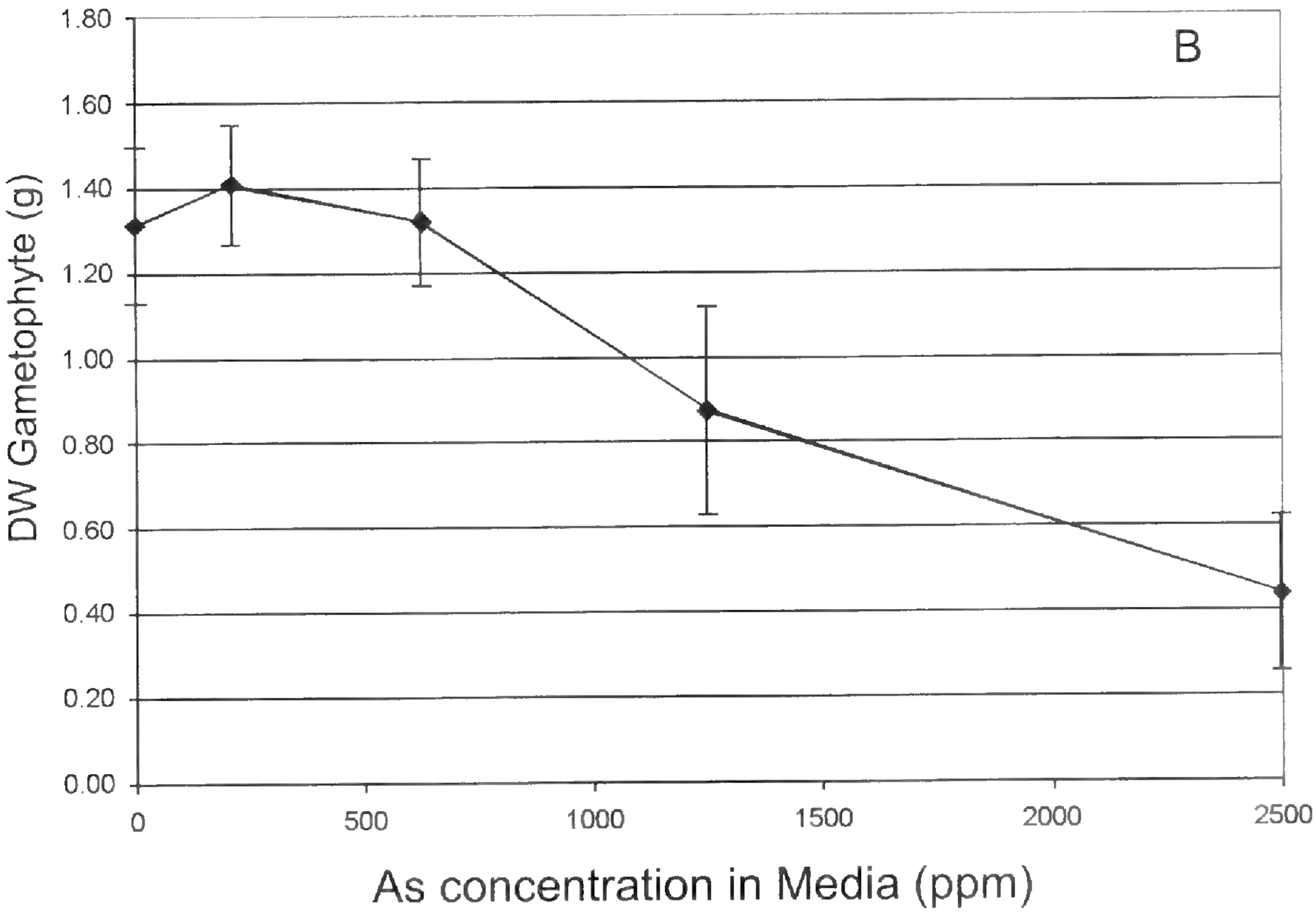




FIG. 2. *Pteris vittata* gametophytic (A) fresh weights (FW) and (B) dry weights (DW) after growth on 0, 200, 625, 1250, and 2500 ppm As media for 10 weeks. FWs increased over the control (0 ppm As) on the 200 and 625 ppm treatments but dropped significantly at higher concentrations (A). DWs (B) at 200 and 625 ppm As were similar to control but dropped at higher concentrations. Bars represent ±SDs.

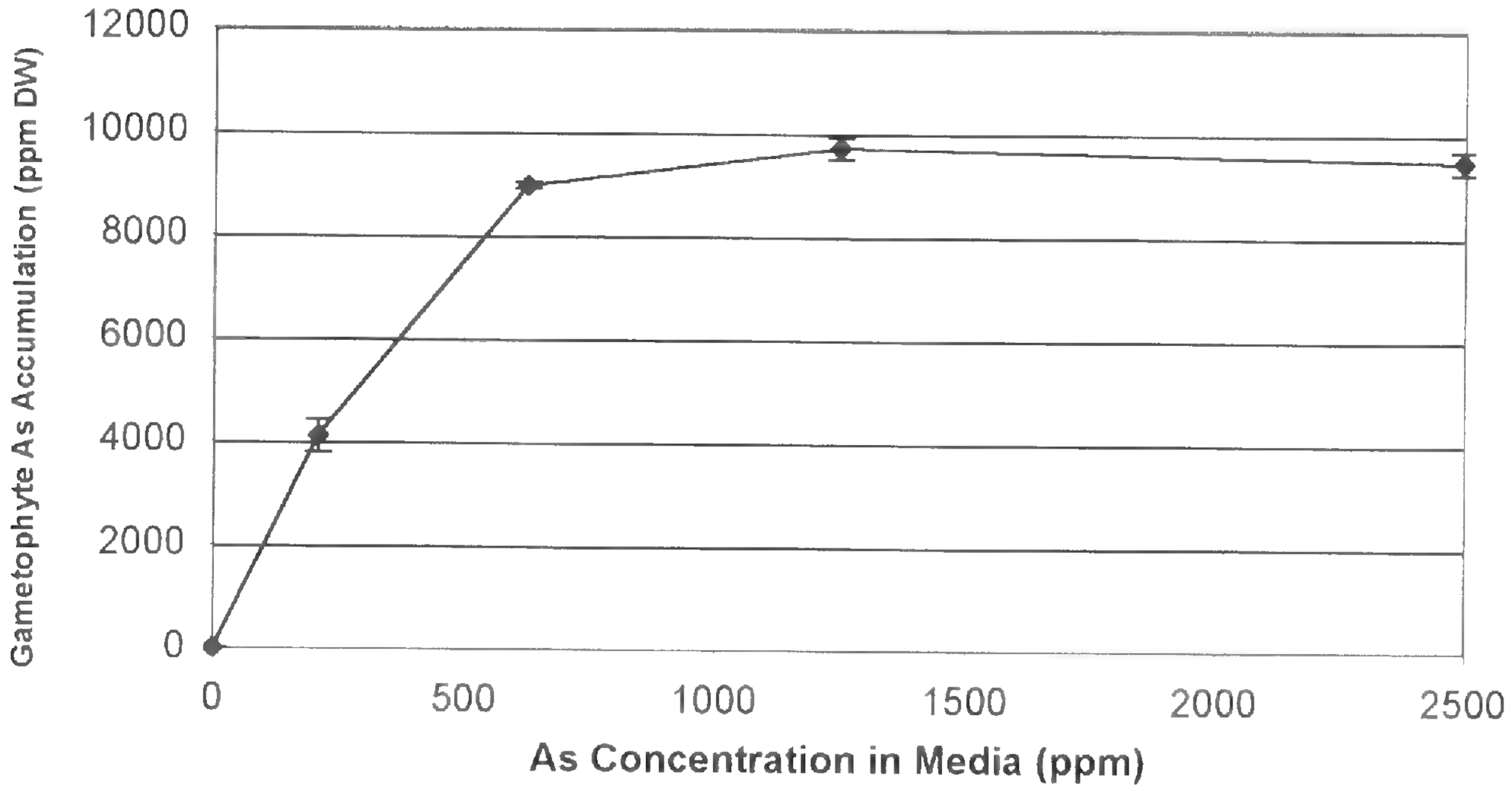



FIG. 3. *Pteris vittata* gametophytic As accumulation after growth on 0, 200, 625, 1250, and 2500 ppm As media for 10 weeks. Values for As accumulations are based upon gametophyte DW analyses. Bars represent ±SDs.

The second application of CAMA resulted in additional chlorophyll and carotenoid loss from all treatment groups. By day 15, chlorophyll and carotenoid contents were significantly lower for all CAMA-treated sporophytes when compared to the control. While the fronds showed signs of toxicity at all treatment levels, regrowth of healthy fronds occured in each treatment during and after experimentation.

## DISCUSSION AND CONCLUSIONS

The ability of spores from *P. vittata*, *P. gallinopes*, *P. cretica* var. *albolineata*, and *P. cretica* 'Major' to germinate on all As treatments tested indicates that

TABLE 2. Arsenic accumulation in *P. vittata* fronds after 2, 6, and 10 weeks of exposure to 0 ppm soil (control) and artificially-spiked soil containing 550 ppm As, 1100 ppm As, 1650 ppm As, and 550 ppm As + 500 ppm phosphate ($PO_4$). Least significant difference (LSD) test ($P = 0.05$) was used for separation of means. Lower case letters denote differences between treatments within a day, and upper case letters denote differences within a given treatment throughout the experiment.

| | Frond As accumulation (ppm DW) | | |
|---|---|---|---|
| | Exposure time (weeks) | | |
| Treatment | 2 | 6 | 10 |
| **Control** | 22 Bd | 27 Bd | 44 Ae |
| **550 ppm As** | 121 Cc | 3330 Bb | 4140 Ac |
| **1100 ppm As** | 947 Ca | 5320 Ba | 6500 Aa |
| **1650 ppm As** | 711 Cb | 3600 Bb | 5740 Ab |
| **550 ppm As + 500 ppm $PO_4$** | 124 Cc | 1120 Bc | 2610 Ad |

TABLE 3. Effects of foliar-applied arsenical herbicide (CAMA) concentration on total chlorophyll content (mg $L^{-1}$) of *P. vittata* pinnae at day 0, 7, and 15. Herbicide was not applied to the control group with 0.25% As, 0.50% As, and 1.0% As applied to respective treatment groups. Least significant difference (LSD) test (P = 0.05) was used for separation of means. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day, and upper case letters denote differences within a given treatment throughout the experiment.

| | **Total chlorophyll content (mg $L^{-1}$)** | | |
|---|---|---|---|
| | **Length of exposure (days)** | | |
| **% As** | **0** | **7** | **15** |
| **Control** | 28.7 Aa | 31.7 Aa | 28.7 Aa |
| **0.25%** | 22.7 Aa | 22.0 Ab | 10.0 Bb |
| **0.50%** | 22.8 Aa | 13.6 Bc | 3.5 Cc |
| **1.0%** | 28.8 Aa | 14.5 Bc | 1.7 Cc |

they are the most As-tolerant species of this study regarding spore germination. *Pteris cretica* 'Parkeri' also proved very tolerant, germinating on tested treatments below 1900 ppm As. However, lack of germination of *P. cretica* 'Parkeri' spores on media containing increased phosphate, even with relatively low arsenic, suggests interactions that are not currently understood. Through observation of mature *P. vittata* sporophytes, we have established that increased phosphate application, possibly through fertilizers, may lead to decreased arsenic accumulation in some species. Further testing is needed to examine the interactive and competitive nature of elevated phosphates in soil medium and its impacts upon all stages of the fern life cycle. *Pteris pacifica* and *P. ensiformis* var. *victoriae* both germinated relatively poorly on arsenic-laden media, indicating that development may be impeded in phytoremediation efforts. Further work on the phylgenetic relationships of these species may offer insight into why some species seem to possess abilities not shared with others within the genus Pteris.

TABLE 4. Effects of foliar-applied arsenical herbicide (CAMA) concentration on total carotenoid content (mg $L^{-1}$) of *P. vittata* pinnae at day 0, 7, and 15. Herbicide was not applied to the control group and 0.25% As, 0.50% As, and 1.0% As applied to respective treatment groups. Least significant difference (LSD) test (P = 0.05) was used for separation of means. Same letter denotes no statistical difference. Lower case letters denote differences between treatments within a day, and upper case letters denote differences within a given treatment throughout the experiment.

| | **Total carotenoid content (mg $L^{-1}$)** | | |
|---|---|---|---|
| | **Length of exposure (days)** | | |
| **% As** | **0** | **7** | **15** |
| **Control** | 4.22 Bab | 4.37 Ba | 5.44 Aa |
| **0.25%** | 3.54 Ab | 3.72 Aa | 2.88 Ab |
| **0.50%** | 3.57 Ab | 2.48 Ab | 1.02 Bc |
| **1.0%** | 4.43 Aa | 1.66 Bb | 0.51 Cc |

Increased fresh weights of gametophytes in the presence of 200 and 625 ppm As indicate a growth strategy for *P. vittata* that has been adapted to environments high in As, apparently even benefiting from the presence of As. In a similar study, Gumaelius *et al.* (2004) found gametophytes of *P. vittata* exposed to low arsenate concentrations (0.2 mM) to be larger than control gametophytes, while cellular shape and organization were relatively unaffected. Ma *et al.* (2001) reported sporophytes of *P. vittata* increased in biomass by 40% when exposed to 100 ppm soil As. Additional observations of dense rhizoids on 200 and 625 ppm As treatments may be analogous to increased root production of sporophytes in As-spiked soil, as seen in rhizotron experiments. Decreases in biomass at higher As concentrations (≥1250 ppm As) indicate a limit of gametophytic tolerance to arsenic. In the germination experiments few sporophytes developed after maturation of gametophytes in the presence of As and then only at the 200 ppm As level.

In comparing gametophytic and sporophytic tolerance of As, gametophytes appear to be less equipped to tolerate As in the growth media of these experiments. Despite this, in the germination experiments, spores of *P. vittata* germinated on each treatment. The extent of As accumulation by gametophytes and sporophytes varied. Sporophytes exposed to 550 ppm soil As accumulated, on average, 4140 ppm As (DW) in fronds, whereas gametophytes exposed to 625 ppm As accumulated up to 8960 ppm As (DW). Greater hyperaccumulation of As by gametophytes is likely responsible for eventual negative effects on biomass and may relate to availability of As in the media. The availability of As to organisms is often far less than indicated by total concentrations in soil (Tu and Ma, 2002). This is due in part to As occurring in a number of relatively insoluble forms such as $FeAsO_4$ and $AlAsO_4$ (Ma *et al.*, 2001). Fitz *et al.* (2003) stated that iron oxides and hydroxides are the primary sorption sites of As in soils. Gametophytes of this experiment were in direct contact with highly available As and accumulated it in high concentrations, likely in response to the relatively few sorption sites in the nutrient agar. Although the mechanism of gametophytic uptake of As remains unknown, the phosphate transport system implicated in sporophytic As uptake is likely involved (Wang *et al.*, 2002).

Tu *et al.* (2002) has estimated up to 5 harvests over 80–100 weeks would be the minimum required for *P. vittata* to remediate soil contaminated with 100 mg As $kg^{-1}$. Self-sustaining populations of *P. vittata* on As-contaminated sites would alleviate costs associated with initial planting and future plantings of sporophytes after each harvest. Based upon this work with spore germination and gametophyte development, it is probable that germination and then fertilization could occur on soil contaminated with up to 200 ppm As and possibly more. It is highly improbable that gametophytes would be adversely affected by soil As concentrations in which sporophytes could grow.

Observations in the rhizotron experiments of 63% greater root biomass within soils spiked with 250 ppm As suggest that *P. vittata* has an affinity for soils with high As content. Tu *et al.* (2002) found *P. vittata* root biomass to exceed that of aboveground tissues after growth on As-amended soil. Many

plants respond to internal and external nutrient status and proliferate roots in areas of higher soil nutrient content (Malamy and Ryan, 2001; Williamson *et al.*, 2001). Schwartz *et al.* (1999) and Whiting *et al.* (2000) found roots of the hyperaccumulator *Thlaspi caerulescens* proliferated in soils with elevated levels of Zn and Cd. The preferential proliferation of roots in zones of contamination is especially beneficial in soils where contamination is heterogeneously distributed (Goodson *et al.*, 2003). Boyd and Martens (1992) proposed that metal uptake and subsequent hyperaccumulation could act as a defense mechanism against herbivory. Whether the uptake of As is a defense mechanism or if it serves an as yet unascertained metabolic function remains unknown.

Phosphate additions to As-contaminated soils increase solubility, mobility, and phytoavailability of As by anion exchange with adsorbed arsenate, increasing uptake of As by some species (Davenport and Peryea, 1991; Peryea, 1998; Abedin *et al.*, 2002). However, in this study, sporophytic accumulation of As was 37% less in the phosphate-enriched As treatment than the 550 ppm As counterpart. Inhibition of As uptake by phosphate has been reported in other plants (Asher and Reay, 1979; Meharg *et al.*, 1994; Pickering *et al.*, 2000).

Arsenate, a phosphate analog, is commonly the inorganic As form most abundant in aerobic environments (Silver and Misra, 1988; Abedin *et al.*, 2002; Meharg and Hartley-Whitaker, 2002; Tu *et al.*, 2003). Arsenic is likely taken up as arsenate via a phosphate transporter protein in root plasmalemma (Silver and Misra, 1988; Abedin *et al.*, 2002; Meharg and Hartley-Whitaker, 2002); however, this transporter has a higher affinity for phosphate than As (Meharg and Macnair, 1990; Meharg and Macnair, 1992; Hartley-Whitaker *et al.*, 2001). Effects of competition between As and phosphate are plant-specific and are therefore important to consider when a species such as *P. vittata* is used for phytoremediation efforts.

Differences in competition between As and phosphate likely relate to characteristics of the growth media and root/media interactions. In natural soils arsenate and phosphate may not only compete for uptake but also for sorption sites on soil particles. Therefore, if an As-contaminated soil is amended with phosphate, displacement of adsorbed As may occur, resulting in greater As uptake when compared with unamended soil. If a soil has relatively few or weak sorption sites, or if As is primarily unadsorbed, direct competition between arsenate and phosphate for uptake by roots may result in decreased uptake of As due to the higher affinity of the phosphate transporter for phosphate (Meharg and Macnair, 1990; Meharg and Macnair, 1992; Hartley-Whitaker *et al.*, 2001).

Phosphate additions initially had a greater impact on As hyperaccumulation, and at the end of week six 66% less uptake of As was observed compared to the 550 ppm As treatment. This was possibly due more to the abundant phosphate at the beginning of the experiment being at a competitive advantage for uptake by the plant (Meharg and Macnair, 1990). Throughout the experiment, the available phosphate pool decreased as it was taken up, and the rate of As uptake increased. Gradual increases in As uptake on phosphate-

enriched soils may relate to decreased competition and/or greater production of roots due to phosphate nutrition. Contrasting those of the phosphate treatment, the sporophytes of the 550 ppm As treatment (without phosphate additions) exhibited greater initial As uptake. Arsenic accumulation progressively slowed, likely due to the depletion of available As.

A key characteristic of plants useful to phytoextraction is efficient uptake and translocation of contaminants to harvestable tissue (Lasat *et al.*, 1998; McGrath *et al.*, 2001). Meharg and Macnair (1992) reported that many plants are tolerant to As by suppression of the high affinity phosphate transport system. Burlo *et al.* (1999) discovered that tomato plants limit As transport to shoots, accumulating As primarily in roots. In *P. vittata*, however, As was translocated aboveground and in higher than ambient concentrations; up to 6940 ppm As in fronds on 1100 ppm As soil.

The apparent ease of the movement of As to aboveground portions may relate to the reduction of arsenate to arsenite once inside the plant (Meharg and Hartley-Whitaker, 2002). According to Meharg and Hartley-Whitaker (2002), arsenite is easily translocated since it does not compete with phosphate, and this decreased competition may result in moderate tolerance of As by the plant. Huang *et al.* (2004) used EXAFS to conclude arsenate reduction occurs in roots following uptake, and Webb *et al.* (2003) found arsenite to be the main As form in both stems and fronds. Zhao *et al.* (2003), Bondada *et al.* (2004), and Chen *et al.* (2004) supported the fronds and leaves as the primary sites of arsenate reduction.

Translocation of contaminants to harvestable parts is crucial to phytoextraction (Pickering *et al.*, 2000). In this study, increased As concentrations in fronds corresponded with higher As concentrations in soil. Hyperaccumulation by sporophytes was significantly greater when grown on the 1100 ppm As treatment than on the 1600 ppm As treatment. Tu and Ma (2002) reported a similar finding for *P. vittata* when sporophytes exposed to >100 mg As $kg^{-1}$ soil accumulated less As in fronds when compared to sporophytes grown on soil with less As. Decreased As uptake at higher ambient concentrations may result from toxicity to roots, thus inhibiting uptake (Tu and Ma, 2002). Despite the possibility of toxicity, fronds of sporophytes grown on all As treatments contained greater As concentrations than the surrounding soil, and no visible signs of toxicity were observed.

In contrast to the remarkable tolerance of gametophytes and sporophytes to As in the growth media, sporophytes were sensitive to foliar-applied CAMA. Tu and Ma (2002) grew *P. vittata* on soils with varying concentrations and species of As and found that, though sporophytes were tolerant of up to 500 ppm inorganic arsenicals, sporophyte death occurred on soils containing 50 ppm sodium dimethylarsonate (NaDMA), an organic arsenical. Similarly, CAMA is an organic compound and may circumvent most tolerance mechanisms of *P. vittata*. Bondada *et al.* (2004) foliar-applied both arsenate and arsenite, without a surfactant, to fronds of *P. vittata*, and discovered the fern was tolerant and accumulated As through the leaves. The surfactant in the solutions of the present study likely aided unregulated passage of CAMA

through the cuticle into the leaves, leading to chlorophyll and carotenoid decreases. *Pteris vittata* apparently has limited tolerance to foliar-applied CAMA based upon toxicity varying with CAMA concentration. Though some fronds died as a result of foliar-applied CAMA, new, healthy fronds emerged as the experiment progressed, thus no plant death was observed.

Germination of spores, tolerance of gametophytes, and emergence of sporophytes in the presence of high concentrations of As indicate successional populations of *Pteris vittata* on sites contaminated with As even at high concentration are possible. Competition between arsenate and phosphate is important to consider with phytoremediation, especially if fertilizers are to be used to enhance efforts. Impressive As hyperaccumulation and cellular tolerance coupled with extensive root systems are further evidence for the potential of *P. vittata* as a phytoremediator of As contaminated soils.

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# Shorter Notes

**New Pteridophyte Records for the State of Morelos & México, México.**—Four new pteridophyte records are reported as a result of herbarium work done during the examination of the Prof. Jankiewicz's collection. The collection is located in Herbarium of the Institute of Botany of the Jagiellonian University (KRA). It was gathered in 1982 in central and southern México and includes 135 sheets of pteridophytes. The identifications were made according to the keys from The Pteridophytes of México (Mickel and Smith. 2004. The Pteridophytes of México. Mem. New York Bot. Gard. 99:1–1054.).

***Argyrochosam pallens*** (Weath. *ex* R. M. Tryon) Windham—State of Morelos, mun. Tepoztlán, near Tepoztlán, sandy mountains, 1500 m, *Jankiewicz 0298630* (KRA).

***Campyloneurum angustifolium*** (Sw.) Fée—State of México, mun. Villa Guerrero, rock-bottom of valley, *Jankiewicz 0298631* (KRA).

***Selaginella landii*** Greenm. *et* N. Pfeiff.—State of Morelos, mun. Tepoztlán, near Tepoztlán, sandy mountains, 1500 m, *Jankiewicz 0298632* (KRA).

***Selaginella reflexa*** Underw.—State of Morelos, rocky hills, 900 m, *Jankiewicz 0298633* (KRA).

We would like to thank Alan R. Smith for the identification of *Selaginella* specimens, and for confirmation of our identifications of other specimens. We also thank Wacław Bartoszek, the curator of the Herbarium of the Jagiellonian University (KRA), for making available to us his specimens for study.—
Przemysław Naks, Department of Plant Taxonomy and Phytogeography, Institute of Botany, Jagiellonian University, Kopernika 27, 31-512 Kraków, Poland, and Krzysztof Piątek, Department of Plant Ecology, Institute of Botany, Jagiellonian University, Kopernika 27, 31-512 Kraków, Poland.

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## American Fern Journal

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# Characterization of Four Members of the Alpha-Tubulin Gene Family in *Ceratopteris richardii*

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ABSTRACT.—Four members of the alpha-tubulin gene family were examined in *Ceratopteris richardii*. Genetic linkage mapping based on a population of nearly 500 Doubled Haploid Lines was able to position three or four members of this gene family on linkage groups 17, 24, and 28, respectively (two of the four observed polymorphic restriction fragments containing alpha-tubulin genes are either identical or map too close to each other on linkage group 17 to be distinguishable in map distance). Non-mapable monomorphic bands observed on probed Southern blots suggest that the alpha-tubulin gene family in this species is large. Four alpha-tubulin genes from *C. richardii* were sequenced and found to be fairly similar to each other in terms of their amino acid sequences, with their greatest diversity at the carboxy-terminal ends. BLAST comparisons found each of these four amino acid sequences more similar to an alpha-tubulin from a dicot, gymnosperm, or alga species than it was to any other alpha-tubulin sequence presently known from *Ceratopteris* or from the fern *Anemia phyllitidis* or the moss *Physcomitrella patens*. Bayesian phylogenetic analysis of nucleotide sequences placed three of the four *Ceratopteris* alpha-tubulin gene copies in a clade with copies from *Pseudotsuga* and *Anemia*, consistent with a history of two gene duplication events, one following and one preceding the divergence of ferns and seed plants. The fourth copy is robustly separated from the preceding three and placed in a clade of algal alpha-tubulin genes, suggesting its divergence from the ancestor of the other three before the divergence of algae and land plants. As characterized thus far, the alpha-tubulin gene family of *C. richardii* is relatively large as compared to the six copies known from fully sequenced *Arabidopsis thaliana*, a condition that may be correlated with the large genome size and diverse life history constraints of this homosporous fern species. These findings suggest several new opportunities for research into the evolution, function, and regulation of the alpha-tubulin gene family in *Ceratopteris*.

This report describes the application of DNA sequencing and genetic linkage mapping to the alpha-tubulin genes of *Ceratopteris richardii* and shows how such studies can further enhance the utility of this model system. Initial successes with *Ceratopteris* were in the areas of cytogenetics (e.g., Hickok, 1976, 1977a, 1977b, 1978, 1979a, 1979b; Hickok and Klekowski, 1973, 1974), physiology and development, and Mendelian genetics (reviewed in Hickok, 1987; Hickok *et al.*, 1987, 1995). More recent studies have focused on the molecular genetics of *Ceratopteris* (Munster *et al.*, 1997; Hasebe *et al.*, 1998; Aso *et al.*, 1999; Stout *et al.*, 2003; Rutherford *et al.*, 2004; Salmi *et al.*, 2005). These recent studies that employ robust molecular methods are especially encouraging, since the lack of a technique to induce stable genetic trans-

formation in *Ceratopteris* has undoubtedly impeded its function as a unifying model system. In this report, DNA sequences from four unique alpha-tubulin genes of *C. richardii* are described, and the phylogenetic relationships of these genes to other plant alpha-tubulin genes are inferred. Molecular linkage data for three polymorphic alpha-tubulin loci of *C. richardii* are used to position these loci on the new genetic linkage map of this species, and several potential strategies for using this new information in molecular studies are outlined. These perspectives should provide further encouragement for those seeking to utilize *Ceratopteris* as a model system.

The alpha-tubulin gene family was selected for these studies because of the biological significance and tractability of tubulin in the gametophyte generation of ferns and because of the new insights that may be gained for genomics by studying them. Inferring evolutionary relationships among members of gene families is generally problematic because distinguishing paralogues and orthologues is difficult without appropriate known outgroups to polarize the gene phylogeny. Tubulin genes are ideal for studying gene family evolution because 1) alpha-, beta-, and gamma-tubulin genes are known to have diverged well before the divergence of plant lineages, and 2) the highly conserved nature of the genes allows their sequences to be aligned. These features allow inference of gene phylogenies within a given tubulin gene group, using sequences from other tubulin groups as outgroups.

Tubulins and the microtubules they form are obviously essential components of all eukaryotic cells. In fern gametophytes, however, their roles are directly observable in various stages of development that can be easily studied. For example, one of the first events that signals preparation for spore germination is the migration of the nucleus within the cytoplasm (Banks, 1999). This event, which is critical to the continued development of the gametophyte, is inhibited in *Onoclea sensibilis* by several microtubule inhibitors, including colchicine (Vogelmann *et al.*, 1981). An important role for microtubules at a later stage of development has been suggested by the studies of Murata *et al.* (1997) on blue light-induced inhibition of cell growth in dark-grown *Ceratopteris* gametophytes. These investigators found that cortical microtubules reorient in response to blue light at the same time that inhibition of cell elongation occurs. Microtubules also serve a key role in the organization and function of fern sperm (Raghavan, 1989), and *Ceratopteris* sperm has been used extensively in studies characterizing these roles (Hoffman and Vaughn, 1995a, 1995b, 1996; Hoffman *et al.*, 1994; Renzaglia *et al.*, 2004).

Because homosporous ferns compose most of the sister group to seed plants (including flowering plants; Pryer *et al.*, 2001), increased knowledge of genome structure and organization in the genomes of homosporous ferns will significantly broaden our knowledge of genome structure and evolution in vascular plants. Furthermore, characterization of the alpha-tubulin gene family in *Ceratopteris* will ultimately help to answer questions specifically related to the origin and function of gene families in organisms with large genomes. At 11,294 Mb (Jo Ann Banks, personal communication), the genome

of *C. richardii* is ca. 110 times the genome size of *Arabidopsis thaliana*, 24 times that of rice, 12 times that of tomato, 4 times that of maize, and 2 times that of barley. Some relevant questions include the following. Do organisms with larger genomes have larger gene families? Do such organisms (with their apparent excess amounts of DNA) have more pseudogenes among the members of their gene families? How are the various members of gene families regulated during development in organisms with large genomes? The latter question is an especially interesting one as it relates to the ferns, whose alternation of generations features fully independent sporophyte and gametophyte generations.

This study employed two distinct strategies for characterizing alpha-tubulin genes from *C. richardii*. The first approach involved identifying, isolating, and sequencing alpha-tubulin genes that were expressed in the gametophyte generation. This was accomplished by using an antibody-based screening method with a cDNA library derived from the gametophyte generation. Bayesian Inference analyses determined the phylogenetic relationships of these newly obtained sequences to other plant alpha-tubulin genes. The second approach utilized a mapping strategy developed for the recently completed project of Nakazato *et al.* (2006) to generate a high-resolution linkage map for *C. richardii*. This mapping project used genetic polymorphisms present in a population of nearly 500 Doubled Haploid Lines (DHLs) generated from an initial cross between diploid inbred lines of highly diverged geographic races of *C. richardii* (Hickok *et al.*, 1995): ΦN8 (derived from a Nicaraguan collection) and Hα-PQ45, a mutant of Hn-n (derived from a Cuban collection). Together these two approaches provide detailed insights regarding a previously uncharacterized gene family in *C. richardii*. These new insights suggest novel strategies for studying the alpha-tubulin gene family at the level of development and gene regulation and also at the level of the genome.

## Materials and Methods

*cDNA library screening.*—The cDNA library used in this study was a gift from Jo Ann Banks (Purdue University). It was made from 12-day-old cultures of *C. richardii* gametophytes of the Hn-n strain containing both males and hermaphrodites. The cDNA was cloned into the *Eco*RI site of the lambda ZipLox bacteriophage vector (Life Technologies). The aliquot used to screen for alpha-tubulin genes was from a sample that had been amplified from the original library. The library was screened using standard methods for detecting specific proteins via antibody labeling (Young and Davis, 1991). In brief, the bacteriophage vector was first grown on a lawn of bacteria and, after plaques were produced, gene expression was induced with IPTG. Proteins from the library were adhered to nylon membranes and detected by hybridization to an anti alpha-tubulin monoclonal antibody (Sigma, catalogue number T5168). The presence of this antibody was detected with a labeled secondary antibody. After initial detection and isolation of plaques that tested positive for expression of alpha-tubulin, several rounds of re-screening were conducted

to eliminate contaminating vectors that were not positive for alpha-tubulin. Bacteriophage vectors containing alpha-tubulin cDNAs were converted to plasmids via an *in vivo* excision process as described in technical reference materials supplied by Life Technologies.

*DNA sequencing.*—Plasmids with the cDNA inserts were used as the sequencing templates. For each plasmid, at least two sequencing reactions were conducted using the two primer sites that flank the *Eco*RI site where the cDNA is inserted. When the results from two sequencing reactions indicated that the insert was longer than 1220 base pairs, two additional pairs of primers were constructed based on the new sequence data. These new internal sequencing primers provided more reliable results for the central regions of these longer inserts. The sequence reads from either two or four reactions were assembled using the software program ContigExpress (NTI Vector). Ambiguities that resulted from discrepancies between overlapping sequences were resolved by relying on the sequence(s) with the clearest chromatogram.

*BLAST comparisons.*—The sequences were compared using the standard BLAST program for proteins (protein-protein BLAST) provided on the NCBI web site to compare individual sequences with other sequences contained in the GenBank database as described in the Results section. The Composition-based statistic option and the filter options were not enabled so that the results of the BLAST comparisons would be based solely on similarities and/or differences of individual amino acids. Since the GenBank database is constantly being updated, it should be noted that the reported BLAST comparisons were last confirmed on July 29, 2005.

*Phylogenetic analysis of alpha-tubulin gene relationships.*—A Bayesian Inference analysis of nucleotide sequences was used to infer the phylogenetic relationships of the four *C. richardii* alpha-tubulin genes with regard to other plant alpha-tubulin gene nucleotide sequences available in GenBank for Viridiplantae. Sequences used were from a broad selection of plants, including those corresponding to the results of the amino acid BLAST search above, except that nucleotide sequences precisely corresponding to the amino acid sequences of *Gossypium hirsutum* Q6VAG0 and *Chlamydomonas reinhardtii* P09204 could not be located in GenBank. Prior to analysis, nucleotide sequences were aligned visually. The first three nucleotides, which were invariable, and the last 45 nucleotides, which caused ambiguous alignments, were removed. MrModeltest 2.2, a modified version of Modeltest 3.6 (Posada and Crandall, 1998) determined that the suitable nucleotide substitution model for the analyzed sequences is GTR+I+G. Bayesian analysis was conducted using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with three million generations and a sample frequency of 1000. The first 300 "burn-in" trees were discarded after the analysis. Five independent runs using the same setting converged to identical trees, except that one node was resolved in only one run. Two beta-tubulin genes were used as an outgroup to root the resulting tree. The species used and their GenBank accession numbers are identified in the Results section, as are the Bayesian posterior probability confidence values for the tree's clades.

*Linkage mapping of the alpha-tubulin genes.*—A genetic linkage map of the *Ceratopteris richardii* genome was developed independently of this alpha-tubulin study (Nakazato *et al.*, 2006). The mapping population of ~500 Doubled Haploid Lines (DHLs) was generated by intragametophytic selfing of gametophytes derived from spores of an initial cross between diploid inbred lines of highly diverged geographic races of *C. richardii* (Hickok *et al.*, 1995): *Φ*N8 (derived from a Nicaraguan collection, *Nichols 1719*, GH) and Hα-PQ45, a mutant of Hn-n (derived from a Cuban collection, *Killip 44595*, GH). The map is based on analysis of Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and allozyme markers. RFLPs used in the general mapping project were generated from genomic DNA of parental and DHL sporophytes digested with *Eco*RI and *Hind*III, separated on 0.8% agarose gels in 1X TAE, and Southern blotted to nylon membranes. Further details of the methods used in development of mapping materials and map construction are in Nakazato and Gastony (2006) and Nakazato *et al.* (2006). Alpha-tubulin gene copies were located on the linkage map in the following way. RFLPs containing the alpha-tubulin genes were detected by probing the Southern blots with an alpha-tubulin probe made cheiluminescent by digoxigenin (DIG)-labeling according to a protocol optimized for the *C. richardii* genome mapping project. The alpha-tubulin probe used is *C. richardii* cDNA clone Cri_10_E18_SP6 (GenBank sequence number BQ086953), from the cDNA library of *C. richardii* gametophytic tissue provided by Jo Ann Banks (Purdue University). This clone sequence corresponds to GenBank sequence AY231146 of TuaCR1 in Table 1, according to an NCBI BLAST search using blastn, which found sequence identity at 651/652 (99.85%) of the bases compared. Probing of the mapping project's Southern blots with the alpha-tubulin probe was carried out toward the end of the mapping project when the Southern blots were beginning to wear out. Thus the quality of the autorads presented here are suboptimal but nevertheless scorable. Parental alpha-tubulin RFLPs segregating in the DHL mapping population were scored and placed on the linkage maps by MAPMAKER/EXP 3.0 (Lander *et al.*, 1987) at a UNIX workstation at Indiana University, Department of Biology, at settings used for the general mapping project.

## Results

*Sequencing results.*—A cursory comparison of the amino acid sequences for the four *Ceratopteris* alpha-tubulin genes (Fig. 1) suggests immediately that they are relatively similar to one another. Although only one of the four sequences is complete, it is possible to note at least two trends directly from the comparison presented (Fig. 1). First, for the portion where sequences are available for all four genes (i.e., residues 195 to 332 of TuaCR1), TuaCR3 and TuaCR4 are the most distinct. In this region, TuaCR3 contains five unique amino acid residues and TuaCR4 contains four, while the other two sequences each possess only a single unique amino acid. The second observation that can be made directly from these data relates only to the three sequences that

TABLE 1. A comparison of the ten *Ceratopteris* alpha-tubulin sequences identified in this study to other alpha-tubulin sequences recorded in GenBank.

| Names for new gene | GenBank Accession number | Number of bases sequenced in DNA[1] | Size of Predicted ORF | Accession number, description, and identity for amino acid sequence(s)[2] most similar to new sequences[3] |
|---|---|---|---|---|
| TuaCR1 | AY231146 | 1,676 | 450 | 1) Q6VAG0, Tubulin alpha-2 chain *Gossypium hirsutum*, 436/450 (96.2%) |
| | AY862561 | 1,159 | 306 | |
| | AY862563 | 1,564 | 450 | |
| TuaCR2 | AY862565 | 1,015 | 266 | 1) AAK81858.1, alpha tubulin subunit *Rosa* Hybrid cultivar, 260/267 (97.4%) |
| | AY862566 | 1,016 | 267 | |
| | AY862567 | 1,018 | 267 | 2) AAV92379.1, alpha tubulin 1 *Pseudotsuga menziesii*, 260/267 (97.4%) |
| | AY862569 | 1,016 | 267 | |
| TuaCR3 | AY862562 | 938 | 257 | 1) BAA03955, alpha-tubulin 1 *Chlorella vulgaris* 245/257 (95.3%) |
| | AY862564 | 1044 | 257 | 2) P09204, Tubulin alpha-1 chain *Chlamydomonas reinhardtii*, 245/257 (95.3%) |
| TuaCR4 | AY862568 | 975 | 325 | 1) AAV92379.1, alpha tubulin 1 *Pseudotsuga menziesii*, 316/325 (97.2%) |

[1]This includes reliable DNA sequences before and after the open reading frame if present, but not the predicted polyA tail.
[2]If two or more versions of the same gene occurred in GenBank, only the most recent one is listed here. If several identical sequences were submitted by the same author(s) and are listed with the same submission date, only the one with the last accession number in the numerical sequence is listed here. The descriptions are given in the form in which they were submitted to GenBank.
[3]Sequence similarities were determined using the standard BLAST program for protein sequences to search the GenBank database. The Composition-based statistic option and the filter options were not enabled. The BLAST searches were performed on July 29, 2005.

include complete carboxy-terminal ends (i.e., TuaCR1, 2, and 3). In this region, these three sequences are highly diverse, with TuaCR3 once again exhibiting the greatest number of unique residues.

An additional sequence identified as an alpha-tubulin sequence from *Ceratopteris* has been deposited in GenBank by a separate research group (Salmi *et al.*, 2005). This 825 base-long sequence (GenBank accession BE642799) is a single pass sequence from a collection of expressed sequence tags. The bl2seq tool on the NCBI web site was used to compare this sequence with the four alpha-tubulin sequences described here (data not shown). A large region of this sequence (ranging from 574 to 640 bases) is 77 or 78% identical to the beginning portions of TuaCR1 and TuaCR4 respectively. It has no significant similarity to TuaCR3. The highest identity (88% for a region that is 195 bases long) occurs between this sequence and TuaCR2. This region of similarity occurs at the beginning of TuaCR2 and at the end of the sequence described by Salmi *et al.* (2005). Because this sequence was derived from a single sequencing experiment, it may be expected to contain a relatively large number of incorrect bases. Furthermore, the authors did not provide a deduced

```
TuaCR1   MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPSDKTVGGGDDAFNT
TuaCR2   ----------------------------------------------------
TuaCR3   ----------------------------------------------------
TuaCR4   -------**********************H********************

TuaCR1   FFSETGAGKHVPRAIFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANN
TuaCR2   ----------------------------------------------------
TuaCR3   ----------------------------------------------------
TuaCR4   **************V******************F**********N********

TuaCR1   FARGHYTIGKEIVDLCLDRIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLL
TuaCR2   ----------------------------------------------------
TuaCR3   ----------------------------------------------------
TuaCR4   **************************************H**********A***

TuaCR1   LERLSVDYGKKSKLGFTVYPSPQVSTSVVEPYNSVLSTHSLLEHTDVAVLL
TuaCR2   ----------------------------------**************S***
TuaCR3   ------------------------------------------********M*
TuaCR4   ****************************************************

TuaCR1   DNEAIYDICRRSLDIDRPTYTNLNRLVSQVISSLTASLRFDGALNVDVTEF
TuaCR2   ****************E***********************************
TuaCR3   ****************E*************************************I***
TuaCR4   ****************E****N*****I************************

TuaCR1   QTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSSMMAKCD
TuaCR2   ****************************************************
TuaCR3   ****************************************************A******
TuaCR4   **********************A***************S*************

TuaCR1   PRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINY
TuaCR2   ****************************************************
TuaCR3   **********M*************S****************************
TuaCR4   **********************------------------------------

TuaCR1   QPPTVVPGGDLAKVQRAICMISNSTSVAEVFSRIDFKFDLMYCKRAFVHWY
TuaCR2   ******************V*******************Y******A********
TuaCR3   ******************V********I******L*H******A********
TuaCR4   ----------------------------------------------------

TuaCR1   VGEGMEEGEFSEAREDLAALEKDYEEVGAEGQDDDEPGD DEY
TuaCR2   **************************DEG**GE*G***
TuaCR3   **********************F*****DSTEG*GEDEGE**
TuaCR4   ------------------------------------
```

FIG. 1. Alignment of the predicted amino acid sequences of four alpha-tubulin proteins from *Ceratopteris richardii*; the standard symbols for amino acids are used. Only the sequence for TuaCR1 is complete, and all other proteins are compared to it. Dashes represent unknown amino acids, asterisks represent known amino acids that are identical to those shown for TuaCR1, bold letters represent known amino acids that differ between TuaCR1 and at least one other TuaCR copy, and a blank space represents an apparent gap in the alignment.

protein sequence for this gene. For these reasons, and because it may represent an otherwise uncharacterized portion of one of the genes described here, it was not included in any of the comparisons described below.

A BLAST comparison of the four protein sequences for the *Ceratopteris* genes with other alpha-tubulin sequences recorded in GenBank reveals a somewhat diverse pattern (see the last column of Table 1). First, each of the four sequences was more similar to an alpha-tubulin from a different plant species than it was to any other *Ceratopteris* alpha-tubulin sequence. Second, the sequences from other plants that are most similar to the *Ceratopteris* sequences are quite diverse. The first two *Ceratopteris* amino acid sequences (those of TuaCR1 and TuaCR2) are most similar to sequences derived from dicotyledonous plants and/or a gymnosperm, the third is most similar to two different algal sequences, and the fourth is most similar to a sequence from a gymnosperm. These observations are perhaps more noteworthy in light of the fact that two distinct sequences for alpha-tubulin genes from the fern *Anemia phyllitidis* (one is a partial sequence) and two from the moss *Physcomitrella patens* are deposited in GenBank. When compared to the *Ceratopteris* genes (data not shown), some of the four lower plant genes have nearly as many identical amino acid sequences as do the sequences from the higher plants and algae shown in Table 1. However, when these high levels of identity exist, the lower plant sequences also have at least one additional amino acid or at least one less amino acid than the *Ceratopteris* sequences, resulting in gaps in the sequence homologies. The homologies indicated in Table 1 do not have such gaps and therefore the sequences they refer to are considered to be more similar to the *Ceratopteris* sequences.

For comparative purposes, BLAST searches were conducted for each of the known alpha-tubulin genes of *Arabidopsis* to determine what sequences from *Arabidopsis* or other organisms would show the highest similarities to each of these genes (data not shown). The entire genome of *Arabidopsis* has been sequenced, and six separate alpha-tubulin genes (TUA1–TUA6) have been located in its genome. The patterns of similarity for these genes are dramatically different from those of *Ceratopteris*. In general, the six *Arabidopsis* alpha-tubulin genes show much more similarity to one another than do the four *Ceratopteris* genes. Among these six genes are two pairs whose protein sequences are identical. The proteins of TUA2 and TUA4 are identical, and those of TUA3 and TUA5 are identical. Furthermore, TUA6 is almost identical to TUA2/4, sharing 448 out of 450 amino acids (99.6% identity). The amino acid sequences that are the next most similar to these five *Arabidopsis* genes (after comparing them to other *Arabidopsis* genes) are all sequences from angiosperms. The sequence of TUA2/4 is 98.7% identical to a sequence from *Brassica napus*, the sequence of TUA6 is 99.1% identical to the same sequence from *B. napus*, and the sequence of TUA3/5 is 97.1% identical to a sequence from *Oryza sativa*. Only the sequence of the remaining *Arabidopsis* gene product, that of TUA1, is more similar to the sequence of a different plant than it is to another *Arabidopsis* gene. The TUA1 protein shares 414 amino acids out of 450 (92% identity) with the grass *Miscanthus*.

Carboxy termini of the four protein sequences of alpha-tubulins from *Arabidopsis thaliana*:

| | |
|---|---|
| TUA1 | AREDLAALEKDYEEVG**G**EG**AE**D**DDE**EG**DE**Y |
| TUA2/4 | AREDLAALEKDYEEVG**A**EG**GD**D**EDD**EG**EE**Y |
| TUA3/5 | AREDLAALEKDYEEVG**A**EG**GD**D**EED**EG**ED**Y |
| TUA6 | AREDLAALEKDYEEVG**A**EG**GD**D**EDD**EG**EE**Y |

Carboxy termini of three inferred protein sequences of alpha-tubulins from *Ceratopteris richardii*:

| | |
|---|---|
| TuaCR1 | AREDLAALEKD**Y**EEVGA**EGQDDD**E**PGD D**EY |
| TuaCR2 | AREDLAALEKD**Y**EEVGA**EGDEG**D**EGEDGD**EY |
| TuaCR3 | AREDLAALEKD**F**EEVGA**DSTEG**D**GEDEGE**EY |

FIG. 2. Comparison of the carboxy terminus regions of *Arabidopsis* and *Ceratopteris* alpha-tubulin genes. Bold letters represent regions where the sequences within each set are not identical; a blank space represents an apparent gap in the alignment.

To further compare the relative diversity of the *Ceratopteris* and *Arabidopsis* alpha-tubulin proteins, the sequences from the carboxy terminus region, which is considered to be highly variable in alpha-tubulin proteins (e.g., see Sullivan, 1988; Fosket and Morejohn, 1992), were aligned with one another (Fig. 2). Since only three of the four *Ceratopteris* sequences contain the coding region for the carboxy terminus portion of the protein, TuaCR4 was omitted from this analysis. For both species the amino acid sequence is relatively conserved until the last 14 residues at the carboxy terminus end, or in the case of TuaCR1, which appears to have a single amino acid deletion in this region, the last 13 amino acids. In this region the *Arabidopsis* sequences share 6 identical amino acids out of 14, while the *Ceratopteris* sequences share only 3 identical amino acids.

*Gene phylogeny results.*—The Bayesian tree (Fig. 3) infers phylogenetic relationships of nucleotide sequences of the four sequenced *C. richardii* alpha-tubulin genes in relation to alpha-tubulin genes from other plant species. This figure depicts the tree from the single run noted in Materials and Methods as resolving one of the nodes, the resolved subnodes being toward the top of the tree with probabilities of 0.80, 0.50, and 0.81. The central clade from *Zea mays* 22149 to *Oryza sativa japonica* cultivargroup 1136121 was not resolved in any of the Bayesian runs. Posterior probability values show that many clades of this gene tree are strongly to moderately supported, although some deeper branches had weak support. The unresolved and weakly supported clades, however, are inconsequential to this paper, which focuses instead on the four copies of *C. richardii* genes and their placement in strongly supported clades. All nodes separating the four *Ceratopteris* genes are strongly supported. Three *Ceratopteris* copies (TuaCR1, TuaCR2, and TuaCR4) toward the top of Fig. 3 are relatively closely related to each other in a strongly supported subclade that is part of the major clade stretching from *C. richardii* TuaCR1 29423812 to

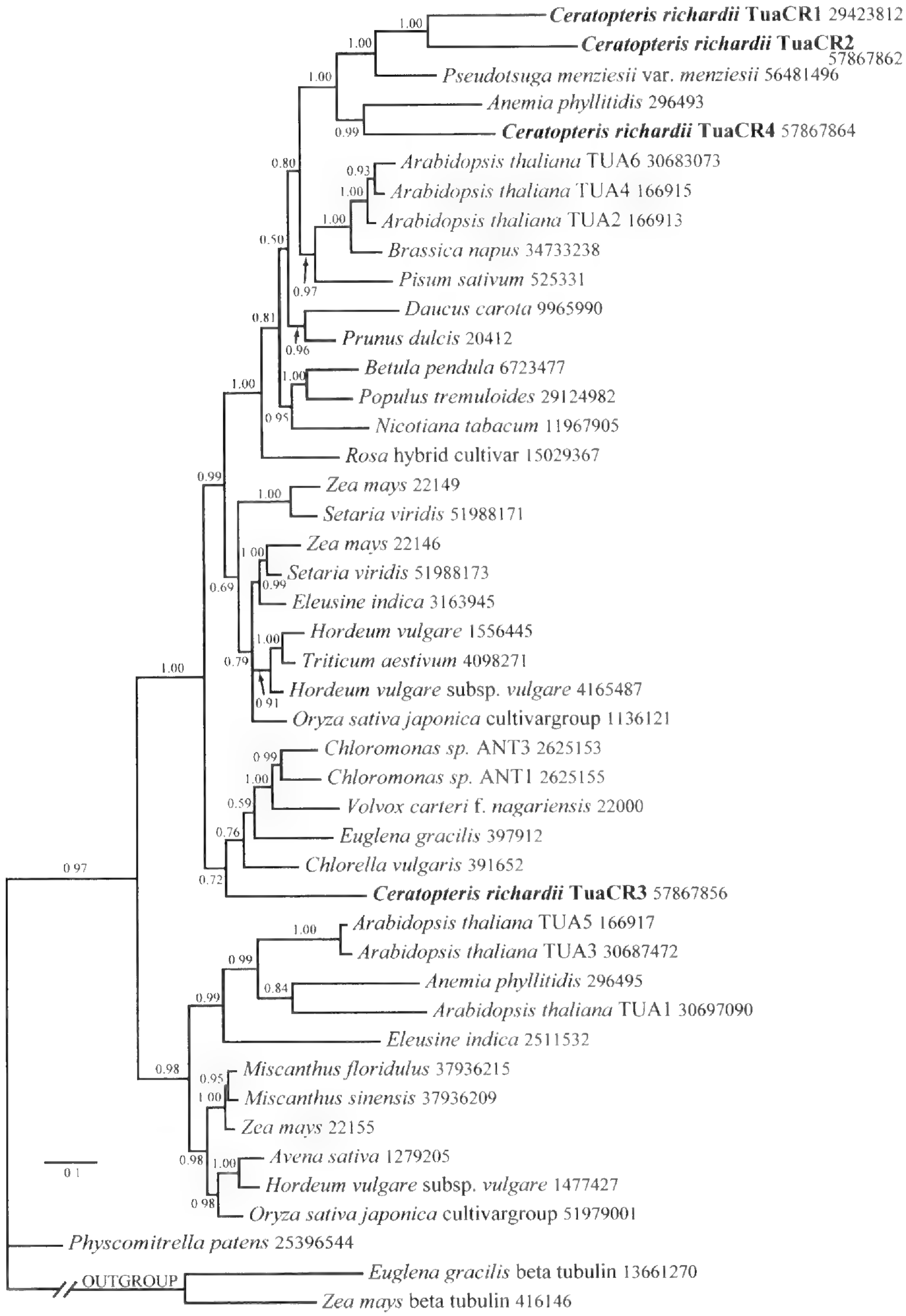


FIG. 3. Bayesian inference gene tree based on aligned nucleotide sequences of alpha-tubulin genes of Viridiplantae in GenBank (excluding the invariant first three nucleotides and the non-alignable last 45 nucleotides), showing relationships of the four sequenced *C. richardii* alpha-tubulin gene copies discussed. Taxon sources and GenBank gi numbers are given for each gene copy. Bayesian posterior probability values indicate support for clades.

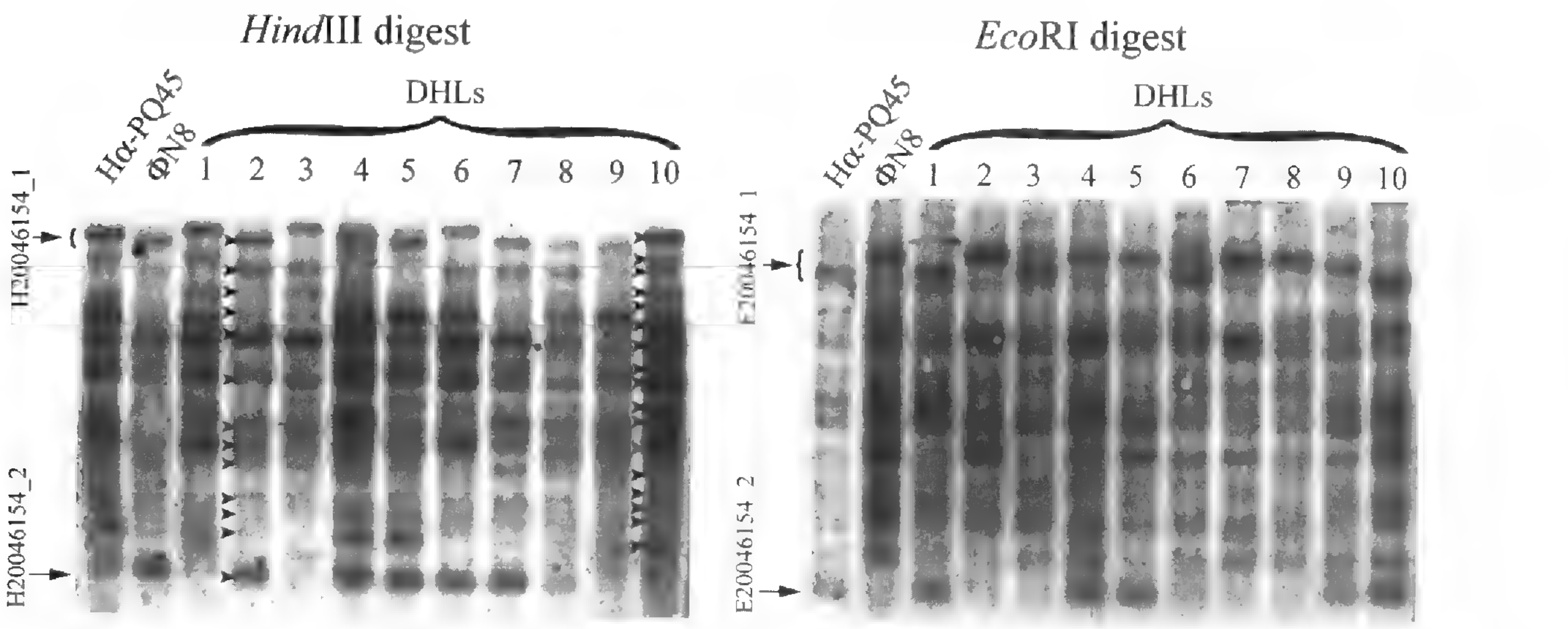


FIG. 4. Example autoradiograms of total genomic DNAs from the two parental *C. richardii* races and 10 DHLs derived from their cross, all probed with a DIG-labeled alpha-tubulin Cri_10_E18_SP6 cDNA clone after respective digestions of the genomic DNAs with *Hind*III or *Eco*RI. Arrowheads in DHL lanes 2 and 10 indicate at least 13 and 17 probable bands respectively. Segregating co-dominant and dominant RFLP bands containing alpha-tubulin genes are identified by arrows to the left of each autoradiogram and are discussed in the text and mapped in Fig. 5.

*Oryza sativa japonica* cultivargroup 1136121. This major clade is separated from its sister clade of miscellaneous algae and *C. richardii* TuaCR3 57867856 with a posterior probability of 1.00, although the precise positioning of *C. richardii* TuaCR3 in the algal clade is less strongly supported.

*Mapping results.*—Probings of parental and DHL DNAs with the DIG-labeled alpha-tubulin Cri_10_E18_SP6 cDNA clone yielded at least ca. 13–17 restriction fragment bands per DHL. The total number of bands cannot be determined with precision because of overlap and faintness of some of the bands. As an example, Fig. 4 shows the probing results for parental sporophytes Hα-PQ45 and ΦN8 and for ten DHLs whose genomic DNAs were cut with restriction enzymes *Hind*III and *Eco*RI, respectively. The DHL in lane 2 of the *Hind*III digest, for example, probably exhibits at least 13 bands (arrowheads) containing sequence to which the alpha-tubulin probe anneals and the DHL in lane 10 shows at least 17 bands. In some cases multiple bands may represent a single alpha-tubulin gene sequence that has been cut by the *Hind*III or *Eco*RI restriction enzyme. Although the sequence of the cDNA probe used in this alpha-tubulin study contains no *Hind*III or *Eco*RI restriction site, the coding region of the target DNA might contain such sites, and introns within the genes of the target DNA may contain *Hind*III or *Eco*RI sites. No data are available to address these possibilities.

Mapping can be performed only for those parental alpha-tubulin gene copies contained in restriction fragments that are polymorphic between the two parents and that therefore segregate in the DHLs. Four such unequivocal segregating sets of restriction fragments were observed in the genomic DNAs digested with *Hind*III and *Eco*RI in our mapping population. These are identified (Fig. 4) as H20046154_1 and H20046154_2 scored from the *Hind*III

digest and E20046154_1 E20046154_2 scored from the *Eco*RI digest. Polymorphic restriction fragment markers are usually expressed as co-dominant bands, meaning that the gene's presence is visualized in respective bands from both parents. In Fig. 4, this is exemplified in the *Hind*III digest by locus H20046154_1 where the larger fragment from parent Hα-PQ45 (also seen in DHL lanes 1, 3, 6, 10) is ca. 1 mm closer to the top of the figure than is the smaller fragment from parent ΦN8 (also seen in DHL lanes 2, 4, 5, 7, 8, 9). Locus E20046154_1 in the *Eco*RI digest in Fig. 4 shows a similar pattern. In this case the smaller fragment in parent Hα-PQ45 (also seen in DHL lanes 1, 3, 6, 10) is ca. 1.5 mm farther from the top of the autorad than is the larger fragment from parent ΦN8 (also seen in DHL lanes 2, 4, 5, 7, 8, 9). The two bands from the *Hind*III digest co-segregate with the two bands from the *Eco*RI digest in all of the mapping population's DHLs, indicating that they either mark the same identical locus visualized in the two different digests or mark two loci so closely linked that they show no crossover distance between them (i.e., they map to the same location). Locus H20046154_2 on the *Hind*III digest, on the other hand, is visualized as a dominant band in parent *Φ*N8 (also seen in DHL lanes 2, 4, 5, 6, 7, 8) meaning that no alternative band expression is visualized in the Hα-PQ45 parent (and in DHL lanes 1, 3, 9, 10). The reason for this dominant pattern is presently unknown but may be because the H20046154_2 gene copy in the Hα-PQ45 parent has been lost or has been moved to a different part of the genome where it cannot be scored because it overlaps with a different band, etc. In the *Eco*RI digest, locus E20046154_2 is also expressed as a dominant marker, present in parent Hα-PQ45 (and in DHL lanes 1, 4, 5, 9, 10), but with an alternative band lacking in parent *Φ*N8 (and in DHL lanes 2, 3, 6, 7, 8). Clearly the bands marking H20046154_2 and E20046154_2 do not co-segregate in the DHLs of the two digests, indicating that they mark different loci. The mapping program places H20046154_2 on linkage group (LG) 28 and E20046154_2 on LG 24 (Fig. 5 at arrows), whereas H20046154_1 and E20046154 1 map to the same position on LG 17 (Fig. 5 arrow).

## Discussion

*Map positions of alpha-tubulin genes.*—The genetic linkage mapping project for *C. richardii*, fully described by Nakazato *et al.* (2006), has identified 41 linkage groups that partly correspond to the 39 chromosomes per haploid set in this species. Alpha-tubulin gene copy H20046154_2 is located on LG 28, H&E20046154_1 is on LG 17, and E20046154_2 is on LG 24 (Fig. 5). Also seen on these three linkage groups are a large number of AFLP markers, each identified by a lowercase "a" followed by the primer pair used to generate it, and additional RFLP markers whose code numbers are the GenBank gi numbers of the cDNA library clones used as probes, prefaced with an "H" or an "E" to indicate whether that locus was visualized on the *Hind*III or the *Eco*RI digests. On LG 17 is also found the isocitrate dehydrogenase (IDH) isozyme locus, which maps to virtually the same position as H9960276 2. In

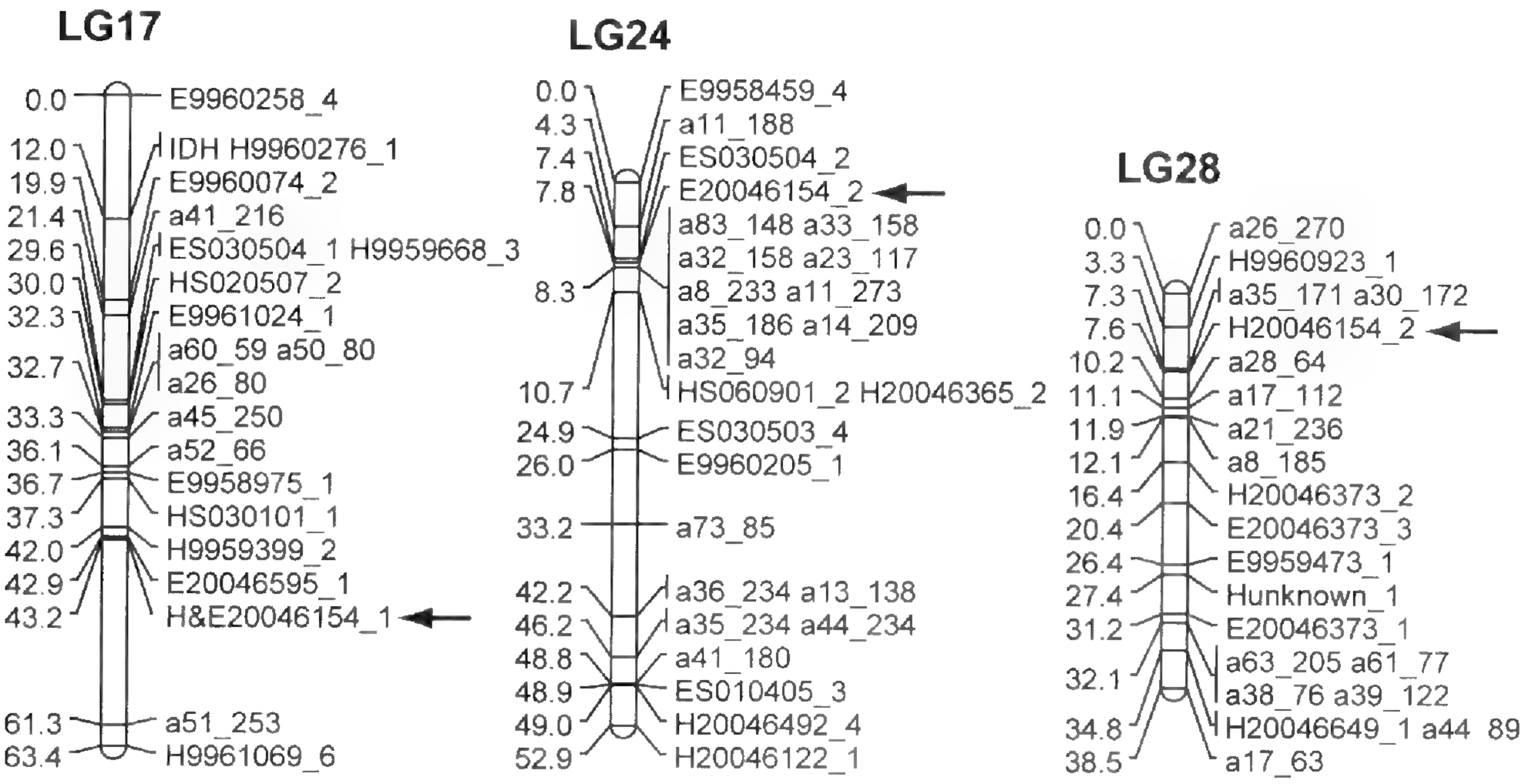


FIG. 5. Three of the 41 linkage groups presently identified in the *C. richardii* genome, showing at arrows the positions of the four segregating RFLP bands that contain alpha-tubulin genes identified in Fig. 4.

all cases, the cumulative distance in centiMorgans from the topmost maker is given to the left of each linkage group.

Because the alpha-tubulin clone used as a DIG-labeled probe here anneals to every alpha-tubulin gene copy with sufficient sequence identity, given the stringency conditions of our general mapping project, we cannot determine which of the genes in Table 1 are represented by respective markers H&E20046154_1, H20046154_2, and E20046154_2. The large number of bands (at least 13 to 17 in the indicated lanes in Fig. 4) suggests a large alpha-tubulin gene family in *C. richardii*, even if some genes are represented by more than one band. We were unable to map more than three or four alpha-tubulin loci because only four loci were contained in restriction fragments polymorphic in the two parents in *Hind*III and *Eco*RI digests.

Ceratopteris *alpha-tubulin gene phylogeny*.—The phylogenetic relationships of the four *C. richardii* genes in this paper are illustrated in Fig. 3. Bayesian posterior probability values show that copies TuaCR1 and TuaCR2 at the top of the tree are very strongly grouped as sister to each other and that both are in turn robustly sister to a copy from the conifer *Pseudotsuga menziesii*. Also with very strong support, the preceding are separated from TuaCR4 which is sister to an alpha-tubulin gene copy from the fern *Anemia phyllitidis*. The most parsimonious hypothesis to explain these present data is that TuaCR1 and TuaCR2 result from a recent duplication, perhaps after the divergence of ferns and seed plants, and that the gene duplication leading to the TuaCR4 lineage and the TuaCR1+TuaCR2+*Pseudotsuga* lineage preceded the divergence of ferns and seed plants. *Ceratopteris richardii* TuaCR3 (Fig. 3), on the other hand, is grouped in a clade of algal alpha-tubulin gene copies below the center of the tree. Although the precise relationships within this

clade of algal genes+TuaCR3 are not strongly supported, a 1.0 posterior probability value strongly separates this clade from the other three *Ceratopteris* genes in the major sister clade of miscellaneous seed plant alpha-tubulin gene copies above it. This indicates that TuaCR3 had already diverged from the common ancestor of the other three *Ceratopteris* alpha-tubulin genes by the time of the common ancestor of the ferns and algae, preceding the divergence of algae and land plants. The sampling of alpha-tubulin genes from plants in general, however, is currently very incomplete. The four *C. richardii* copies discussed here are simply those expressed in a cDNA library derived from 12-day-old gametophytes. Surely they do not represent the full range of alpha-tubulin gene copies expressed in the life cycle of *C. richardii*, just as the two alpha-tubulin gene copies sequenced thus far from the fern *Anemia phyllitidis* in Fig. 3 must represent only a fraction of the copies from that species. Inferred timings of duplications will likely change when more alpha-tubulin gene sequences become available. It may be noteworthy that *C. richardii* and the algae have motile gametes whereas all of the seed plants in Fig. 3 (including *Pseudotsuga*) lack motile sperm. This suggests TuaCR3 as a candidate for the gene copy functioning in sperm motility microtubules.

The occurrence of distantly related beta- and gamma-tubulin genes resulting from ancient duplications and the large number of alpha-tubulin gene copies seen in the *C. richardii* genomes in Fig. 4 indicate that there has been extensive and continuous duplication of tubulin genes throughout the evolution of organisms. It is also likely that deletion/silencing of tubulin gene copies is frequent, perhaps in lineage-specific ways. For example, despite intensive sequencing of alpha-tubulin genes in seed plants, gene copies from seed plants are absent from the lineage containing the TuaCR3 and algal gene copies in Fig. 3. This suggests that silencing/deletion of this copy may have occurred specifically in the seed plant lineage.

*Diversity of the* C. richardii *alpha-tubulin genes.*—In terms of amino acid sequences, particularly the carboxy termini, the four *Ceratopteris* alpha-tubulin sequences appear to be relatively diverse both when compared to one another (Figs. 1 and 2) and when compared to sequences from other plants (see Table 1, Fig. 2, and text of the Results section). The structural similarity of the *Ceratopteris* alpha-tubulin protein sequences to those of other diverse species is not surprising. This kind of similarity is often seen for alpha-tubulin proteins when sequences are compared using the BLAST algorithm (data not shown). The phylogenetic relationships of these four *C. richardii* sequences determined by Bayesian analysis of their nucleotide sequences excluding the invariant first codon and the non alignable 45 carboxy terminus nucleotides, however, indicates less diversity (Fig. 3). Phylogenetic analysis shows that TuaCR1 is closest to TuaCR2 and that, except for the alpha-tubulin copies from *Pseudotsuga* and *Anemia*, TuaCR4 is most closely related to TuaCR1 and TuaCR2. TuaCR3, on the other hand, is quite distant from the other three *C. richardii* copies and is most closely related to algal alpha-tubulin genes.

*Potential insights from further studies of the* Ceratopteris *alpha-tubulin gene family.*—Two main types of insights may be gained by studying the

diverse genes of organisms like *Ceratopteris*, and these are discussed in turn below. First, the diversity maintained in gene families like that of alpha-tubulin may provide new insights into the evolutionary history of specific genes as well as the mechanisms by which they evolve. The conventional wisdom regarding gene families is that the multiple versions of related genes develop by gene duplication and random mutation that produces variants subject to varying degrees of selection. In some cases, the different forms of these related genes assume different roles over the course of evolutionary history (e.g., see Zhang, 2003; Irish and Litt, 2005).

Consider the comparison presented earlier between the alpha-tubulin genes of *Arabidopsis* and those of *Ceratopteris*. The relative similarity in amino acid sequence of the six alpha-tubulin genes of *Arabidopsis thaliana* noted above suggests at least two likely interpretations. First, selection pressure may be very high for alpha-tubulin genes in this species, such that very little deviation is tolerated. Second, there may be relatively fewer potential roles for alpha-tubulin in the development and life history of *Arabidopsis* (for example, sperm are non-motile, unlike the situation in ferns) and therefore evolutionary factors have resulted in less diversity. In this respect, it is noteworthy that the *Arabidopsis* alpha-tubulin gene that differs most from the other five, TUA1, appears to be preferentially expressed primarily in pollen grains (Carpenter *et al.* 1992), while the others are apparently expressed in various tissues throughout the plant (Kopczak *et al.*, 1992).

By comparison with the alpha-tubulin genes of *Arabidopsis*, amino acid sequences of the four known alpha-tubulin genes of *Ceratopteris* appear somewhat diverse, suggesting selective pressures favoring the origin or maintenance of alpha-tubulin variation in this fern. This may relate in part to its fully independent gametophyte generation with perhaps more potential roles for alpha-tubulin (for example, as a component of the flagella in swimming sperm) than are found in its angiosperm counterpart, *Arabidopsis*. If these considerations prove correct and generally applicable to other genes, new insights regarding evolutionary mechanisms could be gained from studies focusing on *C. richardii* as a model homosporous vascular plant that could never be gained by studying plants like *Arabidopsis* alone.

A second type of information to be gained by studying diverse members of gene families, such as those of the *C. richardii* alpha-tubulin family, relates to gene expression. For example, what are the specific roles of the various members of the gene family? When during development and under which environmental conditions are these genes expressed? How is the expression of each gene controlled? To provide some insight into how *C. richardii* can be utilized to answer such questions, two potential research strategies utilizing the new sequence data presented in this report are described below.

A large number of morphological mutants exist from classical mutagenesis and selection screens using *Ceratopteris* (reviewed in Hickok, 1987; Hickok *et al.*, 1987, 1995). Some affect cell shape, like the *dwarf* or *bubbles* mutant (Hickok, personal communication), others affect intracellular organization, like the *polka-dot* mutant (Vaughn *et al.*, 1990), while yet others, like *sleepy*

*sperm* (Renzaglia *et al.*, 2004) affect the mobility of the sperm. Based on the phenotypes of the above mutants, it is likely that the defect associated with some of them may involve either an alpha-tubulin gene itself, a gene that controls expression of an alpha-tubulin gene, or a gene for another protein that interacts with alpha-tubulin. With the four sequences presented in this report, it will be relatively simple to assess the first two possibilities, using PCR to obtain sequence data and techniques like reverse transcription-PCR (RT-PCR) and/or real-time PCR to study relative levels of gene expression.

One may also study the roles of the various alpha-tubulin genes by utilizing recently developed methods for inducing gene silencing via RNA interference (RNAi) in spores and gametophytes of *Ceratopteris* (Stout *et al.*, 2003; Rutherford *et al.*, 2004). RNAi is a technique whereby specific genes may be silenced by the introduction of short single- or double-stranded RNA sequences into an organism's cells. These short RNA sequences, which must be highly similar to or identical to the complementary coding sequences within the targeted gene, trigger a cellular response that causes the degradation of the mRNA transcribed from the targeted gene.

In the two studies published so far using RNAi in *C. richardii*, the short RNAs have been introduced either by simply soaking spores directly in a solution containing the RNAs (Stout *et al.*, 2003) or by using the biolistic method to deliver the RNA into gametophytic cells (Rutherford *et al.*, 2004). The effects of gene silencing in *C. richardii* appear to be transmissible to cells descended from the cell into which the inhibiting RNA was initially introduced. In experiments where the inhibitory RNA was introduced into cells of young gametophytes by biolistic delivery, an observable phenotype was sometimes even transmitted to developing sporophytes (Rutherford *et al.*, 2004).

Each of the four alpha-tubulin genes described here has at least several portions of its sequence that are not shared by the other known members of this gene family (this is especially evident in the 3' regions of TuaCR1, 2, and 3). These sequences can be used to design short inhibitory RNAs that should be able to silence specifically the expression of their respective genes. Alternatively, it should be possible to silence two or more of these genes at the same time by using short RNA sequences that are shared by two or more genes. In either approach, such silencing can be confirmed by demonstrating an absence of (or reduction in) the targeted mRNA by using RT-PCR (Stout *et al.*, 2003) or real time PCR (Rutherford *et al.*, 2004), and phenotypes resulting from the silencing may also be observed in some cases (Rutherford *et al.*, 2004).

Several potential phenotypes could be easily observable as a result of silencing alpha-tubulin expression in *Ceratopteris*. Some may include such developmental defects as the inhibition of spore germination, or the generation of abnormal rhizoids, prothalial cells, or specialized cells such as those of antheridia or archegonia. Cytoskeletal defects generated by this approach may also lead to observable phenotypes—some may be associated with cell size or shape, while others may be as striking as the phenotype of the *polka-dot* mutant. Other observable abnormalities may affect the function of spermata-

zoids, generating phenotypes similar to the *sleepy sperm* mutant. The obvious advantage to generating such phenotypes through RNAi is that in each case, the phenotype will be directly associated with the silencing of one or more specific alpha-tubulin genes. This in turn may lead to the assignment of specific roles for each member of this gene family.

The linkage positions of the three mapped loci together with the specific sequencing data provided here should facilitate future cloning of genomic sequences containing alpha-tubulin genes in *C. richardii*. This will enable characterization of the organization and controlling elements of these genes, enhancing our understanding of the evolution, function, and regulation of the alpha-tubulin gene family in vascular plants in general. The research possibilities discussed here illustrate how robust molecular techniques can be applied to the *C. richardii* system, furthering its usefulness as a model system.

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# Ten New Species and Two New Combinations of *Blechnum* (Blechnaceae, Pteridophyta) from Bolivia

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ABSTRACT.—We describe ten new species of *Blechnum* (Blechnaceae, Pteridophyta) from Bolivia and provide a key to the Bolivian species of the genus. The new species are: *B. bicolor*, *B. bolivianum*, *B. bruneum*, *B. cochabambense*, *B. pazense*, *B. reflexum*, *B. repens*, *B. smilodon*, *B. squamatum*, and *B. vallegrandense*. *Blechnum gracilipes* and *B. squamipes* are elevated to species rank.

*Blechnum* (Blechnaceae) has about 150–200 recognized species but is still taxonomically very incompletely known, especially in the Andes. During our studies of the genus for a forthcoming guide to the ferns of Bolivia, we have encountered ten unnamed species that are here described.

Most of the species named here belong to sect. *Parablechnum* (C. Presl) T. Moore, one of the morphologically most distinctive species groups in *Blechnum*. This section is characterized by dimorphic leaves, usually erect or suberect rhizomes, ovate to ovate-lanceolate rhizome and petiole base scales, these bicolorous or concolorous and one cell thick near their apices, and a chromosome base number of x = 28. Section *Parablechnum* is also highly supported in molecular studies and perhaps should be treated as a distinct genus (Cranfill, 2001). The distinctiveness of the section contrasts with the difficulty in distinguishing the included species. This is partly due to the lack of monographic studies on the section and partly the result of poorly defined species boundaries and possible hybridization.

**Blechnum bicolor** M. Kessler *et* A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Nor Yungas, Unduavi, 3400 m, November 1910, *Buchtien 2690* (holotype: UC). **Fig. 1, A–C**.

Species ex grege *Parablechnuo* rhizomatibus brevibus erectis, squamis rhizomatum bicoloris cellulorum elongatorum translucentiumque, squamis costalibus bicoloribus, pinnis coriaceis marginarum parce reflexarum.

Terrestrial; rhizomes stout, erect, ca. 3 cm in diameter including petiole bases, scales 6–10 × 1–2.2 mm, lanceolate, translucent, reddish brown to

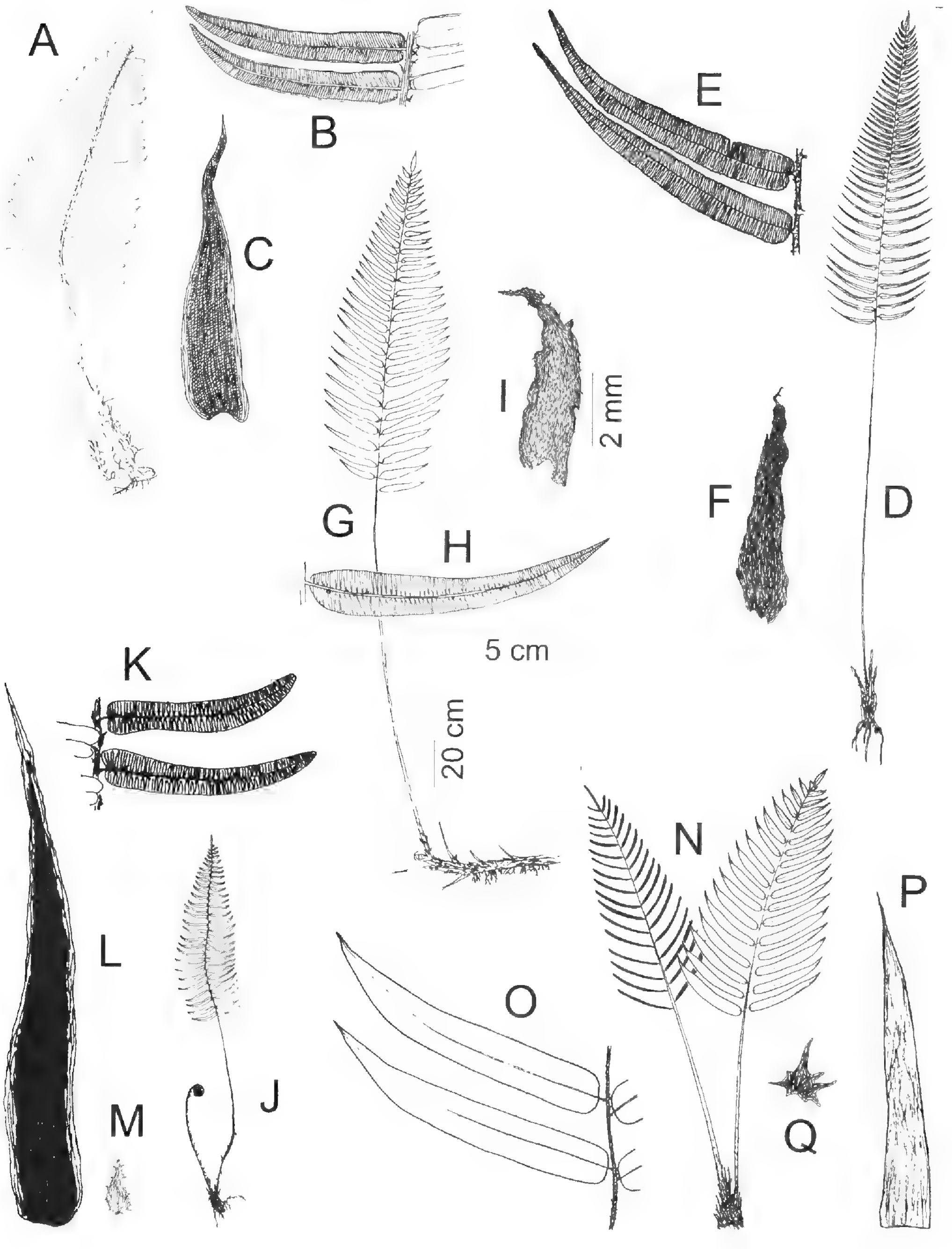


FIG. 1. *Blechnum bicolor*: A. Habit, B. Median pinnae, C. Rhizome scale. *Blechnum bolivianum*: D. Habit, E. Median pinnae, F. Rhizome scale. *Blechnum pazense:* G. Habit, H. Median pinna, I. Rhizome scale. *Blechnum cochabambense*: J. Habit, K. Median pinnae, L. Rhizome scale, M. Costal scale. *Blechnum squamatum*: N. Habit, O. Median pinnae, P. Rhizome scale, Q. Costal scale. The drawings of habit, pinnae, and scales, respectively, are to the same scale for all species.

blackish with narrow, sharply defined pale margins, cells elongate, margins entire; sterile leaves to 90 × 15 cm, erect; petioles to 45 cm long and 3 mm thick, reddish brown to distally stramineous, moderately scaly (but scales often abraded), scales basally similar to those of the rhizomes but soon grading into a mixture of two types of smaller, paler scales, one 2–10 × 0.2–2 mm, lanceolate, spreading, translucent, yellowish brown, entire or sparsely denticulate, the other 0.1–0.8 × 0.1–0.7 mm, roughly ovate, appressed, yellowish brown, ciliate; sterile laminae broadly lanceolate, 1-pinnate with an abruptly reduced base and gradually reduced apex with a conform apical pinna; pinnae ca. 15–28 pairs, to 9 × 1 cm, oriented at roughly 90° to the rachises or slightly ascending, the longest pinnae located in the proximal third of the laminae, the lowermost pinnae ca 90% as long as the longest ones, pinnae petiolulate (0.5–2 mm), falcate, apices acuminate, margins narrowly hyaline, finely serrulate, reddish brown with slightly paler marginal areas; laminar tissue rigidly chartaceous, drying to a similar pale olive green on both sides, adaxially glabrous, abaxially with scattered, tiny scales on the veins, the scales 0.05–0.2 × 0.05 × 0.2 mm, arachnoid, appressed, pale brown; veins simple or 1-forked, slightly immersed on both sides, ending in small hydathodes; fertile fronds similar to the sterile ones, but pinnae narrower, 3–5 mm wide; sori limited to the central part of the pinnae, i.e., lacking in the proximal 5–10 mm and the distal 10–20 mm; indusia dark, entire to erose at maturity.

PARATYPES.—Bolivia. **Cochabamba:** Prov. Ayopaya, Comunidad Pampa Grande, entre Achira y Pampa Grande, zona de pastoreo, 16°40′S, 66°28′W, 2130 m, 09 November 2002, *Jimenez 1552* (GOET, LPB, UC). **La Paz:** Prov. F. Tamayo, PN-ANMI Madidi, senda Keara-Mojos, abajo de Chunkani, 14°38′S, 68°57′W, 2910 m, 11 August 2001, *Jimenez 942* (LPB); Prov. Nor Yungas, Estación Biólogica de Tunquini, Bajo Hornuni, senda del campo de Don Pedro al camino de la mina, 16°12′S, 67°53′W, 2300 m, 25 July 2000, *García 4479* (GOET, LPB, UC); Prov. Nor Yungas, camino desde Unduavia a Chulumani, 16°18′S, 67°52′W, 3150 m, 22 December 1991, *Schmit 168* (LPB, UC); same general locality, 3115 m, 24 December 1991, *Schmit 170 pp* (LPB).

The epithet alludes to the bicolorous costal and rhizome scales.

This species forms a natural group with *B. bolivianum* and *B. pazense*, all of which are described here. Only in the last few years have enough new collections been made to allow the evaluation of the variability within the species and the consistency of the differentiating characters. As a group, they are perhaps most closely related to *B. cordatum* (Desv.) Hieron., which is a much larger species with paler, larger, concolorous rhizome scales. *Blechnum cordatum* is highly variable and probably comprises a species complex. A detailed study of this complex is needed to understand the impressive radiation it has undergone in the Bolivian Andes.

*Blechnum bicolor* is similar to *B. pazense* in having bicolorous costal scales and relatively thick-textured pinnae with slightly revolute margins, but differs by having less elongate rhizomes, bicolorous rhizome scales with translucent

cells, and stramineous costae and rachises. It is also similar to *B. bolivianum*, but the latter has concolorous costal scales, darker rhizome scales with shorter, occluded cells, and thinner-textured leaves.

On the type, Buchtien indicated that this species has a trunk up to 1 m tall, but this is probably due to confusion with one or both of two trunk-forming species of *Blechnum*, namely *B. loxense* (Kunth) Hook. *ex* Salomon and *B. auratum* (Fée) R. M. Tryon & Stolze, that co-occur with *B. bicolor* at the type locality. None of the additional specimens seen and no related species of *Parablechnum* have tall trunks. The type was studied by C. V. Morton in the late 1960s. On a note attached to the specimen he wrote that he was unable to name the specimen.

*Blechnum bicolor* is fairly common in humid montane forests at 2300–3400 m in Cotapata National Park, Bolivia. A specimen (*Jimenez 1552*) from Cochabamba, about 140 km east of the type locality may also belong with this species, but the rhizome and costal scales are less clearly bicolorous.

**Blechnum bolivianum** M. Kessler *et* A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Nor Yungas, Estación Biológica de Tunquini, senda cafetal (cerca del campamento de Don Pedro) al camino de la mina, 16°12′S, 67°53′W, 2500 m, 8 March 2001, *Bach 1332* (holotype: UC; isotypes: GOET, LPB). **Fig. 1, D–F**.

Differt a *Blechno cordato* (Desv.) Hieron. squamis rhizomatis atrobrunneis cellulorum occlusorum, pinnibus angustioribus tenuioribusque, rhachidibus cum squamis densiore vestitis.

Terrestrial; rhizomes stout, erect, ca. 5 cm in diameter including petiole bases, scales 4–6 × 1.2–2.5 mm, ovate-lanceolate, dark brown to blackish with narrow, pale brown margins, cells small, elliptical, mostly occluded, margins entire, erose or with a few distinct teeth; fertile leaves unknown; sterile leaves to 120 × 26 cm, erect; petioles to 60 cm long and 4 mm thick, reddish brown to distally stramineous, moderately scaly (but scales often abraded), scales basally similar to those of the rhizomes but these soon grading into a mixture of two types of smaller, paler scales, one 1.5–4 × 0.5–1.2 mm, ovate to ovate-lanceolate, spreading to appressed, dull pale brown, finely denticulate, the other 0.3–1 × 0.2–0.8 mm, ovate, dense, appressed, dull pale brown, ciliate; sterile laminae narrowly deltate, 1-pinnate with a rather abruptly reduced base and gradually reduced apex with a conform apical pinna; pinnae ca. 25–30 pairs, to 14 cm × 16 mm, oriented at 80–90° to the rachises, the longest pinnae located in the proximal 1/3 of the laminae, the proximal pinnae ca. 85% as long as the longest ones, pinnae petiolulate (1–2 mm), slightly curved forward, apices long-acuminate, margins narrowly hyaline, finely serrulate, very slightly revolute; aerophores tuberculiform; rachises reddish brown to stramineous, adaxially grooved, moderately scaly, scales similar to those of petioles but slightly smaller; costae reddish brown to stramineous, adaxially grooved, glabrous, abaxially densely scaly, the scales appressed to the laminae, 0.5–1.2 × 0.3–0.8 mm, ovate-lanceolate, denticulate, pale brown; laminar

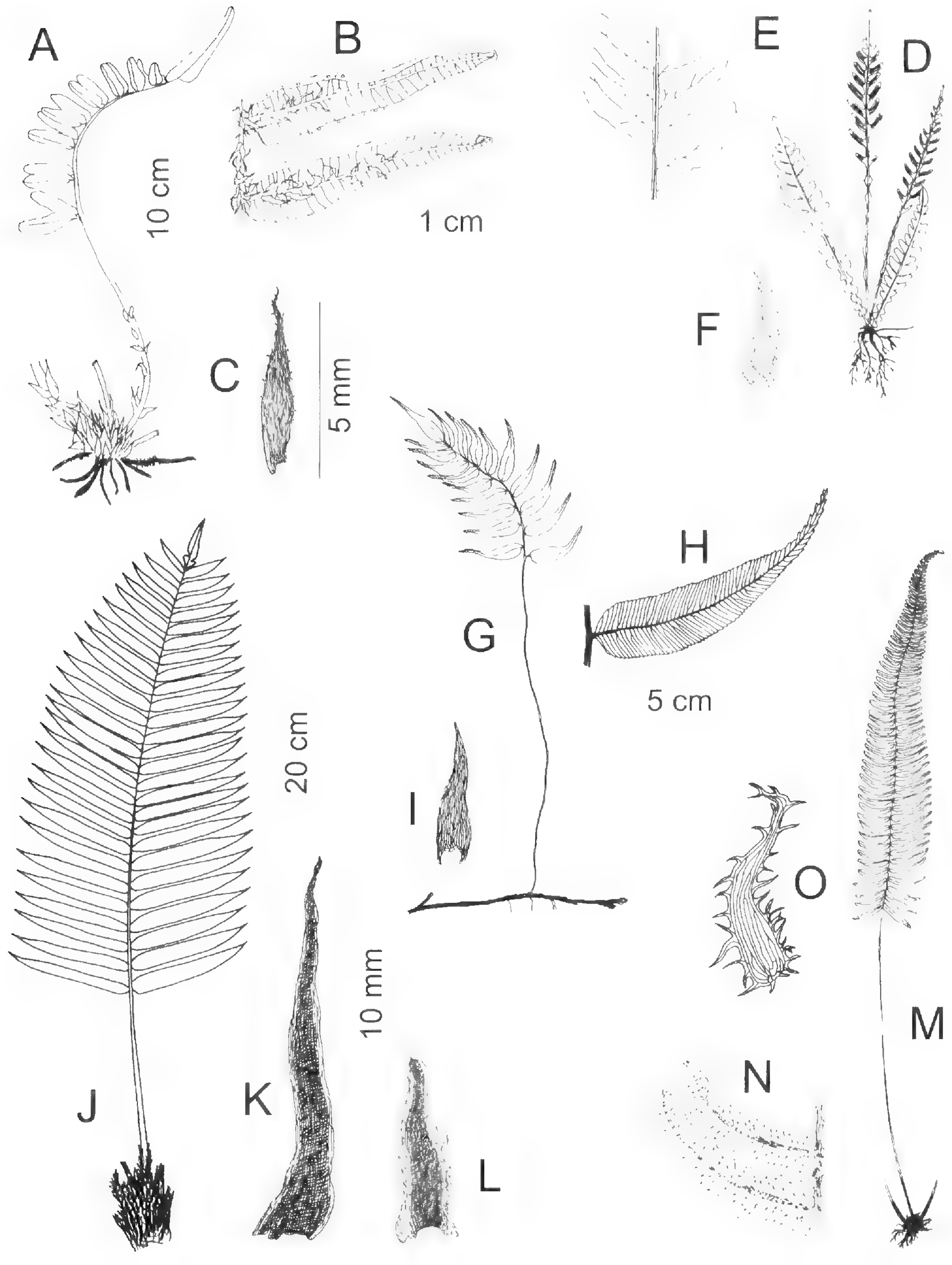


FIG. 2. *Blechnum reflexum*: A. Habit, B. Median pinnae, C. Proximal petiole scale. *Blechnum vallegrandense*: D. Habit, E. Median pinnae, F. Rhizome scale. *Blechnum repens*: G. Habit, H. Median pinna, I. Rhizome scale. *Blechnum bruneum*: J. Habit, K. Rhizome scale, L. Petiole scale. *Blechnum smilodon*: M. Habit, N. Median pinnae, O. Rachis scale. The drawings of habit, pinnae, and scales, respectively, are to the same scale for the upper two and lower three species.

tissue chartaceous, drying to a similar pale olive green on both sides, adaxially glabrous, abaxially with scattered, tiny scales on the veins, the scales 0.05–0.2 × 0.05 × 0.2 mm, arachnoid, appressed, pale brown; veins simple or 1-forked, adaxially slightly immersed, abaxially slightly raised and slightly paler than the laminar tissue, ending in small hydathodes.

PARATYPES.—Bolivia. **Cochabamba:** Prov. Carrasco, km 10 desde Siberia a Comarapa, 17°48′S, 64°42′W, 2600 m, 20 October 1996, *Kessler 9166* (LPB, UC). **La Paz:** Prov. Nor Yungas, Estación Biológica de Tunquini, senda cafetal, al camino de la mina, 16°12′S, 67°53′W, 2400 m, 08 April 2001, *Bach 1404* (GOET, LPB); same general locality, 2400 m, 26 July 2000, *García 4459* (LPB).

This new species is perhaps most similar to *B. cordatum*, but differs by having dark brown rhizome scales with occluded cells, narrower, thinner-textured pinnae, and more abundant rachis scales. It is also similar to *B. pazense*, but that species has longer, more creeping rhizomes, paler reddish brown, finely denticulate rhizome scales, narrower, more lanceolate blade outlines, and at least some bicolorous costal scales.

*Blechnum bolivianum* is a fairly common species in humid montane forests at 2400–2600 m in the departments of La Paz and Cochabamba. It mostly occurs at lower elevations than *B. bicolor*.

**Blechnum bruneum** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Bautista Saavedra, 12 km de Charazani hacia Apolo, 15°11′S, 68°46′W, 2500 m, 1 July 1997, *Kessler 10481* (holotype: UC; isotypes: GOET, LPB). **Fig. 2, J–L.**

Differt a *Blechnum brasiliense* Desv. squamis rhizomatis petiolorum basaliumque latioribus brunneisque (vs. nigribus), petiolis longioribus (17–33 cm *vs.* 2–12 cm), presentia squamorum rhachidi.

Terrestrial; rhizomes erect, densely scaly, scales 15–24 × 1.5–2.5 mm, linear to lanceolate, shiny, dull yellowish brown or with narrow dark red-brown centers, entire; fertile leaves unknown; sterile leaves to ca. 120 cm long, erect; petioles 17–33 cm long and to 7 mm in diameter, dark reddish brown, densely scaly at bases, sparsely scale distally, scales similar to those of rhizomes but smaller; sterile laminae to ca. 90 × 30 cm, lanceolate, pinnatisect, basally 1-pinnate, distally pinnatisect; sterile pinnae to ca. 60 pairs, to 15 cm × 16 mm, oriented at ca. 80° to the rachises, the longest pinnae about midblade, proximal pinnae gradually reduced with the lowermost pair to 1.7 cm long, linear-lanceolate, mostly broadly adnate (except the basalmost pinnae), bases somewhat unequal, apices acute, margins cartilageneous, serrulate, not revolute; aerophores absent; rachises dark reddish brown, grooved adaxially, sparsely scaly, the scales similar to those of the petioles but smaller, to 8 × 0.7 mm; costae stramineous, raised on both sides, glabrous; laminar tissue chartaceous, glabrous, drying olive green, slightly paler abaxially than adaxially; veins mostly 1-forked, impressed adaxially, raised abaxially, ending in small hydathodes.

PARATYPES.—Bolivia. **Cochabamba:** Prov. Carrasco, km 7 desde Siberia a Karahuasi, 17°48′S, 64°41′W, 2200 m, 16 October 1996, *Kessler 9094* (GOET, LPB); Prov. Chapare, Incachaca, 2200 m, 23 January 1929, *Steinbach 8914* (UC).

This species has gradually reduced proximal pinnae as well as lanceolate, brown rhizome and basal petiole scales (hence the species epipthet). In laminar shape it closely resembles *B. brasiliense*, but that species and its close Old World relatives have narrower, entirely or mostly black rhizome scales. *Blechnum bruneum* further differs from *B. brasiliense* by having longer petioles (17–33 cm *vs.* 2–12 cm) and by bearing scattered scales on the rachises. *Blechnum bruneum* grows along streams and in swampy places in humid montane forests at 2200–2500 m and appears to replace *Blechnum brasiliense* (up to 1600 m) at higher elevations in the Bolivian Andes.

**Blechnum cochabambense** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **Cochabamba:** Prov. Carrasco, Km 95–96 on old road between Cochabamba and Villa Tunari, 17 12.64′S, 65 41.95′W, 3400 m, 24 October 1999, *Ståhl 5156B* (holotype: AAU; isotypes: BOLV, S). **Fig. 1, K–M.**

Species ex sectione *Parablechno* rhizomatibus erectis squamas lanceolatas integres, 14–30 mm longas ferentibus, mixtura squamarum costalium partim ovatarum, spadicearum, ciliatarum, partim lanceolatarum, brunnearum, integrium usque ad denticulatarum, pinnis coriaceis falcatis basaliter cordatis, venis adaxialiter immersis, squamis laminarum satis densis preastans.

Terrestrial; rhizomes erect, ca. 3–4 cm in diameter including petiole bases, scales 14–30 × 2–3 mm, linear-lanceolate, reddish brown with narrow paler margins, entire; leaves to 70 × 15 cm, erect to arching, fertile leaves unknown; petioles to 30 cm long and 5 mm thick, shiny red-brown, densely scaly, scales of two types, one similar to those of the rhizomes but smaller, basally erose, and deciduous except at petiole base, the other appressed, ovate to lanceolate, 1–3 × 0.3–1.5 mm, dull pale brown, erose-denticulate; laminae lanceolate, 1-pinnate with a gradually reduced apex and an elongate apical segment; pinnae 21–28 pairs, to 9 cm × 11 mm, oriented 90 to the rachis, the longest pinnae the 2nd or 3rd pair from the laminar base, proximal pinnae 80–90% the length of the longest pinnae, pinnae petiolulate (1–2 mm), falcate, bases cordate, apices acuminate, margins narrowly hyaline, serrulate, revolute; rachises red-brown to stramineous, grooved, densely scaly, scales of two types, one 1–3 × 0.3–0.6 mm, spreading, lanceolate, brown, entire to denticulate, the other 0.3–1 × 0.2–0.8 mm, loosely appressed, ovate, tan, erose to ciliate; aerophores at pinna bases tuberculiform, dark; costae adaxially stramineous, grooved, moderately scaly, the scales similar to those of the rachis but smaller, abaxially stramineous, densely scaly, the scales similar to the large rachis scales; blades coriaceous, drying pale olive green, slightly paler abaxially, adaxially sparsely scaly, the scales 0.1–0.7 × 0.1–0.5 mm, loosely appressed, ovate, tan, erose to ciliate, abaxially sparsely scaly, the scales 0.1–1 × 0.05 × 0.5 mm, loosely arranged, lanceolate, tan, ciliate; veins simple, adaxially

immersed in leaf tissue, abaxially slightly raised and slightly paler than the laminar tissue, ending in small hydathodes.

PARATYPE.—Bolivia. **Cochabamba:** Prov. Carrasco, Lope Mendoza, km 110 camino a Santa Cruz, 17°32′S, 65°22′W, 3110 m, 05 May 1995, *Fernández 521* (BOLV, LPB).

This species belongs to sect. *Parablechnum*. It is recognized by its erect rhizomes with bicolorous, lanceolate, entire rhizome scales 14–30 mm long; tan, denticulate costal scales, coriaceous, falcate, basally cordate pinnae with adaxially sunken veins; and rather dense blade scales. It is probably most closely related to *B. cordatum s. lat.*, but that has concolorous, tan, translucent rhizome scales, whitish to pale tan, translucent, entire to erose costal scales, and broader, flat, thinner-textured pinnae. Other related species may be *Blechnum lima* Rosenst., which has similar coriaceous pinnae with sunken veins, but much larger, denticulate costal scales, and *B. squamatum* (which grows with *B. cochabambense*), which is larger overall and has broader, thinner-textured pinnae and much smaller rhizome scales.

*Blechnum cochabambense* is known from two collections in humid montane forests with *Weinmannia* or *Polylepis lanata* (Kuntze) M. Kessler & Schmidt-Leb. at 3100–3400 m in a limited area of Dept. Cochabamba (hence the species epithet).

**Blechnum pazense** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Franz Tamayo, PN-ANMI Madidi, sendero Keara-Mojos, a 1 hora y media aproximadamente de caminata desde Tokuaqe por la senda del inciensal, 14°36′S, 68°57′W, 2490 m, 11 March 2001, *Jimenez 752* (holotype: UC; isotype: LPB). **Fig. 1, G–I**.

Species ex sectione *Parablechno* rhizomatibus elongatis, prostratis usque ascendentibus, squamis rhizomatum uniforme atrorufescentibus denticulatisque, squamis costalibus fere 1 mm longis partimque bicoloribus preastans.

Terrestrial; rhizomes elongate, creeping to suberect, ca. 2–3 cm in diameter including petiole bases, scales 5–8 × 1.2–2 mm, lanceolate, dark reddish brown, denticulate; leaves to 175 × 21 cm, erect to arching, fertile and sterile leaves similar; petioles to 100 cm long and 5 mm thick, dark reddish brown, adaxially densely scaly, scales basally similar to those of the rhizomes but distally mixed with appressed, ovate to lanceolate, dull pale brown, denticulate scales 1–3.5 × 0.3–1 mm; laminae lanceolate, 1-pinnate, each with a gradually reduced apex; pinnae ca. 40 pairs, to 11 cm × 12.5 mm, oriented at 75–90° to the rachis, the longest pinnae medial, the proximal pinnae ca. 85% as long as the longest ones, pinnae petiolulate (1–3 mm), falcate, apices long-acuminate, margins hyaline, serrulate, slightly revolute; aerophores tuberculiform; rachises adaxially dull brown, grooved, glabrous, abaxially dark reddish brown to atropurpureous, densely scaly, scales 1–3 × 0.3–1 mm, appressed, ovate to lanceolate, pale brown, dull, denticulate; costae adaxially stramineous, grooved, glabrous, abaxially atropurpureous, bearing

dense scales, the scales appressed, 0.5–1 × 0.3–0.7 mm, ovate, entire to denticulate, pale brown to (at the base of the costae) bicolorous with dark brown centers and whitish margins; laminar tissue chartaceous, drying pale olive green on both sides, adaxially glabrous, abaxially with scattered, tiny scales mostly on the veins, the scales 0.05–0.2 × 0.05 × 0.2 mm, arachnoid, appressed, pale brown; veins mostly 1-forked, some simple, adaxially immersed, abaxially slightly raised and slightly paler than the laminar tissue, ending in small hydathodes; sori linear, continuous, parallel to the costae, leaving 2–4 mm of green laminar tissue between the sori and pinna margins, usually not reaching the pinna apex; indusia to 1 mm wide, translucent, brown, entire to slightly erose.

PARATYPES.—Bolivia. **La Paz:** Prov. F. Tamayo, Apolo, Pinalito, 14°31′S, 68°16′W, 2200 m, 26 April 2003, *Cayola 365* (LPB); Prov. F. Tamayo, PN-ANMI Madidi, sendero Keara-Mojos, a 1 hora y media aproximadamente de caminata desde Tokuaqe por la senda al inciensal, 14°36′S, 68°57′W, 2490 m, 11 March 2001, *Jimenez 751* (LPB, UC); Prov. F. Tamayo, PN-ANMI Madidi, senda Keara-Mojos, desde Tokuaqe, antes de llegar a Chunkani, 14°38′S, 68°57′W, 2830 m, 11 October 2001, *Jimenez 1008* (GOET, LPB, UC); Prov. Nor Yungas, Estación Biólogica de Tunquini, Bajo Hornuni, senda del campo de Dn Pedro al camino de la mina, 16°12′S, 67°53′W, 2400 m, 25 July 2000, *García 4460* (LPB, UC).

This species belongs to sect. *Parablechnum*. It is recognized by its elongate, creeping to suberect rhizomes, uniformly dark reddish brown, denticulate rhizome scales, and partly bicolorous costal scales about 1 mm long. It is most similar to *B. bolivianum*, which, however, has shorter, erect rhizomes, darker, entire, erose or spinulose rhizome scales, shorter and broader, basally more abruptly reduced laminae, and concolorous costal scales.

*Blechnum pazense* is fairly common locally in humid montane forests at 2400–2850 m in Dept. La Paz, where it co-occurs with *B. bicolor* and *B. bolivianum*.

**Blechnum reflexum** Rosenst. ex M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **Cochabamba:** Prov. Carrasco, Km 104 antigua carretera Cochabamba-Villa Tunari, 17°11′S, 65°40′W, 3250 m, 25 June 1996, *Kessler 6697a* (holotype: UC; isotype: LPB). **Fig. 2, A–C.**

Unicum in sectione *Parablechno* pinnis lateralibus ovato-lanceolatis distincte petiolulatis (1–2 mm) marginibusque forte reflexis

Terrestrial; rhizomes erect, ca. 1.5 cm in diameter including petiole bases, scales probably similar to those on the petiole bases (but not discernable on the small rhizomes available); fertile leaves unknown; sterile leaves to 33 cm long, arched; petioles to 18 cm long and 2.5 mm in diameter, slightly longer than the laminae, dark reddish brown, densely scaly at the bases with a few similar but smaller scattered scales distally, the scales 3–7 × 0.6–2.2 mm, lanceolate to oblong-lanceolate, spreading, flaccid, translucent, not shiny, light orangish

brown, with finely dentate margins; sterile laminae to 15 × 5 cm, lanceolate, 1-pinnate with a distinct, elongate apical pinna; sterile lateral pinnae to 15 pairs, to 2.6 cm × 5.5 mm, oriented at right angles to the rachis, strongly folded towards the adaxial rachis, the longest pinnae about 1/3 from the base of the laminae, the proximal pinnae ca. 60–70% as long as the longest ones ovate-lanceolate, often with unequal bases, petiolules 1–2 mm long, apices rounded, margins slightly cartilaginous, paler, serrulate, and strongly revolute; apical pinna of each blade to 4.4 cm × 7 mm, triangular with an abruptly reduced, unequal base and acute apex; rachises dark reddish brown, grooved adaxially, densely scaly, the scales 1.5–5 × 0.2–1.2 mm, lanceolate to oblong-lanceolate, appressed to slightly spreading, flaccid, translucent, orangish brown, with finely dentate margins; costae pale brown, deeply sunken adaxially and strongly raised abaxially, moderately scaly, the scales similar to those on rachis but somewhat smaller and relatively broader; laminar tissue coriaceous, drying dark olive adaxially and paler brown abaxially, glabrous; veins mostly simple, rarely 1-forked, impressed on both sides, abaxially conspicuously darker than the laminar tissue, ending in small hydathodes.

PARATYPE.—Bolivia. **La Paz:** Prov. Nor Yungas, Unduavi, 3300 m, November 1910, *Buchtien 2681* (UC).

This distinctive species was recognized by Rosenstock as undescribed and annotated accordingly on the herbarium sheet now at UC. However, Rosenstock never published it. To date it is known from only two sterile collections. Therefore, the measurements given in the description may not reflect the potential size of this species. Nevertheless, we do not hesitate in describing it as new because it has unique, petiolulate, ovate-lanceolate, and strongly reflexed lateral pinnae (hence the species epithet) with strongly revolute margins. No other known species of *Parablechnum* has similarly reflexed pinnae. The pinnae are somewhat reminiscent of *Blechnum auratum*, but that species has very different, long, falcate, and shiny rhizome scales and belongs to sect. *Lomariocycas* (J. Sm.) C. V. Morton.

*Blechnum reflexum* is apparently a rare and local species that has been found on rock faces in humid habitats around the present timberline at 3250–3300 m on the eastern Andean slope in Bolivia.

**Blechnum repens** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Nor Yungas, Estación Biológica de Tunquini, Bajo Hornuni, senda del campo de Don Pedro al camino de la mina, 16°12′S, 67°53′W, 2200 m, 16 August 2000, *Jimenez 453* (holotype: LPB). **Fig. 2, G–I.**

Unicum in genere *Blechno* rhizomatibus gracilibus, 3–4 mm latis, nigribus, longe prostratis pinnisque maximam partem liberis.

Epiphytic; rhizomes long-creeping, 3–4 mm thick, lustrous red-black, moderately scaly, scales 4–5 × 0.8–1.4 mm, linear to lanceolate, shiny, orange- to red-brown with slightly paler margins, margins subentire to remotely denticulate; fertile leaves unknown; sterile leaves to 75 cm long;

petioles ca. 45 cm long and to 1.5 mm in diameter, dark reddish brown, sparsely scaly, scales similar to those of the rhizomes but smaller; sterile laminae to ca. 30 × 15 cm, deltate, 1-pinnate, distal two pinna pairs basally somewhat adnate; sterile pinnae 7–9 pairs, to 8 cm × 16 mm, the proximal pinnae the longest, lanceolate with elongate, falcate apices, margins finely serrate, very narrowly cartilaginous, not revolute; aerophores tuberculiform; rachises dark reddish brown, grooved adaxially, sparsely scaly, the scales fibrillose, 0.2–1 mm long, orange; costae stramineous, slightly raised on both sides, basally with a few scales resembling the rachis scales; laminar tissue chartaceous, glabrous, drying olive green, slightly pale abaxially than adaxially; veins mostly 1-forked, impressed adaxially, raised abaxially, ending in small hydathodes.

This striking species has slender, 3–4 mm wide, blackish, long-creeping rhizomes unlike most other species of *Blechnum*. We lack fertile material, but the laminar aspect and the scale characters suggest that it belongs in sect. *Parablechnum*. It is perhaps closest to *B. pazense* but that species has erect to short-creeping, 2–3 cm thick rhizomes, and ca. 40 pinna pairs (*vs.* 7–9 pairs in *B. repens*). Interestingly, another epiphytic species of *Blechnum* with blackish, creeping rhizomes, *B. anthracinum* R.C. Moran, has recently been described from Cotapata, about 10 km south of the type locality of *B. repens* (Moran, 1992). Despite the apparent similarity in the rhizome characters, *B. anthracinum* is not closely related to *B. repens*, belonging to the species group of *B. occidentale*. It has monomorphic sterile and fertile leaves, fully adnate pinnae, and different scale characters. The apparently independent, convergent evolution of two epiphytic species of *Blechnum* with blackish, creeping rhizomes at the same site is puzzling.

**Blechnum smilodon** M. Kessler & M. Lehnert, ***sp. nov.*** TYPE.—Bolivia. **La Paz:** Prov. Nor Yungas, Trocha al Valle de Coscapa, Parque Nacional Cotapata, 16°12′S, 67°53′W, 3250 m, 11 Sep 1997, *Kessler 11814* (holotype: GOET; isotypes: LPB, UC). **Fig. 2, M–O**.

Differt a *Blechno stipitellato* (Sodiro) C. Chr statura minore (frondibus usque 90 × 13 cm vs. 120 × 22 cm), pinnis creberioribus per fronde (40–55 vs. 20–40 iugis), venis complanis vel parce prominentibus (vs. immersis), squamibus costarum rhachidumque magis ciliatis.

Terrestrial; rhizomes stout, erect, ca. 3 cm in diameter including petiole bases, scales 8–12 × 1.5–2 mm, lanceolate, translucent, blackish to reddish brown with slightly paler marginal areas, cells elongate, margins irregularly dentate; sterile leaves to 90 × 13 cm, erect; petioles to 45 cm × 0.6 cm, dark red-brown with atropurpureous bases, sparsely scaly (scales often abraded), basal scales similar to those of rhizomes; fertile blades unknown; sterile blades lanceolate, 1-pinnate, each with an abruptly reduced base and gradually reduced apex; pinnae ca. 40–55 pairs, to 7.5 cm × 9 mm, proximally deflexed, at midblade oriented at roughly 90° to the rachis, distally slightly ascending, the longest pinnae located in the proximal third of the blade, these ca. 60–80%

as long as the longest ones, petiolulate (0.5 mm), slightly falcate, apices acuminate, margins narrowly hyaline, serrulate, slightly to strongly revolute; aerophores tuberculiform; rachises atropurpureous, reddish brown or stramineous, adaxially grooved, densely scaly, the scales of two types, one 3–7 × 0.2–0.8 mm, lanceolate, spreading, translucent yellowish brown with irregular median lines of dark red-brown to blackish cells, the margins setose, the other 0.2–2 × 0.1–0.8 mm, ovate to broadly lanceolate, appressed, yellowish to dark brown, setose, with the setae about as wide as the scale body; costae stramineous, adaxially grooved, on both sides bearing sparse scales resembling the smaller rachis scales; blade tissue thinly coriaceous, drying olive green on both sides, adaxially glabrous, abaxially with scattered, tiny scales on the veins, the scales 0.05–0.2 × 0.05 × 0.2 mm, arachnoid, appressed, pale to medium brown; veins simple, flush or slightly raised on both sides, ending in small hydathodes.

PARATYPES.—Bolivia. **Cochabamba:** Prov. Ayopaya, comunidad Pampa Grande, sendero a Incacasani Grande, 16°40′S, 66°28′W, 3240 m, 18 September 2002, *Jimenez 1679* (GOET, LPB, UC); Prov. Chapare, ca. 8 km al N de Maycamayu, ca. a 70 km de Sacaba, 17°12′S, 65°57′W, 3350 m, 08 December 1991, *Kessler 2917* (LPB). **La Paz:** Prov. Nor Yungas, Estación Biológica de Tunquini, senda nueva del camino de la mina (curva al lado oeste) al pantanón, 16°11′S, 67°53′W, 3000 m, 14 September 2000, *Bach 1038* (GOET, LPB); same locality, 13 July 2002, *Bach 1839* (GOET, LPB, UC); Prov. Nor Yungas, Estación Biólogica de Tunquini, Bajo Hornuni, senda del campo de Don Pedro al camino de la mina, 16°11′S, 67°53′W, 2550 m, 17 August 2000, *Jimenez 474* (GOET, LPB); Prov. Murillo, valle de Zongo aprox. 15 km desde la cumbre, 16°9′S, 68°7′W, 3150 m, 04 July 1979, *Beck 1126* (LPB).

The name, taken from the sabre-toothed lion, alludes to the strongly dentate scales of the species.

The specimens placed here have previously been determined as *Blechnum stipitellatum* (Sodiro) C. Chr., and the range of that species was accordingly indicated as including Bolivia by Tryon & Stolze (1993). Recent field experience with both typical *B. stipitellatum* in Ecuador and the Bolivian plants, and study of type material of *B. stipitellatum* has, however, convinced us that two different and actually quite distinct species are involved. *Blechnum smilodon* differs from *B. stipitellatum* mainly in its smaller size (leaves to 90 × 13 cm *vs.* to 120 × 22 cm), higher number of pinnae per blade (40–55 *vs.* 20–40 pairs), flush or prominulous (*vs.* immersed) veins, and much more strongly dentate rachis and costal scales. *Blechnum smilodon* is known only from Bolivia, where it is fairly locally common at the humid timberline ecotone and less commonly found in cloud forests at 2500–3350 m.

True *B. stipitellatum* (syn.: *B. rubicundum* Hieron.) is most similar to *B. lima* Rosenst., from which it differs primarily in blackish to reddish brown (*vs.* tan) scales on the rhizomes, petioles, rachises, and costae, and somewhat less deeply sunken veins. These two species appear to replace each other geographically, with *B. lima* being known only from Bolivia, and *B.*

*stipitellatum* found from southern Peru north to Colombia. Previous reports of *B. lima* from Ecuador (Jørgensen and León Yanez, 1999) are based on misidentified material of *B. stipitellatum*.

**Blechnum squamatum** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia, Cochabamba, Carrasco, 94 km antigua carretera Cochabamba-Villa Tunari, 17°08′S, 65°38′W, 3500 m, 28 June 1996, *Kessler 6790* (holotype: UC; isotypes: GOET, LPB). **Fig. 1, N–Q**.

Differt a *Blechno cordato* (Desv.) Hieron. squamis costalibus valde majoribus, squamis rhachidium densioribus, majoribus partimque bicoloribus, aerophorisque majoribus.

Terrestrial; rhizomes erect, 15+ cm tall, ca. 10–20 cm in diameter including petiole bases, scales 8–14 × 2–24.5 mm, lanceolate, pale orangish brown, on some specimens with darker centers, margins entire or very slightly and remotely denticulate; sterile leaves to ca. 100 × 30 cm, erect; petioles to 50 cm long and 4 mm thick, proximally reddish brown, distally stramineous, moderately scaly (scales often abraded), scales of two types, one similar to those of rhizomes, but averaging somewhat smaller, the other 0.2–1.2 × 0.2–0.9 mm, roughly ovate to broadly lanceolate, appressed, pale, ciliate; sterile laminae broadly lanceolate, 1-pinnate, each with a rather abruptly reduced base and gradually reduced apex with a conform apical pinna; pinnae ca. 15–18 pairs, to 21 × 2.5 cm, ca. 70° to the rachises, the longest about a third from the base of the laminae, the proximal pinnae ca. 80% as long as the longest ones, petiolulate (3–5 mm), falcate, apices long-acuminate and serrulate, margins narrowly hyaline, entire, flat; fertile leaves known only from fragments; pinnae to 9 cm × 4 mm, linear, thicker-textured and drying of darker color than the sterile pinnae; sori covering the entire pinnae except the distal 1–3 mm; indusia dark brown, entire but erose after opening; rachises pale reddish brown to straw-coloured, adaxially grooved, moderately to densely scaly, scales similar to those of the petioles, but smaller, with partly fibrillose and arachnoid scales; aerophores at pinna bases dark reddish brown, peg-like, 1–5 mm long and 1–2 mm in diameter; costae reddish brown to straw-coloured, adaxially grooved, glabrous, abaxially with spreading scales 1–3.5 × 0.4–1.7 mm, triangular to lanceolate with cordate to truncate bases, margins denticulate to ciliate, pale orangish brown, larger scales often with dark reddish brown centers; laminar tissue chartaceous, drying dark olive green adaxially and pale olive green abaxially, adaxially glabrous, abaxially with scattered, small scales on the veins, the scales 0.05–1 × 0.05 × 0.5 mm, arachnoid to triangular, appressed, pale brown; veins simple or 1-forked, slightly immersed adaxially, ending in small hydathodes.

PARATYPE.—Bolivia. **La Paz:** Prov. Saavedra, Charazani, Richtung Carijana, 2600 m, 17 January 1994, *Herzog 438* (LPB); Prov. Caranavi, Serranía Bellavista, 47 km Caranavi a Sapecho, 15 39′S, 67°28′W, 1150 m, 31 August 1997, *Kessler 11661* (LPB, UC).

This species differs from *B. cordatum* in having much larger costal scales, much denser, larger and partly bicolorous rachis scales, and larger aerophores. *Blechnum cordatum* is a highly variable species with respect to size, number and shape of pinnae, and scale color and density, and presumably comprises a species complex. We have been unable to subdivide this complex in any meaningful way, with the exception of the most distinct form, which is described here. *Kessler 11661* differs from the type in having uniformly tan costal scales and relatively small (1 mm *vs.* 2 mm diameter) aerophores. More material is needed to evaluate the variability of this species.

*Blechnum squamatum* appears to be uncommon in humid montane forests at 1150–3500 m.

**Blechnum vallegrandense** M. Kessler & A.R. Sm., ***sp. nov.*** TYPE.—Bolivia, Depto. Santa Cruz, Prov. Valle Grande, 13 km de Loma Larga a Valle Grande, 18°39′S, 63°55′W, 2300 m, *Kessler 6474* (holotype: UC; isotypes: GOET, LPB). **Fig. 2, D–F**.

Differt a *Blechno mochaene* Kunkel var. *squamipede* (Hieron.) de la Sota frondibus sterilibus petiolos deficientibus, frondibus valde brevioribus, squamis rhizomatum atrorufescentibus.

Terrestrial; rhizomes erect, stout, ca. 1 cm in diameter including petiole bases, scales 2–3.5 × 0.8–1.3 mm, lanceolate, subclathrate, dark red-brown, entire; sterile leaves to 11.5 cm × 17 mm, erect, lacking petioles, sterile laminae lanceolate, pinnatisect with a slightly elongate apical pinna; sterile pinnae 15–17 pairs, to 10 × 4 mm, at ca. 65–70° to the rachis, the longest about 2/3 from the base of the laminae, the proximal pinnae ca. 15% as long as the longest ones, broadly adnate, slightly falcate, apices obtuse to slightly acuminate, margins with one row of hyaline cells forming a very finely serrulate margin, sometimes slightly revolute; apical pinna to 10 × 5 mm, triangular in outline, confluent at the base to one or two distalmost pinnae, apex subacute; rachises stramineous, grooved adaxially, glabrous; costae greenish to stramineous; laminar tissue fleshy, drying olive green on both sides, glabrous; veins ill-defined, mostly simple, partly 1-forked, ending in small hydathodes; fertile leaves to 16 cm × 14 mm, erect, petioles to 15 mm, proximally with 4–5 pairs of greatly reduced, broadly triangular, winglike sterile pinnae, distally the laminae lanceolate, with an elongate apical pinna; fertile pinnae 11–12 pairs, to 11 × 1.2 mm (excluding protruding indusia), oriented at ca. 55–65° to the rachises, broadly adnate, linear, falcate, apices acuminate, margins with one row of hyaline cells, drying olive green on both sides, glabrous, the fertile apical pinna to 12 × 1.2 mm, linear; rachises and costae as on sterile leaves; sori covering the entire pinnae except the apical 0.8–1.5 mm; indusia to 0.8 mm wide, dull brown, erose, spreading and extending beyond laminar tissue at maturity.

With its dimorphic sterile and fertile leaves, short-creeping to erect, stoloniferous rhizomes, adnate pinnae, and concolorous rhizome scales this species belongs to the species group of *B. penna-marina* (Poir.) Kuhn and *B.*

*divergens* (Kunze) Mett. It is most similar to *Blechnum squamipes* (Hieron.) M. Kessler & A.R. Sm. but differs by lacking petioles on the sterile leaves, having much smaller leaves, and by the dark red-brown rhizome scales. The type specimen of *B. vallegrandense* was originally identified as the Chilean species *B. blechnoides* (Bory) Keyserl. by Smith *et al.* (1999), but *B. vallegrandense* is much smaller, lacks petioles on the sterile leaves, and has thinner-textured blades. Species related to *B. blechnoides* and *B. mochaenum* Kunkel constitute a complex that is in need of detailed monographic study.

## New Combinations

The following two taxa were originally described as varieties, but we consider them to be distinct at species rank:

**Blechnum gracilipes** (Rosenst.) M. Kessler & A.R. Sm., ***comb. nov.*** – *Blechnum blechnoides* Keyserl. var. *gracilipes* Rosenst., Repert. Spec. Nov. Regni Veg. 9: 343. 1911.

Endemic to Bolivia; saxicolous in humid montane forests at 700–2200 m.

**Blechnum squamipes** (Hieron.) M. Kessler & A.R. Sm., ***comb. nov.*** – *Blechnum lanceolatum* (R. Br.) J. W. Sturm var. *squamipes* Hieron., Bot. Jahrb. Syst. 22: 381. 1896.

Southern Bolivia and northwestern Argentina; humid forests at 1850–2300 m.

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# *Pteridium caudatum* (L.) Maxon Behaves as a Potassium Plant and Accumulates Aluminum in the Subterranean Organs

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ABSTRACT.—The purpose of this study was to investigate the nutritional status of *Pteridium caudatum* (bracken fern) in a Neotropical region where this species occurs in acid leached soils. In this region there is a high availability of Al in soluble toxic forms, rendering *P. caudatum* an important weed associated with wildfire regimes. Water-soluble Ca, exchangeable Ca fraction, Ca bound to pectate + phosphate, and bound to oxalate were evaluated from *P. caudatum* sampled from a burned parcel of land 94 and 270 days after an accidental fire, as well as from an unburned control parcel. Both sites were located in a tropical secondary savanna community in a successional mosaic of a cloud forest. The concentrations of total Ca, N, P, K, Mg, Fe, Mn, Zn, Cu, Ni and Al, and their distribution in the plant organs were investigated. The study addressed the hypothesis that shoots should show low concentrations of Ca because a low cation capacity exchange has been reported in roots of *Pteridium*. We expected a low water-soluble Ca fraction because bracken has been defined in the literature as a non-calcicole plant. The exchangeable fraction and pectate + phosphate bound Ca constituted 60 to 85% of the total Ca in pinnae and rhizomes, while the oxalate bound Ca constituted only 3 to 14% of the total Ca. Concentrations of Al as high as 248.3 mmol $kg^{-1}$ were found in roots. Pinnae showed only 84.53 mmol Ca $kg^{-1}$ and 5.62 mmol Al $kg^{-1}$, and their Ca/Al ratio was 15 mol $mol^{-1}$ contrasting with *P. aquilinum* from temperate regions where Ca/Al was 1440 mol $mol^{-1}$, however the Ca/P was 2 mol $mol^{-1}$ in both species. We conclude that *P. caudatum* behaves as a potassium plant (soluble K/Ca >>1) such as the grass-like families Poaceae and Cyperaceae and accumulates Al in the subterranean organs.

Bracken ferns of the world-wide genus *Pteridium* appear in the Neotropics on land exposed to human intervention where primary cloud forests have been converted into montane savannas (Alonso-Amelot and Rodulfo-Baechler, 1996). The basic characteristics of tropical montane cloud forests are that they capture water from clouds, have low evapotranspiration rates and add water to the hydrologic system. These forests are threatened by anthropogenic fire usually associated with grazing, agricultural interventions and hunting (Hamilton *et al.*, 1995). *Pteridium* occurs especially widely in fire burned habitats and, unusually, has the ability to pioneer ash-burn surfaces of high potash levels and highly alkaline pH, metamorphosing to become an acidophilus plant once its rhizomes penetrate beyond the initial ash surface layers (Page, 2004).

It is important to understand the mineral nutrition of bracken because this perennial weed causes problems for agriculture, forestry, conservation and

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animal health, despite many attempts to develop control strategies (Papavlasopoulos, 2003). The nutritional status of *Pteridium aquilinum* (L.) Kuhn has been studied in temperate climates (Thompson *et al.*, 1997; Skre *et al.*, 1998) and there are several studies of the Neotropical bracken fern, *P. caudatum* (L.) Maxon, concerning xenobiotic materials such as phenolics, tannins, the cyanogenic glycoside prunasin, and illudanes, which protect the plant from herbivores (Alonso-Amelot and Avendaño, 2002; Alonso-Amelot *et al.*, 2001; 2004). However, as far as we know, bracken's nutritional status in tropical environments has not been investigated.

There is variation in the shoot Ca content of angiosperms (Broadley *et al.*, 2003), the lowest values corresponding to orders of commelinoid monocots (rice, cereals, maize, bananas). According to Broadley *et al.* (2003) root cation-exchange capacity (CEC), pectin and Ca shoot content may be correlated. We expect a lower Ca content in bracken than in dicots based on the low CEC found by Koedam *et al.* (1992) in *P. aquilinum* roots. The root CEC is located in the apoplast and is attributed to the free carboxyl groups of galacturonic acids of cell wall pectins in the middle lamella. The pectin contents of shoot cell walls are comparable to those of root cell walls (Broadley *et al.*, 2003).

According to White and Broadley (2003) plants with a low soluble Ca concentration include the potassium plants, characterized by high shoot K/Ca quotients, and the oxalate plants, which have high tissue oxalate concentrations. Plants that accumulate oxalate can be subdivided into species that contain soluble oxalate and those in which Ca-oxalate is precipitated. Iljin in 1936–1944 (Kinzel, 1983) designated the term calciotrophic to plants which contain appreciable amounts of water-soluble Ca. This physiological terminology refers only to Ca metabolism and is different from the geobotanical label calcicole, which refers to the flora observed on calcareous soils. In such a flora, nutrient acquisition and ecological success depend not only on Ca, but also on other factors such as their root development, microorganism associations, rhizosphere pH, root exudates, etc.

The three distinct physiotypes for Ca nutrition, potassium plants, oxalate plants and calciotrophes, are characteristic of particular angiosperm families (White, 2005), therefore it is interesting to investigate the K/Ca ratio and the total Ca fractionation in a fern. In acid soils of the Instituto Venezolano de Investigaciones Científicas (IVIC) calciotrophes, such as *Clusia multiflora*, are present with a total Ca concentration of 244 mmol $kg^{-1}$, and water-soluble Ca representing 77% of the total Ca, while oxalate comprises only 6% of the total, but this species is also found in dry calcareous forests where it was found to have Ca concentrations of 403 mmol $kg^{-1}$ (Olivares and Aguiar, 1999). Bracken has been defined as an acidiphilous pteridophyte (Koedan *et al.*, 1992; Page, 2004); it usually does not grow on calcareous soils and we may expect a low water-soluble Ca fraction because this fraction is advantageous as an osmotic counter ion in xerophytic, calcareous environments, which is the opposite of bracken habitat. Therefore bracken is predicted to have the physiotype of a potassium or oxalate plant. The Ca physiotype of plants is genetically determined and phylogenetic information has been used to identify crops with

higher Ca content to address Ca malnutrition in humans (White, 2005). It can also be useful to identify if the weed shows a high oxalate fraction and is able to survive excessive Ca in calcareous soils and under liming, or if it behaves as plants in the Poales with a high K/Ca due to lower requirements of Ca than dicots.

In the present study we sampled *P. caudatum* in a tropical secondary savanna community in Venezuela, in a site where tropical montane cloud forest has given way in some areas to secondary savannas due to recurrent human-related fires in the last two centuries. The site is characterized by acid soils (García-Miragaya and Herrera, 1971; Marulanda, 1998). The objectives of this study are: 1) To measure Ca concentrations in pinnae and rhizomes of *P. caudatum* three and nine months after an accidental fire, in May and November, and to contrast these data with control plants growing in a unburned parcel. We expect higher Ca in burned parcels due to the effect of ash fertilization. Similar values of Ca in plants from burned and control parcels will indicate leaching of nutrients during the experimental time. 2) To compare the shoot Ca concentration in the pteridophyte with monocots and dicots reported in the literature, considering that remarkable differences have been found probably as a consequence of their different CEC and pectin contents in the cell wall. 3) To define the physiotype by the soluble K/Ca ratio, related it to its acidiphily, and to evaluate the proportion of soluble, exchangeable pectate + phosphate and Ca-oxalate. 4) To evaluate the distribution of mineral elements in the plant organs.

## Materials and Methods

*Plant material and field sampling.—Pteridium caudatum* [= *Pteridium aquilinum* var. *caudatum* (L.) Domin], supported in the specific rank by Thomson and Alonso-Amelot (2002), was sampled within the grounds of the Instituto Venezolano de Investigaciones Científicas (IVIC), located in Altos de Pipe (10° 24′N, 66° 58′W, 1380 m above sea level). The predominant vegetation at IVIC is primary cloud forest, surrounded by secondary forests and grasslands. Occasionally fires take place during the dry season in the secondary vegetation units. Disturbed areas between the forest and grassland are dominated by ferns. The plants were sampled in a tropical secondary savanna community. The 10-yr average annual precipitation of 1100 mm is relatively well distributed throughout the year, with only two months (February and March) of dry season (Sanhueza *et al.*, 2000). The study was performed in the rainy season (May and November). Soil profiles from Altos de Pipe showed a water pH of 4.7 to 5.2 (García-Miragaya and Herrera, 1971).

Five relatively small plants (< 0.3 m tall) and five larger plants (> 1 m tall), denoted as *s* or *l*, respectively, were collected in a burned parcel (B) in May and November 94 and 270 days after an accidental fire that occurred on February 14, 2003. Sampling was also carried out in a control parcel (C) where fire did not take place. Plants are defined here as fronds and attached underground parts excavated from holes 50 × 50 × 20 cm. Small and large

plants were present in the second sampling in November, but small plants were not found in May in the control parcel. This indicated that vegetative sprouting after May in the control parcel can be attributed to the fact that there were no monitored disturbances of this region. This is different from the fire burned region in which, presumably, there had been activity of small wild herbivores or humans, such as walking or clearing. The area of each parcel was approximately 400 $m^2$ and they were separated from each other by 1 Km. The distance between the samplings was at least 5 m. Sporophytes were sterile at both harvest times and had fronds with three to nine pinnae. The excavated plants were transported to the laboratory and the underground organs were washed with tap water. Aerial and underground organs were dried to constant weight in a ventilated oven for approximately 78 h at 60° C and then ground with a Wiley mill (3383-L10, Thomas Scientific, U.S.A.). The aerial biomass, given by the dry mass of pinnae (green tissue) and rachis + petioles was measured. The total biomass was not calculated because we were not sure if we collected the entire rhizome length in the explored soil area (50 × 50 × 20 cm). Mineral analysis in aerial and underground organs was performed and expressed by dry mass.

*Chemical analyses.*—The Ca fractionation methodology of Kostytschew and Berg described in Kinzel (1989) was used: Hot water-soluble Ca, originally included in the vacuoles, was obtained by heating ground plant material in a proportion of 200 mg dry mass to 5 mL water. After centrifugation at 4.6 G for 10 min, the liquid was collected and the residue treated with the next extracting solution. Calcium adsorbed electrostatically on the cell walls or on polyanions in the vacuoles was extracted by means of hot 10% NaCl solution. The Ca-phosphate + pectate fraction was extracted by means of hot acetic acid (2 mM). In each case the extraction mixture was heated to the boiling point of the solution. Finally, Ca bound to oxalate was extracted overnight with HCl (2 mM) at ambient temperature. The extraction efficiency, calculated by the linear regression of the sum of fractions versus the total Ca, was 95%.

The soluble K in hot water extracts was measured and the K/Ca ratio in pinnae and rhizomes was calculated. The concentration of Ca and K in the water extract and Ca in other extracts were determined on an atomic absorption spectrometer (SpectrAA 55B, Varian Techtron, Victoria, Australia). Rhizomes were used instead of roots for the fractionation because the dry mass of roots per plant was too small for analysis, but concentrations of total Ca, K, Mg, Fe, Al, Mn, Ni, Zn and Cu, were determined from all the organs in nitric-perchloric acid digestions (Miller, 1998).

Total nitrogen concentration was determined by the Kjeldhal method (Tecator Kjeltec Systems from Foss Tecator, Höganäs, Sweden) after digestion of the ground dry plant material with sulfuric acid. Phosphorus was measured colorimetrically (Murphy and Riley, 1962) in the digested material by means of UV/visible spectrophotometer (Ultrospec 2000, Amersham Pharmacia, Cambridge, England). Ash content was determined by heating biomass samples to 510 °C for 8 hours in a muffle furnace (Thermolyne, Iowa, U.S.A.).

Molar ratios (mol N/mol P) for the mineral elements instead of mass ratios (g N/g P) are shown in the present work because they are more common in physiological research, as they reflect the actual stoichiometric relationships. They differ by a factor of 2.21. For comparative purposes mass ratios, common in the literature, were recalculated to molar ratios.

*Statistical analysis.*—Standard errors are used as indicators of sampling error rather than treatment effects. The values from the seven categories shown in Table 1 (May-B*s*, B*l*, C*l* and November-B*s*, B*l*, C*s*, C*l*) were pooled to get the average shown in Table 2. For aerial organs and rhizomes chemical analyses were duplicated (5 plants × 7 categories × 2 chemical analyses = 70), but for roots the dry mass was insufficient for replicate chemical analyses (n = 35).

## Results

*Total Ca concentration in P. caudatum of different size and fire history.*—A clear difference in total Ca concentrations between plants of different size from the burned and control parcels was not observed. Calcium concentration in pinnae was higher than that measured in rhizomes and the highest Ca concentration was found in plants from the control parcel sampled in May (Table 1).

*Ca fractionation and soluble K/Ca ratio.*—The exchangeable fraction represented up to 62% of the total Ca in rhizomes from small plants and up to 53% in the pectate + phosphate fraction in pinnae from large plants, both sampled in the burned parcel in November (Table 1). The exchangeable and phosphate + pectate fractions taken together contributed 60 to 85% of the total Ca in pinnae and rhizomes. The exchangeable fraction was higher than, or nearly equal to, the pectate + phosphate fraction, except in pinnae from the burned parcel in November. The same pattern of low oxalate-Ca was found in pinnae and rhizomes. Water-soluble Ca contributed to the total Ca in a lower proportion in rhizomes than in pinnae and the highest value of this fraction was 28% in pinnae from small plants from the burned plot in November. Pinnae had higher soluble K concentrations than rhizomes. The highest concentration of soluble K and K/Ca ratio were found in pinnae from the burned parcel.

*Biomass, N, P and ash content in P. caudatum of different size and fire history.*—Aerial biomass appears to be higher in non-burned plots samples (Table 2). The highest value of ash content in *P. caudatum* was found in rhizomes. Pinnae from burned and control parcels were similar in ash content, but rhizomes from the burned parcel showed a higher ash percent of dry mass. Contents of P were higher in burned plot samples of pinnae and rhizomes. Large plants sampled in May in the non-burned plot had the lowest contents of N and P found in this work for pinnae.

*Distribution of metals in the plant organs and ratio of total mineral elements in pinnae.*—Underground organs showed higher concentrations of Al, Fe, Zn, Cu and Ni than aerial ones, and roots showed higher concentrations of these metals than the rhizomes (Table 3). The concentration of Mn was higher in

TABLE 1. Total Ca concentration and fractions of Ca, expressed in per cent of total, extracted with hot water (water-soluble), hot NaCl solution (exchangeable), hot acetic acid (Ca-phosphate and Ca-pectate) and HCl (Ca-oxalate), water-soluble K and soluble K/Ca ratio in relatively small (*s*) and larger (*l*) *P. caudatum* from a burned (B) field, 94 and 270 days after fire, and from a control (C) plot. For each category values given are means ± SE (n = 5).

| | Calcium | | | | | | |
|---|---|---|---|---|---|---|---|
| | total | water-soluble | hot NaCl | hot acetic | HCl | Sol. K | Sol. K/Ca |
| | mmol kg$^{-1}$ | (% total Ca) | | | | mmol kg$^{-1}$ | mol mol$^{-1}$ |
| **PINNAE** | | | | | | | |
| **May, 94 days after fire** | | | | | | | |
| B*s* | 54 ± 12 | 16 | 42 | 34 | 8 | 431 ± 38 | 101 ± 22 |
| B*l* | 80 ± 6 | 18 | 36 | 38 | 8 | 419 ± 29 | 41 ± 6 |
| C*l* | 116 ± 9 | 18 | 48 | 29 | 5 | 309 ± 37 | 22 ± 2 |
| **November, 270 days after fire** | | | | | | | |
| B*s* | 101 ± 12 | 28 | 23 | 37 | 12 | 514 ± 92 | 37 ± 17 |
| B*l* | 59 ± 4 | 21 | 23 | 53 | 3 | 740 ± 41 | 83 ± 21 |
| C*s* | 110 + 12 | 22 | 50 | 21 | 6 | 269 ± 20 | 13 ± 2 |
| C*l* | 71 ± 10 | 13 | 52 | 27 | 9 | 260 + 20 | 44 ± 15 |
| Burned (n = 20) | 73 ± 6 | 21 ± 3 | 31 ± 5 | 40 ± 4 | 8 ± 2 | 532 ± 35 | 67 ± 10 |
| Control (n = 15) | 99 ± 7 | 18 ± 3 | 50 ± 1 | 26 ± 2 | 6 ± 1 | 279 ± 16 | 26 ± 5 |
| **RHIZOMES** | | | | | | | |
| **May** | | | | | | | |
| B*s* | 49 ± 6 | 18 | 46 | 27 | 9 | 273 ± 20 | 29 ± 5 |
| B*l* | 35 ± 3 | 17 | 44 | 29 | 10 | 217 ± 16 | 31 ± 3 |
| C*l* | 30 ± 4 | 12 | 51 | 24 | 14 | 202 ± 9 | 42 ± 3 |
| **November** | | | | | | | |
| B*s* | 40 ± 3 | 6 | 62 | 24 | 9 | 75 ± 10 | 33 ± 4 |
| B*l* | 26 + 3 | 7 | 53 | 30 | 10 | 111 ± 8 | 63 ± 5 |
| C*s* | 32 ± 2 | 10 | 38 | 43 | 9 | 163 ± 16 | 40 ± 4 |
| C*l* | 25 ± 2 | 13 | 46 | 32 | 9 | 144 ± 10 | 43 ± 6 |
| Burned (n = 20) | 37 ± 3 | 12 ± 3 | 51 ± 4 | 28 ± 1 | 9 ± 0 | 167 ± 15 | 39 ± 3 |
| Control (n = 15) | 29 ± 2 | 12 ± 1 | 45 ± 4 | 33 ± 6 | 11 ± 1 | 170 ± 8 | 42 ± 3 |

rachis + petioles and rhizomes than in pinnae and roots. The quotients calculated with the average of the element concentrations in shoots are given in Table 3 for comparison with the literature.

## DISCUSSION

*Total Ca concentration in P. caudatum of different size and fire history.*— The highest Ca concentration was found in large plants from the control parcel (116 ± 9 mmol kg$^{-1}$; Table 1) which is low compared with the range (25 to 1249 mmol kg$^{-1}$) found in higher plants (Marschner, 1995). Thompson *et al.* (1997) found a Ca concentration of 142 mmol kg$^{-1}$ in pinnae of *P. aquilinum* in England (Table 3). In adult dry leaves of tropical plants, values of up to 955 mmol kg$^{-1}$ Ca have been reported (Olivares and Aguiar, 1999). Zohlen and

TABLE 2. Aerial (pinnae + rachis) and rhizomes biomass. The pinnae contribution in the aerial biomass is indicated. Total N and P concentration and ash content in the same plants of Table 1.

| | Biomass (g dry mass plant $^{1}$) | Pinnae % | N (mmol kg $^{-1}$) | P (mmol kg$^{-1}$) | Ash (% dry mass) |
|---|---|---|---|---|---|
| **PINNAE** | | | | | |
| **May** | | | | | |
| B*s* | 8 ± 2 | 58 | 1646 ± 149 | 53 ± 8 | 6.9 ± 0.2 |
| B*l* | 59 ± 6 | 77 | 1260 ± 51 | 24 ± 3 | 5.9 ± 0.4 |
| C*l* | 52 ± 7 | 82 | 899 ± 27 | 4 ± 1 | 5.5 ± 0.8 |
| **November** | | | | | |
| B*s* | 32 ± 4 | 70 | 1395 ± 118 | 49 ± 11 | 8.7 ± 0.9 |
| B*l* | 62 ± 9 | 68 | 1369 ± 19 | 61 ± 4 | 6.7 ± 0.2 |
| C*s* | 36 ± 7 | 88 | 1590 ± 57 | 33 ± 3 | 8.0 ± 0.9 |
| C*l* | 91 ± 12 | 84 | 1439 ± 76 | 35 ± 6 | 5.7 ± 0.2 |
| Burned (n = 20) | 40 ± 13 | 68 ± 4 | 1425 ± 77 | 47 ± 8 | 7.1 ± 0.4 |
| Control (n = 15) | 60 ± 16 | 85 ± 2 | 1309 ± 210 | 24 ± 10 | 6.4 ± 0.5 |
| **RHIZOMES** | | | | | |
| **May** | | | | | |
| B*s* | 13 ± 1 | | 274 ± 28 | 12 ± 2 | 15.2 ± 3.0 |
| B*l* | 9 ± 2 | | 316 ± 16 | 10 ± 1 | 10.9 ± 0.9 |
| C*l* | 11 ± 2 | | 296 ± 20 | 9 ± 1 | 7.5 ± 0.5 |
| **November** | | | | | |
| B*s* | 9 ± 2 | | 431 ± 27 | 16 ± 4 | 10.3 ± 1.9 |
| B*l* | 7 ± 3 | | 363 ± 33 | 13 ± 2 | 5.5 ± 0.6 |
| C*s* | 5 ± 1 | | 603 ± 21 | 10 ± 2 | 9.2 ± 0.7 |
| C*l* | 8 ± 1 | | 432 ± 30 | 5 ± 1 | 6.8 ± 1.7 |
| Burned (n = 20) | 10 ± 1 | | 346 ± 34 | 13 ± 1 | 10.5 ± 1.2 |
| Control (n = 15) | 8 ± 2 | | 444 ± 89 | 8 ± 2 | 7.8 ± 0.6 |

Tyler (2004) reported 179 to 299 mmol kg$^{-1}$ Ca in calcifuge herbs from Sweden growing on acid soils, and 74 to 142 mmol kg$^{-1}$ Ca in calcifuge grasses. Monocots of the Poaceae take up less Ca than dicots for adequate growth and bracken also shows low Ca.

Ecologists have classified plant species into calcifuges, which occur on acid soils with low Ca, and calcicoles, which grow on calcareous soil (White and Broadley, 2003). The terminology was introduced by Chodat in 1913 and Salisbury in 1920 respectively (Kinzel, 1983). The response of calcifuges to acidic and calcareous soils is important in understanding their ability to compete and survive (Zohlen and Tyler, 2004) because calcifuge plants have a low capacity to solubilize soil P and this is related to low root exudation rates of di- and tricarboxylic organic acids, in particular oxalic acid. Phosphate in plant tissues may also be immobilized when calcifuge plants are forced to grow on a calcareous soil. Under such conditions an excessive uptake of Ca may take place in calcifuges, causing precipitation of Ca-phosphate in their tissues. Zohlen and Tyler (2004) reported calcifuge herbs not only have lower concentrations of soluble inorganic and total P in their leaves compared with calcicoles, but also a lower relative proportion of soluble P when grown on

TABLE 3. Mineral element concentrations in organs of *P. caudatum* (mean ± SE): pinnae, rachis + petioles, rhizomes and roots (n = 35), compared with pinnae of *P. aquilinum* (n = 30) from Thompson *et al.* (1997).

| | ***P. caudatum*** | | | | ***P. aquilinum***[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Pinnae | Rachis+petioles | Rhizomes | Roots | Pinnae | ***P. caudatum P. aquilinum***[a] | | |
| **Element (mmol kg$^{-1}$)** | | | | | | **Quotients in pinnae (mol mol$^{-1}$)** | | |
| **N** | 1374.49 (± 41.64) | 458.11 (± 16.75) | 389.97 (± 15.98) | 527.92 (± 24.45) | 2084.67 (± 16.94) | **N/P** | 37 | 24 |
| **P** | 37.41 (± 2.84) | 11.45 (± 1.05) | 10.71 (± 0.68) | 7.47 (± 0.89) | 87.17 (± 1.18) | **Ca/P** | 2 | 2 |
| **K** | 384.90 (± 14.94) | 244.22 (± 17.03) | 155.44 (± 6.40) | 65.00 (± 9.83) | 555.02 (± 4.67) | **Ca/Mg** | 1 | 1 |
| **Ca** | 84.53 (± 4.58) | 24.27 (± 1.45) | 33.71 (± 1.70) | 57.94 (± 5.02) | 142.22 (± 1.82) | **K/Ca** | 5 | 4 |
| **Mg** | 73.39 (± 2.31) | 21.09 (± 1.61) | 33.73 (± 2.04) | 24.62 (± 2.78) | 98.75 (± 0.75) | **Ca/Al** | 15 | 1440 |
| **Fe** | 2.17 (± 0.25) | 0.95 (± 0.16) | 15.41 (± 1.26) | 49.54 (± 7.02) | 3.58 (± 0.03) | **Al/Fe** | 3 | 0.03 |
| **Al** | 5.62 (± 0.49) | 1.59 (± 0.17) | 65.94 (± 5.51) | 248.30 (± 28.95) | 0.10 (± 0.00) | **Fe/Mn** | 3 | 1 |
| **Mn** | 0.85 (± 0.08) | 3.08 (± 0.32) | 1.18 (± 0.08) | 1.48 (± 0.29) | 4.00 (± 0.13) | **Mn/Zn** | 7 | 28 |
| **Ni** | 0.06 (± 0.01) | 0.03 (± 0.00) | 0.09 (± 0.01) | 0.11 (± 0.02) | nd | **Mn/Cu** | 12 | 7 |
| **Zn** | 0.13 (± 0.01) | 0.04 (± 0.01) | 0.07 (± 0.01) | 0.96 (± 0.11) | 0.14 (± 0.00) | **Mn/Ni** | 14 | nd |
| **Cu** | 0.07 (± 0.01) | 0.04 (± 0.00) | 0.05 (± 0.01) | 0.14 (± 0.04) | 0.56 (± 0.00) | | | |

[a]Mineral element concentrations in *P. aquilinum* from Thompson et al. (1997), in http://www.shef.ac.uk/uni/academic/N-Q/nuocpe/ucpe/nutrient.txt, were recalculated from mass to molar units.

a calcareous soil. This is of importance to their photosynthesis, growth, competition and final survival. Koedam *et al.* (1992) found that the soil preference with respect to soil acidity in *P. aquilinum* sampled in forests from Belgium, France, Luxemburg and Germany was in the acidic range (median pH 4.00) and they did not detect calcium carbonate in the substrate, therefore they considered bracken not to be a calcicole and it was labeled as acidiphilous.

*Ca fractionation and soluble K/Ca ratio.*—In *P. caudatum* the exchangeable fraction is the largest of the Ca fractions, and the pectate + phosphate fraction is also high (Table 1). This is similar to plants in the Boraginaceae in which NaCl and acetic acid extracts represented 56 to 68% of the total Ca (Kinzel, 1989). These fractions are in contrast to those of the calciotrophes in Brassicaceae with 49 to 66% of the total Ca in the water-soluble fraction, or with the oxalate plant, *Ballota nigra* (Lamiaceae), with 91% in the oxalate fraction (Kinzel, 1989). The physiotype of *P. caudatum* does not correspond to a calciotrophe according to the soluble K/Ca ratio (Table 1) nor to oxalate plants as they showed a low acid soluble fraction (Table 1). Therefore, the species behaves as a potassium plant, but with K soluble concentrations <740 $\pm$ 41 mmol $kg^{-1}$, which are lower values than those found in typical potassium plants such as *Carex pendula* (Poales) with soluble K concentrations <2000 mmol $kg^{-1}$ (White, 2005).

*Biomass, N, P and ash content in P. caudatum of different size and fire history.*—When biomass data from Table 2 are recalculated by area units, the aerial biomass from plants in the burned parcel is 161 $\pm$ 51 g $m^{-2}$ contrasting with 239 $\pm$ 65 g $m^{-2}$ in control parcels, however rhizomes have similar biomass in both parcels, 36 $\pm$ 3 and 32 $\pm$ 7 g $m^{-2}$, respectively. We only sampled to a depth of 20 cm and therefore failed to collect the complete rhizome. Alonso-Amelot and Rodulfo-Baechler (1996) reported 53 g $m^{-2}$ and 143 g $m^{-2}$ in fronds and rhizomes of *P. caudatum* in Venezuela.

Ash content in rhizomes of *P. caudatum* was higher than in pinnae, and was similar to that found by Skre *et al.* (1998), who found a maximum ash percentage of 7.2 $\pm$ 1.1 in non-green plant tissue of *P. aquilinum* plants from a burned parcel, contrasting with a lower value of 3.7 $\pm$ 0.5 in plants from an unburned site. However, they reported that the ash percentage of green leaves from a burned parcel and a control site were similar.

The concentration of P in shoots of *P. caudatum* was 4 to 61 mmol $kg^{-1}$, which is low compared with the range (3 to 194 mmol $kg^{-1}$) found in higher plants (Wright *et al.*, 2004). The concentration of N in shoots was 899 to 1646 mmol $kg^{-1}$, and the range found in higher plants is 143 to 4569 mmol $kg^{-1}$ (Wright *et al.*, 2004). Thompson *et al.* (1997) found a P and N concentration of 87 mmol $kg^{-1}$ and 2085 mmol $kg^{-1}$ respectively in pinnae of *P. aquilinum* in England. Differences in N and P in burned and non-burned samples are not clear. Skre *et al.* (1998) reported that the P contents of green parts of *P. aquilinum* from a burned parcel and a control site in Norway did not show significant differences, 284 $\pm$ 58 mmol $kg^{-1}$ and 258 $\pm$ 45 mmol $kg^{-1}$,

respectively. The same was observed for N, 1785 ± 428 mmol $kg^{-1}$ and 1499 ± 214 mmol $kg^{-1}$.

*Distribution of metals in the plant organs and ratio of total mineral elements in pinnae.*—Very high concentrations of Al and Fe were found in underground organs compared to aerial organs (Table 3). Johnson-Maynard *et al.* (1997) reported a lower pH and higher exchangeable Al in a site invaded for 30 years by bracken fern than in an undisturbed forest and suggested that the establishment of bracken fern is responsible for the subsequent alteration of soil properties, which can have significant implications for the growth of other species. Higher organic C and a repartitioning of secondary Al and Fe from inorganic compounds into organic complexes characterize soils supporting bracken fern.

The high N/P ratios in *P. caudatum* (Table 3) may suggest a P-limited biomass production; however, fertilization experiments are necessary to confirm this hypothesis. Güsewell (2004) studied the variation and functional significance of N/P ratios in terrestrial plants and reported that often, but not always, N/P ratios <22 and >44 mol $mol^{-1}$ correspond to N- and P- limited biomass production. Intraspecific variation was reported to be more important than interspecific variation, with N/P ratio variation of individual species of fifty-fold in response to natural or experimental variations in N and P supply. Han *et al.* (2005) found that higher N and P concentrations occurred in seed plants than in ferns, suggesting that these phylogenic groups differed in leaf chemistry. Thompson *et al.* (1997) reported higher P and N concentrations and lower N/P ratio in *P. aquilinum* (Table 3) than found here for *P. caudatum* growing in acid soil. Han *et al.* (2005) studied *P. aquilinum* in China and reported 914 mmol $kg^{-1}$ N, 27 mmol $kg^{-1}$ P and an N/P ratio of 34 mol $mol^{-1}$.

The Ca/P quotient found in shoots of *P. caudatum* was 2 mol $mol^{-1}$ (Table 3). Zohlen and Tyler (2004) reported a Ca/P ratio of 2 - 8 mol $mol^{-1}$ in grasses, which is lower than that found in herbs (4–51 mol $mol^{-1}$). They found the highest value of Ca/P in calcifuge herbs in calcareous soils. Calcifuge herbs with low oxalate exudation by roots and low oxalate precipitation in their tissues may not be able to survive calcareous conditions because of the precipitation of phosphate-Ca in soils and tissues resulting in low inorganic P that is necessary for its metabolism, but calcifuge and calcicole grasses did not differ much in the ratio of Ca to total P (Zohlen and Tyler, 2004).

The Ca/Mg ratio was one in *P. caudatum* and in *P. aquilinum* (Table 3). Broadley *et al.* (2004) reported a higher Ca/Mg ratio than that found in bracken. They studied 117 species from 24 orders and one unassigned family of angiosperms grown hydroponically and 81 species from 20 orders of angiosperms reported in an ecological survey by Thompson *et al.* (1997). They found that if they excluded seven species from the order Caryophyllales, which have relatively higher Mg contents in their shoots than the other species, Ca and Mg concentration regressed significantly and Ca/Mg ratio was 7.7 g $g^{-1}$ ( 4.6 mol $mol^{-1}$).

The differences in Ca/Al and Al/Fe ratios between *P. caudatum* and *P. aquilinum* are remarkable (Table 3) because Al concentration in *P. aquilinum*

in England was low compared to that found in *P. caudatum* in acidic soils, but the concentration of N, P, K, Ca, Mg, Fe, Mn and Cu was higher in *P. aquilinum* shoots. According to Jansen *et al.* (2004) Al accumulators should be defined as plants in which an Al concentration of at least 1000 mg $kg^{-1}$ has been recorded in the dry matter of leaves in at least one specimen growing in its natural habitat. Pinnae of *P. caudatum* only had 152 mg Al $kg^{-1}$ (5.62 mmol $kg^{-1}$) but rhizomes had 1779 mg $kg^{-1}$ (65.94 mmol $kg^{-1}$) and roots had 6700 mg $kg^{-1}$ (248.3 mmol $kg^{-1}$). *Pteridium* was found not to show Al accumulation in above-ground tissues according to Webb (1954). Based on previous observations by Chenery (1949), Al accumulation is mainly characteristic of some groups belonging to the basal leptosporangiate ferns, including some tree ferns, but largely absent in the more derived ferns (the polypodiaceous ferns) and the heterosporous ferns.

Iron is an essential mineral in plants, but Al is only considered beneficial in some cases (Marschner, 1995). However, several tropical plants, such as *Faramea marginata* (Rubiaceae) are Al accumulators (Britez *et al.*, 2002), with Ca/Al<1 and Si/Ca>1. It has been suggested that the formation of an Al-Si complex contributes to the internal detoxification of Al in these plants. Silicon has been reported in bracken (Parry *et al.*, 1985). Hodson *et al.* (2005) reported variation in shoot Si concentration when they compared 735 species, including 59 species of ferns. The relative shoot Si concentration ranged from −2.139 in a Polypodiaceae species to 8.769 in a Poacaeae species. They presented relative units originally in percent dry weight. Data were from 125 studies contained in 54 papers and negative relative shoot Si concentration values arise as a consequence of adjusting for between-studies variation during residual maximum likelihood fitting procedures. In ferns Si content was up to 1.352 in a Woodsiaceae species and *P. aquilinum* was 594 in the total of 735 species ranking, with a mean relative shoot Si concentration of 1.299. In pinnae of *Pteridium caudatum* we found Ca/Al =5 mol $mol^{-1}$ and the Al concentration was low in comparison with Al accumulators, however Ca/Al is 0.51 mol $mol^{-1}$ in rhizomes and 0.23 mol $mol^{-1}$ mol $mol^{-1}$ in roots. The Si content was not measured in *P. caudatum*. A very rough estimate of Si was done by subtracting all the evaluated minerals from the ash mass and Si content was 1.4 times higher in rhizomes than in pinnae.

In *P. caudatum* not only did the roots show high Al concentrations, but also the rhizomes, which are part of the stem, but not of the root system. Liao *et al.* (2004) reported the rhizome of Chinese brake (*Pteris vittata* L.) can be important in the storage of P, Fe and As. They found that in soils with high available As concentrations, such as 1053 μg $g^{-1}$, the pinnae and rhizomes had similar concentrations of As, 1386 ± 161 μ $g^{-1}$ and 1217 ± 96 μg $g^{-1}$ respectively, but the As accumulation (concentration × biomass) was higher in rhizomes than in pinnae because of their higher biomass. However in soils with low available As, < 1.2 μg $g^{-1}$, As concentrations were higher in pinnae than rhizomes.

*Pteridium* is one of the 85 genera of pteridophytes identified by Page (2004) as having species tolerant of growth on low-nutrient substrates, and he defined

the genus as an ancient living vascular plant (ALVP). According to his study edaphic adaptations enable many ALVPs to continue to exist and to occupy diverse edaphically marginal habitats, in which today, plant competition is necessarily low. The present work suggests that in the acid soils where *P. caudatum* was sampled it concentrates more soluble K than Ca in shoots and rhizomes. This fact probably explains why bracken is used in agriculture as a source of potash (K salts). Both organs have low total Ca and P contents, low water-soluble Ca and Ca bound to oxalate, but a higher fraction of exchangeable Ca and pectate and phosphate bound Ca, which are characteristics of calcifuge grasses that do not change their Ca/P ratio much on calcareous soil or under liming, but reduce their growth on those conditions. Aboveground organs did not show Al accumulation as is common in basal ferns (Chenery, 1949) but a much higher Ca/Al ratio in shoots of *P. caudatum* was observed compared with bracken sampled in temperate regions. Accumulation of Al was found in underground organs, which results in *P. caudatum* contributing this metal to the soil when rhizomes and roots decompose. Plugging this weed is necessary before sowing crops that are not tolerant to Al, not only to prevent the weed from reproducing asexually but also to eliminate the storages of Al in the soil.

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# The Complete Plastid Genome Sequence of *Angiopteris evecta* (G. Forst.) Hoffm. (Marattiaceae)

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Abstract.—We have sequenced the complete plastid genome of the fern *Angiopteris evecta*. This taxon belongs to a major lineage (marattioid ferns) that, in most recent phylogenetic analyses, emerges near the base of the monilophytes. We used fluorescence activated cell sorting (FACS) to isolate organelles, rolling circle amplification (RCA) to amplify the plastid genome, followed by shotgun sequencing to 8X depth coverage, and then we assembled these reads to obtain the plastid genome sequence. The circular genome map has 153,901 bp, containing inverted repeats of 21,053 bp each, a large single-copy region of 89,709 bp, and a small single-copy region of 22,086 bp. Gene order is similar to that of *Psilotum*. Several unique characters are observed in the *Angiopteris* plastid genome, such as repeat structure in a pseudogene. We make structural comparisons to *Psilotum* and *Adiantum* plastid genomes. However, the overall structural similarity to *Psilotum* indicates either wholesale conservation of genome organization, or (less likely) repeated convergence to a stable structure. The results are discussed in relation to a growing comparative database of genomic and morphological characters across the green plants.

Vascular plants first appear in the fossil record during the Silurian (Kenrick and Crane, 1997; Pryer *et al.*, 2004a; Stewart and Rothwell, 1993). Although many major lineages are extinct, recent phylogenetic studies (Pryer *et al.*, 2001) indicate that an early split resulted in two extant lineages: seed plants and monilophytes. The latter includes the leptosporangiate ferns, marattioid ferns, horsetails, and a clade that includes eusporangiate ferns and whisk ferns. How these four lineages are related to each other is still poorly understood (Pryer *et al.*, 2001; Pryer *et al.*, 2004b; Wilkström and Pryer, 2005). Resolving these phylogenetic nodes is important for understanding the evolution of morphological, genetic, and developmental systems in monilophytes. As part of an

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effort to provide data for addressing this issue, we sequenced the complete plastid genome of *Angiopteris evecta* (Marattiaceae). Currently, complete plastid genome sequences are available from only one leptosporangiate fern, *Adiantum capillus-veneris* L. (Wolf *et al.*, 2003), and from only one other monilophyte, *Psilotum nudum* (L.) P. Beauv., whereas about 50 seed plant plastid genomes are currently in GenBank. Complete genome sequences can provide information on many levels, including genome structure, gene content, intron content, and nucleotide sequences of targeted regions. We chose *Angiopteris evecta* for our study because it is an easily available representative of an ancient lineage for which no plastid genome has been sequenced. Extant marattioid ferns include about 240 species (see Pryer *et al.*, 2004b) typically treated in four genera and one family (Smith *et al.*, 2006). Marattioid ferns first appeared in the middle carboniferous, and fossils assignable to the extant genus *Marattia* date to the late Triassic (Hill and Camus, 1986). Thus marattioid ferns represent a clade as significant as seed plants or leptosporangiate ferns in terms of age, though not in terms of extant diversity.

Although the plastid genome is generally conserved in overall structure among land plants (Palmer, 1985), there is often sufficient variation for comparative analysis both at the structural and sequence levels. Large rearrangements, spanning several genes, are likely to be rare events that can be used a phylogenetic markers (Raubeson and Jansen, 1992). Early studies of fern chloroplast genomes uncovered a wealth of phylogenetic data and insights into the evolution of the genome (Hasebe and Iwatsuki, 1992; Raubeson and Stein, 1995; Stein *et al.*, 1992; Stein *et al.*, 1989). One significant finding from these studies was that a large portion of the plastid genome has been rearranged in ferns, but the exact series of events has not yet been fully characterized. Subsequently, there was a shift to more focused studies on DNA sequences of a few genes from large numbers of taxa (Hasebe *et al.*, 1994; Hasebe *et al.*, 1995; Pryer *et al.*, 2004b). Thus, our understanding of structural evolution of fern plastid genomes remains limited. This study represents part of a broader investigation into plastid genome evolution by sequencing complete genomes or large portions thereof. Because *Angiopteris* represents a major lineage, details of its plastid genome can provide baseline data for this and other studies. Our objective here is to present the plastid genome sequence of *Angiopteris evecta* and compare it structurally to other monilophytes.

## Materials and Methods

*Preparation and DNA sequencing.*—Pinnules from an immature crozier of *A. evecta* were collected from a plant growing at the University of Washington, Seattle, WA, USA (original source unknown). Voucher specimens (UC 1794629, 1794630, and 1794631) are deposited at the University of California Herbarium at Berkeley (UC). We collected purified fractions of intact chloroplasts from *A. evecta* by fluorescent activated cell sorting (FACS). One hundred milligrams of fresh frond tissue was sliced into 0.25–1 mm segments

in a sterile plastic Petri dish (on ice) in 1.0 mL of an organelle isolation solution containing 0.33 M sorbitol, 50 mM HEPES at pH 7.6, 2 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA, 1% PVP-40, 1.5 M NaCl and 5 mM β-Mercaptoethanol, adjusted to pH 7.6 with KOH. Suspended organelles (chloroplasts, mitochondria, and nuclei) were withdrawn using a wide-bore pipette then filtered through 30 μm nylon mesh. Organelles were then stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and Mitotracker Green (Molecular Probes Inc., Eugene, OR, USA) at final concentrations of 2 μg/mL and 100 nM, respectively. The organelle suspension was incubated on ice for 15 min then analyzed on a FACS DiVa using sterile phosphate buffered solution (Invitrogen Inc., Carlsbad, CA, USA) as sheath fluid. We used a Coherent INNOVA Enterprise Ion laser (Coherent, Inc., Santa Paula, CA, USA) emitting a 488 nm beam at 275 mW to excite chlorophyll and Mitotracker Green, and a UV beam at 30 mW to excite DAPI. Red fluorescence from chlorophyll was passed through a 675±20 nm filter, held within the FL3 photomultiplier tube (PMT). Green fluorescence from Mitotracker Green was passed through a 530±30 nm filter held within the FL1 PMT. DAPI fluorescence from DNA was passed through a 424±44 nm filter held within the FL4 PMT. Organelles were collected into separate sterile 15 ml centrifuge tubes by flow cytometric sorting based on the respective sorting gates. Sorted organelles were pelleted at 3000 g for 15 min, flash frozen in liquid nitrogen, and shipped frozen for DNA isolation and amplification.

The DNA preparation was processed for sequencing by the Production Genomics Facility of the DOE Joint Genome Institute (JGI). Template was first amplified via rolling circle amplification (RCA) with random hexamers (Dean *et al.*, 2001). The RCA product was mechanically sheared into random fragments of about 3 kb by repeated passage through a Hydroshear device (Genemachines, San Carlos, CA, USA). These fragments were then enzymatically repaired to ensure blunt ends, then purified by gel electrophoresis to select for a narrow distribution of fragment sizes. Fragments were ligated into dephosphorylated pUC18 vector and transformed into *E. coli* to create plasmid libraries, using standard techniques (Sambrook *et al.*, 1989). Automated colony pickers were used to select colonies into 384-well plates containing LB medium. After overnight incubation, a small aliquot was processed robotically by RCA of plasmids (Dean *et al.*, 2001), then used as a template for DNA sequencing using Big-Dye chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were cleaned using SPRI (Elkin *et al.*, 2001) and separated electrophoretically on ABI 3730XL or Megabace 4000 automated DNA sequencing machines to produce a sequencing read from each end of each plasmid.

*Assembly and annotation.*—Sequences were processed using Phred (Ewing and Green, 1998; Ewing *et al.*, 1998), trimmed for quality, screened for vector sequences, and assembled using Phrap. Quality scores were assigned automatically, and the electropherograms and assembly were viewed and verified for accuracy using Consed 12 (Gordon *et al.*, 1998). As is typical, manual input was required to reconstruct part of one of the inverted repeat

regions, since automated assembly methods cannot recognize these as different. Regions of low quality or coverage and several gaps were reamplified by PCR and then sequenced. We designed primers from the ends of the longest contigs and used Proofstart long-PCR (QIAGEN, Valencia, CA, USA) to amplify the missing regions. Reagent concentrations and amplification conditions followed the manufacturers instructions and we used PCR extension times of 1 min./kb of estimated PCR product. PCR products were digested with *Tsp*409I (compatible overhang with *Eco*RI) and *Sau*3aI (compatible overhang with *Bam*HI). The fragments were separated in agarose, visualized, and cut from the gels. These fragments were then cloned into puC19, end-sequenced, and added to the previous assembly. If assembly of a gap was incomplete at this stage, then primers were designed from the subclone fragment sequences above and used to sequence the appropriate region using the earlier long-PCR product as a template. In this way we closed all 12 gaps. The final assembly has an average depth of coverage of 8X. We assembled the sequence as a circular genome with two copies of the inverted repeat. We annotated the genome using DOGMA (Dual Organellar GenoMe Annotator) (Wyman *et al.*, 2004). Genes were located by using a database of previously published chloroplast genomes, from which Blast searches (Altschul *et al.*, 1997) are used to find approximate gene positions. From this initial annotation, we located hypothetical starts, stops, and intron positions based on comparisons to homologous genes in other chloroplast genomes. We also took into account the possibility of RNA editing, which can modify the start and stop positions (Kugita *et al.*, 2003). We examined the plastid genome sequence for repeat structure using the program REPuter (Kurtz *et al.*, 2001). We set the minimum repeat size to 20 and analyzed the sequence with only one copy of the inverted repeat.

## Results and Discussion

The plastid genome of *Angiopteris evecta* has 153,901 bp, with inverted repeats ($IR_A$ and $IR_B$) of 21,053 bp each, a large single-copy (LSC) region of 89,709 bp, and a small single-copy (SSC) region of 22,086 bp (Fig. 1). The sequence and annotation is deposited in GenBank as accession number DQ821119. During annotation of the genome, we located the repertoire of genes that is typical of land plant plastid genomes. The overall organization of the *Angiopteris* plastid genome is typical of other vascular plants and most similar to that of *Psilotum nudum* among plastid genomes sequenced to date. Some of the differences between *Angiopteris* and *Psilotum* are possibly a function of autapomorphies in either lineage, but this cannot be determined until more plastid genomes are examined. For example, *Psilotum* lacks three genes (*chlL*, *chlN*, and *chlB*), for subunits of protochlorophyllide, an enzyme involved in the light-independent formation of chlorophyll. These three genes are found in most other plastid genomes, including *Angiopteris*. The ends of the IR also vary considerably among vascular plants. *Psilotum* differs from *Angiopteris* in that the SSC-IR boundary in the former is near *trnL-UAG* and

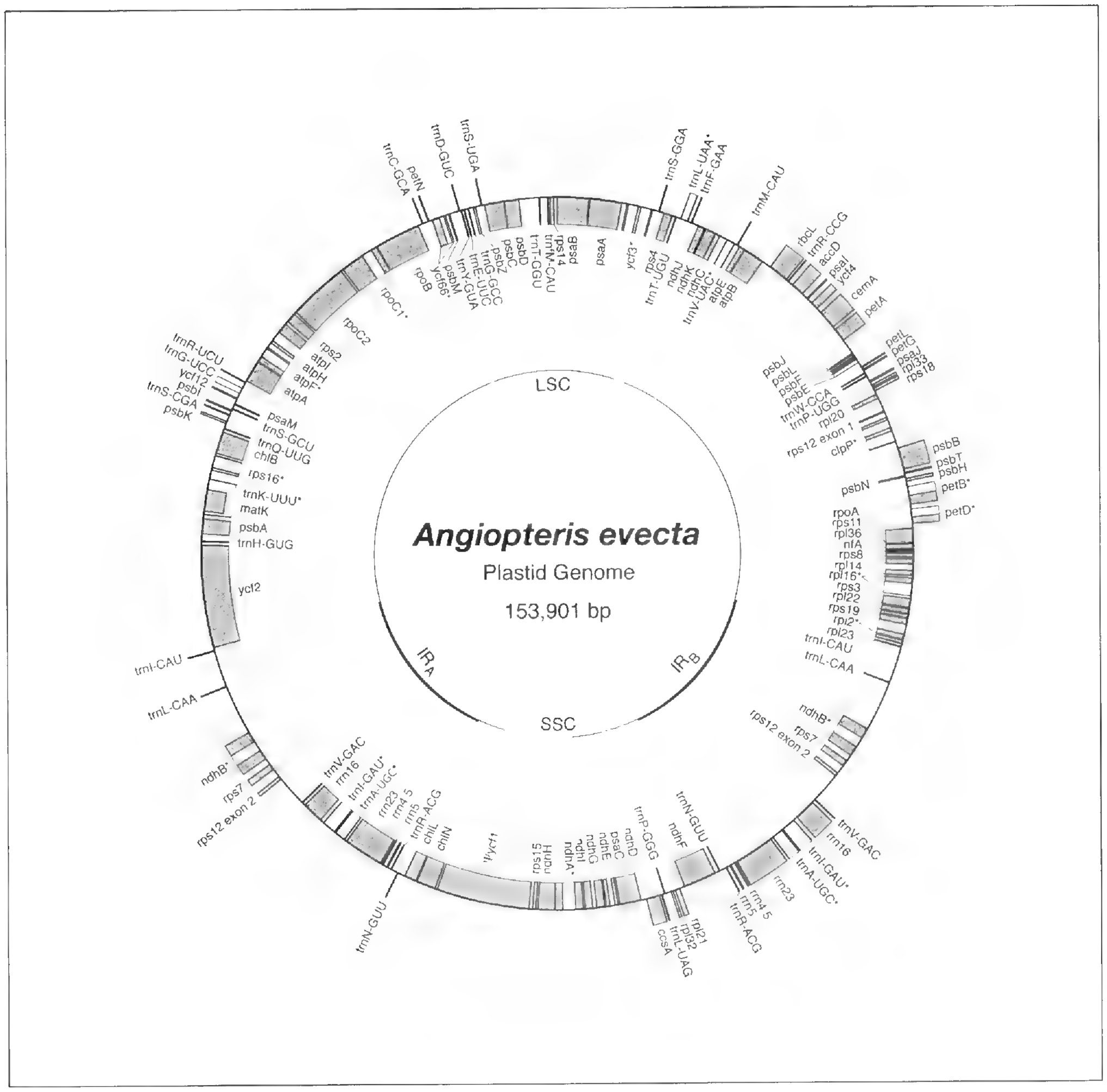


FIG. 1. Circular genome map of the plastid genome of *Angiopteris evecta*. Genes appearing on the outside of the circle are transcribed clockwise; genes on the inside are transcribed counterclockwise. Ψ denotes putative pseudogene. A 10 kb region is marked on the right to show the scale.

the SSC extends from *ccsA* to *ycf1*, whereas in *Angiopteris* the SSC is longer and extends from *ndhF* to *chlL* (Fig. 2). Gene order at the LSC-IR boundary of *Angiopteris* is very similar to that of *Psilotum*, differing only in the sizes of intergenic regions rather than gene positions (Fig. 3). The overall gene order within the IR is similar to that of seed plants and *Psilotum*, consistent with the hypothesis that this region sustained several rearrangements at some time during the diversification of leptosporangiate ferns (Hasebe and Iwatsuki, 1992; Stein *et al.*, 1992). An inversion of about 3Kb, involving *psbD*, *psbC*, and *psbZ*, was previously detected in *Psilotum* and *Adiantum* relative to other land plants, and more recently documented in the plastid genome of *Equisetum* (K. Karol, personal communication). This inversion is also seen in *Angiopteris*, thus providing a potential phylogenetic marker for the monilophyte clade.

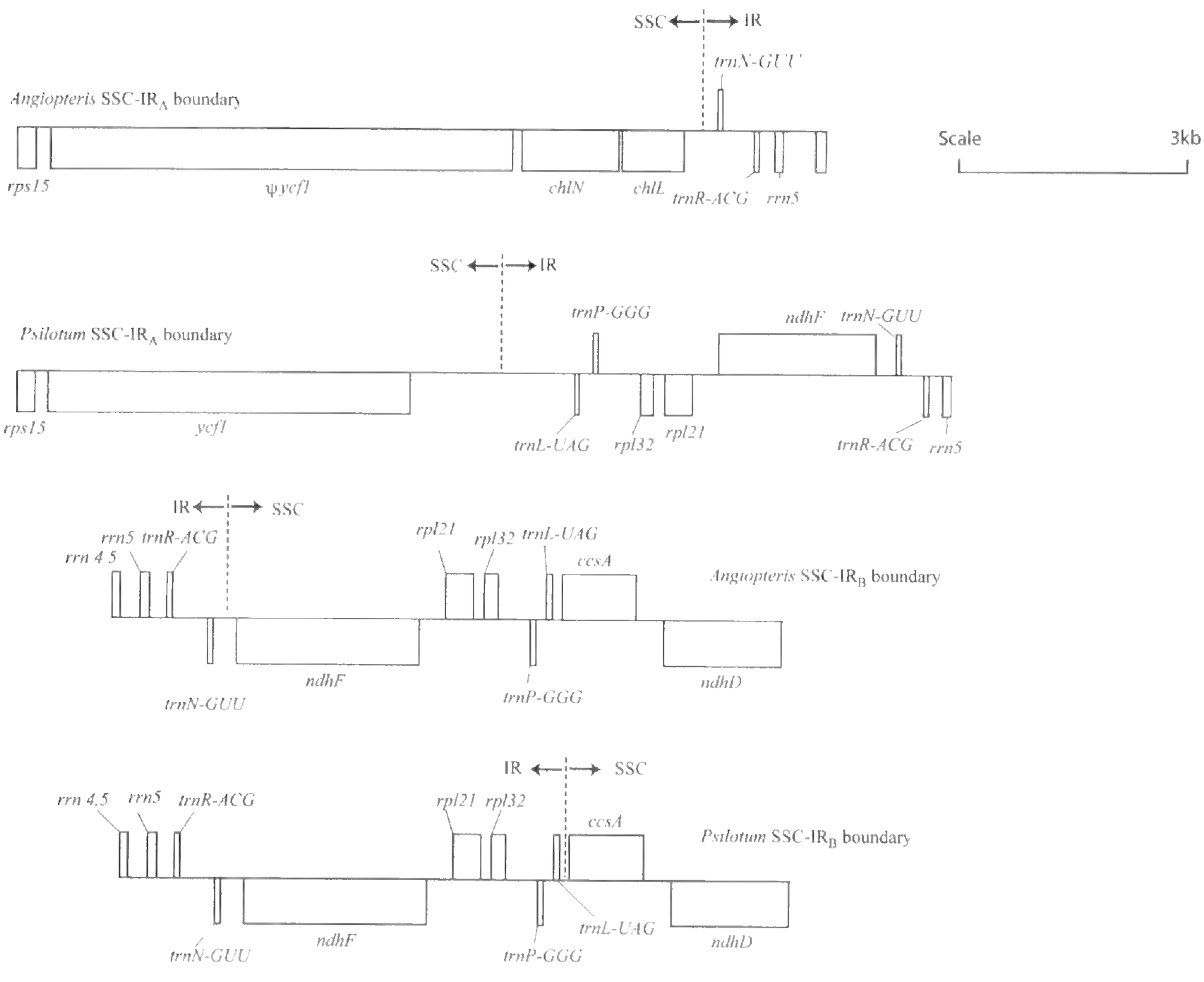


FIG. 2. Diagram of the SSC-IR boundaries, comparing the gene order of *Psilotum nudum* (GenBank accession AP004638) with *Angiopteris evecta* (GenBank accession DQ821119).

Another region of interest is in the LSC between *rpoB* and *psbZ*. This region has the same gene order in *Psilotum* and *Angiopteris* so it is likely to be an ancestral monilophyte organization. The *Adiantum* gene order differs from that of *Angipoteris* and *Psilotum* in this region. However the gene order difference cannot be explained by a single inversion. Instead, at least two overlapping inversions are required to explain the variation. Fig. 4 presents two alternative most-parsimonious pathways from a putative ancestral monilophyte gene order to that of *Adiantum*. Analysis of this region from several clades of leptosporangiate clades may help determine which sequence of events occurred.

One gene that we have not annotated is that for the hypothetical protein ycf68. Although found in several land plant plastid genomes, this gene is usually not annotated, perhaps because it is a relatively short reading frame (approximately 600 bp) and its function is unknown. In *Angiopteris* it is located in the IR at positions 104265–104639 and 139346–138972. However, there are at least three frameshifts, suggesting that ycf68 is a pseudogene.

The *Angiopteris* plastid genome contains several regions with repeat structure. Results from the analysis by REPuter revealed two main regions of

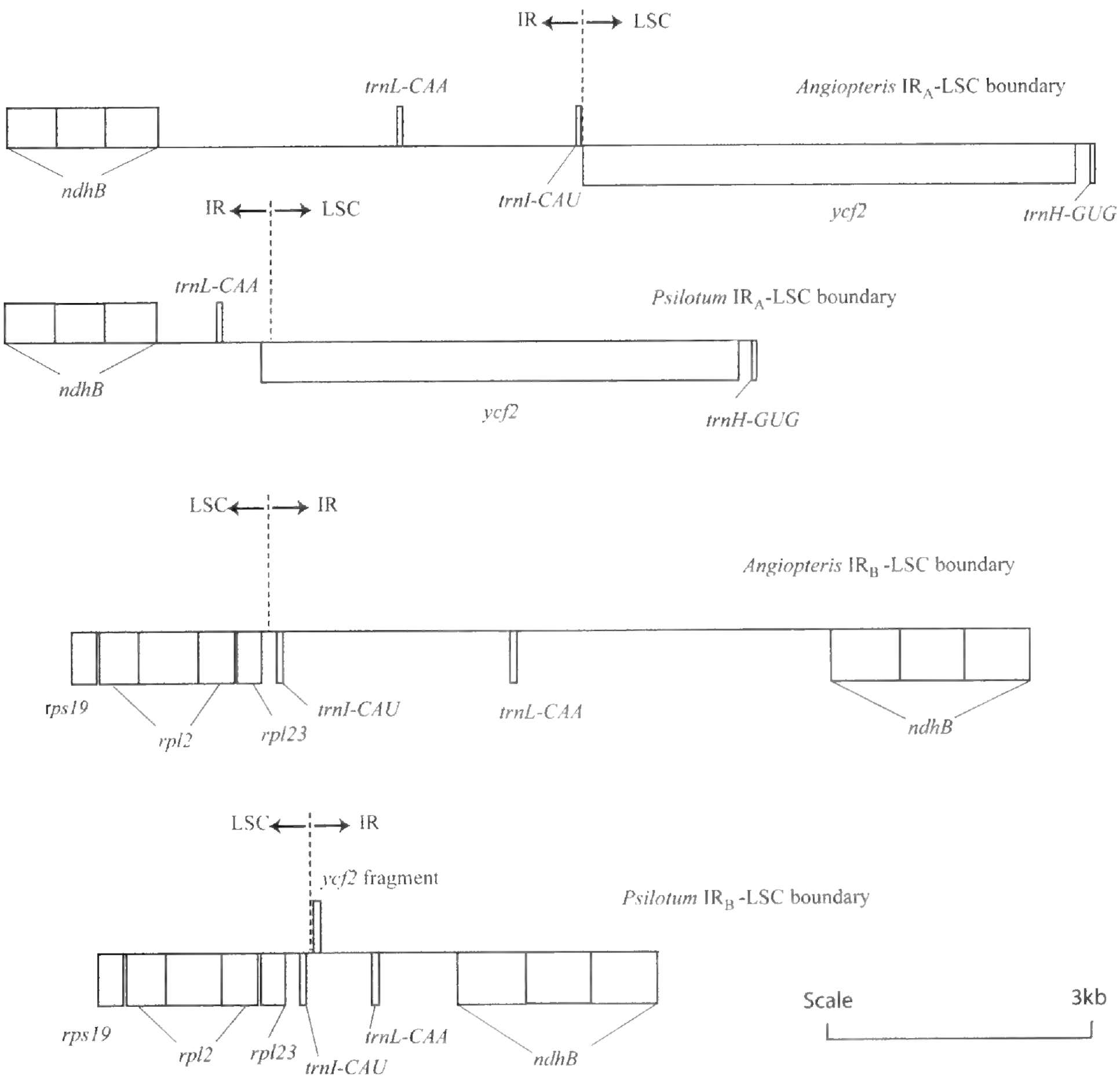


FIG. 3. Diagram of the LSC-IR boundaries, comparing that of *Psilotum nudum* with *Angiopteris evecta*.

long repeats (more than 20 bp). We found an 817 bp direct repeat within the region annotated as the pseudogene for the hypothetical protein *ycf1* in the SSC, as well as a 352 bp string with a 95% similarity to the reverse complement also in the same region. Either several duplications or inversions resulted in *ycf1* becoming a pseudogene, or its loss of function lifted selective constraints against such structural rearrangements. The remaining repeat regions were all at the beginning of the IR between *trnI* and *trnL*. This region is highly variable in several plastid genomes, probably due to the creation of partial genes during expansion and contraction of the IR (Goulding *et al.*, 1996; Palmer, 1991).

We found no stop codons within otherwise open reading frames and no other obvious indications that RNA editing would be required. The *ycf1* pseudogene was too drastically different from heterologous *ycf1* sequences to explain the differences by RNA editing. However, absence of evidence is not evidence of absence; RNA editing can only be tested by sequencing cDNAs.

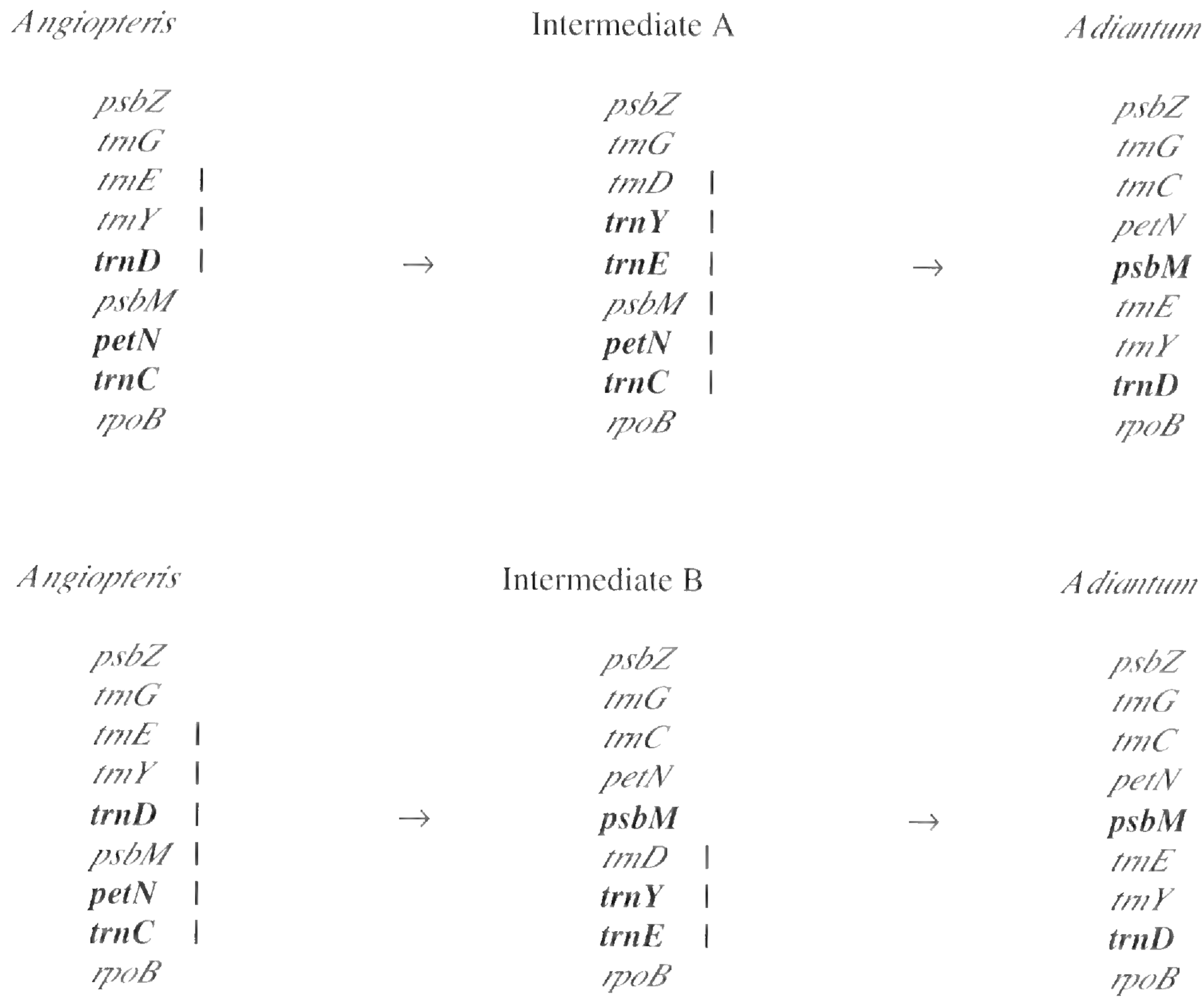


FIG. 4. Two hypothetical pathways to explain the gene order difference in the LSC of *Angiopteris* and *Adiantum* plastid genomes. Vertical bars denote regions that become inverted to produce the next gene order to the right.

This has only been done systematically for two chloroplast genes (*ndhB* and *rbcL*) in all major lineages of land plants (Freyer *et al.*, 1997). The complete set of transcripts from chloroplast genes has only been examined in one liverwort, *Anthoceros*, (Kugita *et al.*, 2003) and one leptosporangiate fern, *Adiantum capillus-veneris* (Wolf *et al.*, 2004), for which 350 RNA edited sites were detected. Thus, it remains unclear whether high levels of RNA editing are derived or ancestral within monilophytes.

Why have the plastid genome structures of *Angiopteris* and *Psilotum* remained so constant over such a long period of evolutionary time? The most-recent common ancestor of *Angiopteris* and *Psilotum* probably lived over 400 million years ago (Pryer *et al.*, 2004a). Plastid genome structure has evolved rapidly in several younger clades such as *Geranium* (Palmer *et al.*, 1987a) and Campanulaceae (Cosner *et al.*, 2004). Some events have been correlated with loss of structural stability, such as loss of the inverted repeat (Palmer *et al.*, 1987b). Clearly, plastid genome structure does not evolve in a clock-like manner. In fact, it is for this reason that structural changes can

provide useful phylogenetic markers. Gene order can take on many possible states whereas DNA sequences have only four states. Thus, structural changes are more complex than nucleotide substitutions: reversion to an ancestral gene order is unlikely compared to reversion to an ancestral base in the DNA sequence. Long evolutionary branches have, on average, more opportunity to accumulate changes. However, the non-clock-like nature of structural changes provides a chance for them to become phylogenetic markers on short branches where signal is weak in DNA sequence data. The conservation of plastid genome structure between *Angiopteris* and *Psilotum* is either a function of long term stability in both lineages, or independent (and perhaps repeated) convergence to a more stable structure and gene order. Distinguishing these hypotheses can only be achieved with more genome structural data from additional clades, and such information is needed if we are to understand more about the levels of homoplasy for structural genomic characters.

If the plastid genome structure of *Angiopteris* has indeed remained constant since the origin of monilophytes, this would correlate with other evolutionary trends in the Marattiaceae. Analysis of rates of molecular evolution for nuclear and plastid genes revealed reduced substitution rates in both the Marattiaceae and in the tree fern clade (Soltis *et al.*, 2002). If genome structure has also been evolving slowly in Marattiaceae this would imply a correlation of evolutionary rates for morphology, DNA sequences, and genome structure. Testing for such a correlation would require more data on genome structure, including nuclear genomes, from more taxa.

*Angiopteris* represents an ancient lineage whose affinities to other monilophytes is currently unresolved. Most analyses of DNA sequence data suggest a sister relationship of Marattiaceae to Equisetaceae, the horsetails (Wilkström and Pryer, 2005). If so, this would be an ancient clade, with little signal remaining. Data are forthcoming on the plastid genome of *Equisetum*, in addition to the mitochondrial genomes of *Angiopteris* and *Equisetum* (K. Karol, personal communication), and it is hoped that additional phylogenetic information will soon be provided.

The circular diagram depicted in Fig. 1 is, like all such genome maps, a visual representation of something far more complex. One unusual feature of plastid genomes is that the LSC and SSC have alternative orientations relative to the IR within a single organelle (Palmer, 1983). This so-called flip-flop recombination has also been documented for plastid genomes in the fern *Osmunda* (Stein *et al.*, 1986). Thus, the relative orientations of the LSC and SSC in any map are arbitrary. Furthermore, experiments with native chloroplast genomes indicate that, at least in some situations, most molecules are linear and some even branched, with few displaying the more familiar circular structure depicted in most maps (Oldenburg and Bendich, 2004).

We provide here the first complete plastid genome sequence from the marattioid clade of plants. Availability of this sequence can enable researchers to design conserved primers to PCR-amplify and sequence new genomic regions that could provide useful phylogenetic information not available from the array of regions usually studied in ferns (Small *et al.*, 2005). In addition,

the structural details of the *Angiopteris* plastid genome join a growing database from other green plants. Ultimately such data can be used to infer phylogeny as well as help understand evolutionary process at both the sequence and genome structural levels.

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# Two New Species of *Pleopeltis* (Polypodiaceae) from Andean South America

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ABSTRACT.—*Pleopeltis orientalis* and *P. oreophila* are described as new. *Pleopeltis orientalis* is restricted to the Cordillera Oriental of Colombia. It resembles the South American species *Pleopeltis fraseri* (Mett. ex Kuhn) A. R. Sm. and *Pleopeltis remota* (Desv.) A. R. Sm. *Pleopeltis oreophila* is found in interandean valleys of northern Peru. It resembles *Pleopeltis pycnocarpa* (C. Chr.) A. R. Sm. and *Polypodium segregatum* Hook.

During taxonomic work on Andean ferns, I found two species of *Pleopeltis* here described as new. Both are members of what was formerly referred to as *Polypodium* subg. *Marginaria* (Bory) C. Chr., that is, the scaly species of *Polypodium*. This group was previously treated in part by de la Sota (1966) and Maxon (1916a, 1916b). Morphological and molecular data (Windham, 1993; Schneider *et al.*, 2004) suggest that these species belong with *Pleopeltis* instead of *Polypodium* sensu stricto. Some new combinations have already been made (Windham, 1993; Kessler and Smith, 2005) and others are pending a revision of the limits of *Pleopeltis* by Alan Smith (A. R. Smith, pers. com.). For this reason, the species described here are compared to other species of *Pleopeltis* and *Polypodium*. The species of *Polypodium* discussed herein will eventually be transferred to *Pleopeltis*.

**Pleopeltis orientalis** Sundue, ***sp. nov.*** TYPE.—Colombia. **Santander**: Mun. Vetas, vic. of Vetas, 3100–3200 m, open rocky hillsides, 16–20 Jan 1927, *Killip & Smith 17295* (holotype: NY; isotype: US). **Fig. 1 A–D**.

Rhizomata dense squamosa, squamis distincte bicoloribus, parte centrali fascia crassa nigrescenti indurata lanceolata 0.6 mm lata ornata, partibus squamae ad fasciam parallelis pallide brunneolis, tenuibus, 0.3–0.5 mm latis, saepe aetate erosis, marginibus squamae denticulatis. Laminae 7.0–19.0 × 6.5–9.5 cm, coriaceae, 1-pinnatisectae, ovatae vel triangulares, ad basem latissimae et truncatae, ad apicem acutae; superficies abaxiales laminae dense squamosae, squamis 1.5–2.0 × 0.7–1.0 mm, peltatim affixis, clathratis, luminibus subocclusis, brunneolis, maximam partem concoloribus praeter insertionem brunneam atque margines subhyalinas, ovatis vel lanceolato-ovatis, ad basem rotundatis, ad apicem acutis, marginibus denticulatis, dentibus simplicibus vel bifidis.

Plants epipetric. Rhizome long-creeping, 3–6 mm wide, densely scaly, the scales 3.5–4.0 × 1.2–1.6 mm, peltate, loosely appressed, lanceolate, distinctly bicolorous, the central portion with a thick, blackish, indurate, lanceolate, stripe 0.6 mm wide, the outer portions of the scale on either side of the stripe

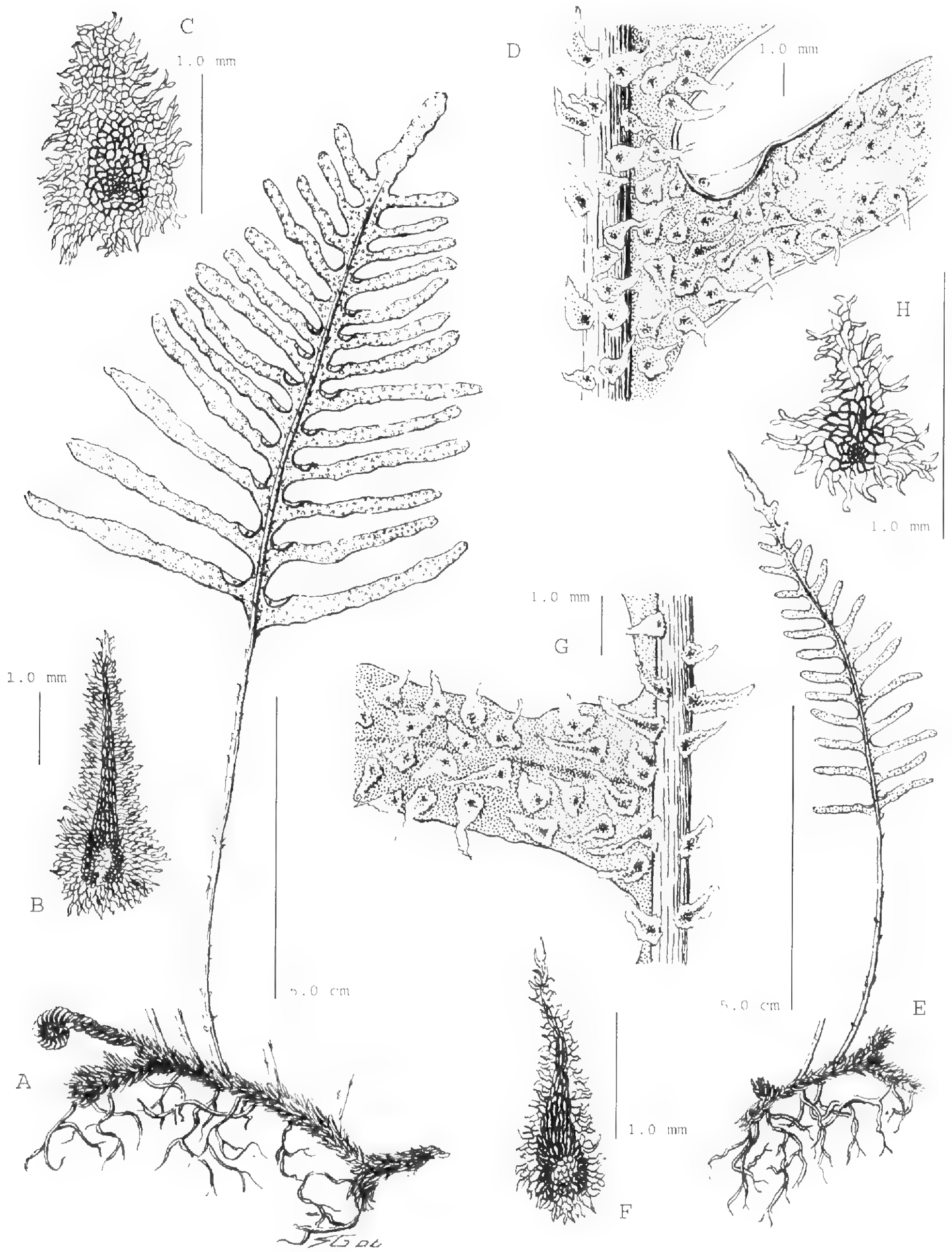


FIG. 1. A–D. *Pleopeltis orientalis* A. habit. B. rhizome scale. C. laminar scale. D. abaxial lamina detail (*Killip & Smith 17295* NY). E–H. *Pleopeltis oreophila* E. habit F. rhizome scale (*Sagástegui et Leiva 12531* MO). G. abaxial lamina detail. H. laminar scale (*Sagástegui & Leiva 15422* UC).

light tan, thin, 0.3–0.5 mm wide, often eroded in age, the margin denticulate. Phyllopodia short, indistinct. Fronds monomorphic. Petioles 5–15 × 0.5–2.0 mm, adaxially grooved, brown to purple-brown, or sometimes stramineous, moderately to densely scaly, the scales similar to those of the laminar axes. Laminae 7.0–19.0 × 6.5–9.5 cm, 1-pinnatisect, coriaceous, yellowish-brown in dried specimens, ovate to triangular, widest at the base, the base truncate, the apex acute; pinnae 2.0–5.5 × 0.3–0.5 cm, 8–16 pairs, linear, even-sided, basal pinnae spreading, the medial and distal pinnae ascending, the pinna bases adnate, the acroscopic margin of the pinna base often slightly folded toward the abaxial side and provided with a dark-colored sunken nectary, the pinna apices acute, the pinna margins entire for most of their length, notched distally, the adaxial lamina surfaces glabrous except for a few scattered scales along the adaxial costae, these scales similar to those of the abaxial axes, hydathodes present, sunken into the laminar tissue, white-encrusted; abaxial lamina surfaces densely scaly, the scales 1.5–2.0 × 0.7–1.0 mm, peltately attached, clathrate, the lumina partially occluded, light-brown, essentially concolorous except for the dark-brown point of attachment and somewhat hyaline edges, ovate to lanceolate-ovate, the base rounded, the apex acute, the margin denticulate, the teeth simple or bifid, the scales of the axes tending to differ by being up to 3.5 ×1.5 mm, lanceolate, bicolorous, the central portion dark-brown, and the scale apices being long-attenuate. Veins obscured by the thick lamina. Sori in one row between the costa and the pinna margins, round, (6)10–14 pairs per pinna, obscured by laminar scales when young, partially confluent at maturity. Spore color unknown.

Distribution and Habitat.—Cordillera Oriental of Colombia, on or among rocks in open rocky slopes and thickets along streams, from 2250–3250 m.

Etymology.—This species is named after the Cordillera Oriental of Colombia to which it is apparently confined.

Additional specimens examined.—COLOMBIA. **Boyacá:** Mun. Ráquira, desierto de la candelaria, 2250 m, 10 Apr 1974, *Acosta-Artega 282* (NY); Mun. Ráquira, slope south of Río Gomeza (Río Arobispo), 7–10 km E of Socha, 6° N, 72° 55–57′W, rock ledge, cleared rocky slope with great boulders, scattered bushes, and brushy ravines, 3050–3070 m, 11 Nov 1944, *Fosberg 22305* (US); **Cundinamarca:** Mun. Suesca, en la parte inferior de las rocas, 2600 m, 2 Jun 1974, *Acosta-Artega 424* (NY); **Santander:** Mun. Vetas, vic. of Vetas, thickets along stream, 3100–3250 m, 16 Jan 1927, *Killip & Smith 17356* (NY).

*Discussion.*—The characteristic sharply bicolorous rhizome scales of *Pleopeltis orientalis* ally it with *Pleopeltis fraseri* (Kuhn) A. R. Sm. and *Pleopeltis remota* (Desv.) A. R. Sm. of South America, and with Central American and Mexican species such as *Polypodium plebeium* Schltdl. & Cham. and *Polypodium madrense* J. Sm. It differs from these by having densely scaly abaxial lamina surfaces, laminar scales that are ovate to lanceolate-ovate and clathrate, and pinnae that are narrow, and spreading to ascending. By

comparison, other South American species in this group are usually sparsely scaly on the laminae abaxially. Some specimens of *Pleopeltis fraseri* from northern Ecuador and the Cordillera Central of Colombia are moderately scaly. These plants could be confused with *Pleopeltis orientalis*, but they differ by having wider pinnae, and darker-brown lamina scales that have a narrow, attenuate apex, arising from an expanded scale base. The spore color of *Pleopeltis orientalis* is unknown because this character is lost in herbarium specimens of *Pleopeltis*. For example, the spores of *Pleopeltis wiesbaurii* (Sodiro) Lellinger are green when fresh (M. Sundue, pers. obs.), but fade to white after drying. *Pleopeltis orientalis* might have green spores like *Pleopeltis remota*, or yellow spores like those of *Pleopeltis fraseri* or *Polypodium plebeium*, all of which appear quite similar to the new species.

**Pleopeltis oreophila** Sundue, ***sp. nov.*** TYPE.—Peru. **Cajamarca**: Prov. Contumazá, de piedras, 2400 m, 13 Jun 1983, *Sagástegui & López 10602* (holotype: NY; isotype: F). **Fig. 1, E–H**.

Rhizomata dense squamosa, squamis bicoloribus, parte centrali fascia conspicua fusca, atrobrunnea vel nigrescenti, opaca, indurata praeter insertionem brunneam, partibus squamae ad fasciam parallelis pallide brunneolis, hyalinis, saepe aetate erosis, marginibus denticulatis, dentibus simplicibus vel bifidis. Laminae 6.2–13 × 1.1–3.5 cm coriaceae, lanceolatae, subaequilaterae, proxime sub medio vel interdum ad basem latissimae, profunde pinnatisectae, ut videtur 1-pinnatae; superficies adaxiales laminae glabrae; superficies abaxiales laminae dense squamosae, squamis 0.6–1.0 mm × 0.4–0.5 mm, clathratis, luminibus apertis vel partim occlusis, ovatis vel lanceolatis, concoloribus atque pallide brunneis vel obscure rubescenti-brunneis, insertione infuscata, marginibus hyalinis, denticulatis, dentibus simplicibus vel plerumque bifidis, squamis secus axes saepe bicoloribus parte distali squamae nigrescentibus, induratis, luminibus occlusis.

Plants epipetric. Rhizome long-creeping, 1.5–3.0 mm wide, densely scaly, the scales 1.7–2.6 × 0.4–0.6 mm, peltate, loosely appressed, linear-lanceolate, long-attenuate, bicolorous, the central portion with a conspicuous dark stripe, the stripe dark-brown to blackish, opaque, indurate except for the point of attachment which is brown, the outer portions on either side of the stripe tan, hyaline, often eroded in age, the margin denticulate, the teeth simple or bifid. Phyllopodia short, indistinct. Fronds monomorphic. Petioles 3.5–8.0 cm × 0.7–1.1 mm, blackish, terete, adaxially grooved, sparsely scaly. Laminae 6.2–13 × 1.1–3.5 cm, coriaceous, lanceolate, nearly parallel-sided, widest just below the middle or sometimes at the base, deeply pinnatisect, appearing 1-pinnate, the lamina base truncate, the apex gradually reduced, attenuate; pinnae 6–15 × 1.5–3.0 mm, (7)10–14(20) pairs, linear, ascending, the bases adnate, the apices rounded to acute, the pinna margins crenate or minutely notched on smaller pinnae, the basal pinnae sometimes with a basal constriction caused by the acroscopic and basiscopic margins folding toward the abaxial side, the acroscopic margin provided with a dark sunken nectary, at

least on basal pinnae; adaxial lamina surfaces glabrous, hydathodes present, sunken, not white-encrusted; abaxial lamina surfaces densely scaly, the scales 0.6–1.0 mm × 0.4–0.5 mm, clathrate, the lumina clear to partially occluded, ovate to lanceolate, concolorous and light brown to dark reddish brown, the point of attachment dark, the margins hyaline, denticulate, the teeth simple or more often bifid, the scales along axes often bicolorous with the distal portion of the scale blackish, indurate, the lumina occluded. Veins obscured by the thick lamina. Sori in one row between the costa and the pinna margin, round, 6–8 pairs per pinna, obscured by laminar scales when young, confluent at maturity on small pinnae. Spore color unknown.

Distribution and Habitat.—Interandean valleys in northern Peru, growing on rocks between 2400–2500 m.

Etymology.—Derived from the Greek "oreophilus", meaning mountain loving.

Additional specimens examined.—PERU. **Cajamarca:** Prov. Contumazá, sobre rocas, 2400 m, 28 Mar 1985, *Sagástegui & Leiva 12531* (MO, UC); Prov. Contumazá, ladera rocosa, 2500 m, 12 Dec 1993, *Sagástegui, Leiva, & Lezama 15107* (F, NY); Prov. Contumazá, Bosque Cachil, bosque húmedo, 2500 m, 12 Nov 1994, *Sagástegui & Leiva 15422* (F, UC).

*Discussion.*—The lamina and rhizome scales of *Pleopeltis oreophila* ally it with *Pleopeltis appressa* M. Kessler & A. R. Sm. *Pleopeltis buchtienii* (H. Christ & Rosenst.) A. R. Sm., *Pleopeltis pycnocarpa* (C. Chr.) A. R. Sm., *Polypodium segregatum* Hook., and *Pleopeltis tweediana* (Hook.) A. R. Sm. This species most closely resembles *P. pycnocarpa*, and most specimens studied were previously identified as this species. *Pleopeltis oreophila* differs from that species by having laminar and rhizome scales with bifid marginal teeth, darker petioles, more elongate and deeply pinnatisect laminae with more widely spaced pinnae (4–10 pairs in *P. pycnocarpa*), and a basal constriction on the basal pinnae which is absent in *P. pycnocarpa*. Both species are epipetric, but *P. pycnocarpa* grows almost exclusively at or above 3000 m, whereas *P. oreophila* is found between 2400–2500 m. *Pleopeltis oreophila* is also similar to *Polypodium segregatum* Hook. which also has laminar scales with bifid marginal teeth and pinnae with basal constrictions. That species differs by having lighter-colored petioles, larger fronds, and more pinnae (13–21), that are more widely spaced, and that have more narrowly constricted pinna bases. *Polypodium segregatum* is known only from Ecuador where it is an epiphyte usually growing between 2400–3000 m. The spore color of *Pleopeltis oreophila* is unknown (see discussion under *Pleopeltis orientalis*), but is expected to be yellow because most *Pleopeltis* have yellow spores, as does its putative close relative *P. pycnocarpa*.

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# New Records of Pteridophytes from Bolivia and Brazil

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ABSTRACT.—Based on herbarium specimens and recent collections made in the last few years, we have found 19 species that are for the first time recorded for Bolivia (9 species) and/or Brazil (12 species). Full specimen citations, comments about previously known distributions, and taxonomic notes are presented for all species. In Bolivia, all new records are for species of *Adiantum*, mostly from lowland regions in northern and eastern Bolivia. In Brazil, most of the new records are for Amazonian Brazil, near the boundaries with neighboring countries. One record is for southern Bahia, showing a clear disjunction between Venezuelan-Guayanan Shield and the Serra do Mar Mountains in northeastern Brazil.

Despite its age, Martius's *Flora Brasiliensis* still remains the most complete reference about the Brazilian flora. Its treatment of pteridophytes by Baker (1870) still represents the most important compilation of Brazilian ferns. However, based on recent nomenclatural changes and several floristic projects, nowadays it is impossible to use only this flora to estimate the diversity of pteridophytes in Brazil. Other literature about Brazilian ferns exists, but it is scattered in monographs of specific families, genera, or groups of species, and often present only a regional diversity of the group. Based mainly on these literature and herbarium data, Prado (1998) estimated 1,200–1,300 species of ferns and lycophytes for Brazil; however, new data suggest even more.

While preparing several local and regional floras in Brazil, as well as some world-wide monographic studies, we found new records of ferns that deserve special mention. Many of the species are well known from neighboring countries (e.g., Venezuela, Guianas, Colombia, Ecuador, and Peru), and their occurrence in boundary regions might be expected. For example, the Venezuelan-Guayanan Shield has many mountains (Roraima, Neblina, Paracaima, etc.) that have high levels of endemics and species richness, including many species recently described based mainly on specimens from the Venezuelan and/or Guianan sides (e.g., Smith, 1990).

For northwestern Brazil, most of the species listed as new records were previously known from neighboring countries that share with Brazil similar lowland vegetation types in the Amazon Basin. For example, the pteridophyte flora of Brazil and Bolivia is similar, and both countries share several species of which some are here presented.

One new record is presented for southern Bahia State, with a clear disjunction between the montane environments in the Andes and/or Venezuelan-Guayanan Shield, and the forests of northeastern Brazil.

At the moment there is no floristic project for the Brazilian pteridophyte flora as a whole, and the species here considered belong to groups that we have recently studied and for which the determinations could be corroborated. Other more poorly studied groups are likely sources of new records for Brazil and Bolivia, but taxonomic revisions are still needed to clarify problems of species circumscription.

For Bolivia, the most recent paper on the pteridophyte diversity was published by Smith *et al.* (1999). They presented 145 species and one variety as new records for the country. The authors commented about the difficulty of estimating the diversity of the group in a floristically poorly known area. An ongoing flora project of Bolivian pteridophytes by M. Kessler & A. R. Smith (pers. comm.) will shortly provide a full treatment of the ca. 1,150 Bolivian pteridophyte species.

As pointed out by Smith *et al.* (1999) among the pteridophytes of Bolivia there are a lot of new records and undescribed species already represented in herbarium collections, which have partly been described by Lellinger & Prado (2001), Prado & Smith (2002), Smith & Prado (2004), Prado (2005a), Kessler & Smith (2005), Kessler *et al.* (2005a), Kessler *et al.* (2005b), Kessler & Mickel (2006), Kessler & Smith (2006), Kessler *et al.* (2006), and Prado (2006).

Here we present some of them as new records for Bolivia corroborating the expectation of Smith *et al.* (1999). Until recently some of these species were considered as endemic to Brazil (*Adiantum ornithopodum*, *A. senae*, and *A. sinuosum*) or Peru (*A. poeppigianum* and *A. scalare*).

Our main goal in this paper is to add taxa not listed before in the older and more recent literature to the pteridophytes of Bolivia and Brazil, as well as to call attention that the taxa cited here have a wider range of distribution than previously known.

**Adiantum anceps** Maxon & C. V. Morton, Amer. Fern J. 24: 15–17. 1934.

The concavely acuminate ultimate segments separate this species from *Adiantum peruvianum*, the most similar species.

Distribution and Ecology.—Previously known from Colombia, Ecuador, and Peru (Tryon & Stolze, 1989); reported here from Bolivia. It grows in forests along meandering streams in valleys.

Specimen Studied.—BOLIVIA. **La Paz:** Prov. Sud Yungas, Río Inicua, 15°29′S, 67°13′W, 550 m, 17 Nov 1996, *D. Lara 95* (LPB).

**Adiantum decoratum** Maxon & Weath., Amer. J. Bot. 19: 165. 1932.

This species can be easily recognized by its atropurpureous petiole and rachises, with dense, spreading, dark brown scales. The scales are entire or denticulate and each has a filiform apex.

Distribution and Ecology.—Previously known from Mexico, Mesoamerica, northern Colombia, French Guiana, and Peru (Lellinger, 1989; Jermy, 1995; Mickel & Smith, 2004; Smith *et al.*, 2005; Smith & Boudrie, pers. comm.). Until

recently, this species was known in South America only from Chocó in Colombia (Mickel & Smith, 2004), but it has recently been reported for Peru (Smith *et al.*, 2005). In Brazil it grows in forests along meandering streams in valleys in steep, hilly terrain.

SPECIMEN STUDIED.—BRAZIL. **Acre:** Mun. Santa Rosa do Purus, Rio Chandless tributary of Rio Purus, right bank, Canamari, 9°22′59″S, 69°56′38″W, ca. 250 m, 28 Mar 1999, *D. C. Daly et al. 10164* (NY).

**Adiantum diogoanum** Glaz. ex Baker, J. Bot. 20: 310. 1882.

This species can be recognized by the rachises with large scales (3–4 cells wide) with ciliate margins and pectinate bases, and pubescent indusia with reddish brown hairs.

DISTRIBUTION AND ECOLOGY.—Colombia, Ecuador, Peru (Smith *et al.*, 2005), and Brazil (Prado, 2000); now recorded from Bolivia, where it was found in forests at 100–1200 m.

SPECIMENS STUDIED.—BOLIVIA. **Beni:** Prov. Yacuma, Campamento Campo Monos, bajando por el Río Curiraba, 14°38′S, 66°4′W, 17 Nov 1996, *A. Acebey 20* (LPB). **La Paz:** Prov. Sud Yungas, Chulumani 107 km hacia NNE, pasando Asunta Alto Charia sobre el río San Jos' afluente del Río Boopi, 15°58′S, 67°10′W, 900 m, 6 Aug 1983, *S. G. Beck 8511* (LPB); Prov. Nor Yungas, puente sobre el Río Beni, al oeste del mismo, 9 km hacia, Litoral, 15°32′S, 67°23′W, 550 m, 30 Mar 1986, *S. G. Beck 13368* (LPB); Prov. Ballivián, Pilón Lajas, Rurrenabaque, Serranía Suse, 14°25′S, 67°31′W, 228 m, 17 Nov 1999, *M. De Boer 1290* (LPB). **Santa Cruz:** Prov. Ñuflo de Chávez, San Ramón Puquio Sur- Ladera NO, 16°38′S, 62°27′W, 505 m, 22 Feb 1991, *R. Quevedo 340* (MO). Prov. Valle Grande, Vallegrande, 18 km a Boyuibe, 18°59′S, 63°44′W, 550 m, 9 Jul 1995, *M. Kessler 5265* (LPB).

**Adiantum dolosum** Kunze, Linnaea 21: 219. 1848.

The entire pinnae with approximately equilateral bases, the single, long sori on each side of the pinna, and the partialy areolate veins are good features to distinguish this species.

DISTRIBUTION AND ECOLOGY.—Venezuela, Guyana, Suriname, French Guiana, and Brazil (Smith & Lellinger, 1995; Cremers, 1997); reported here from Bolivia, where it was found in Amazonian forests at 130 m.

SPECIMEN STUDIED.—BOLIVIA. **Pando:** Prov. Manuripi, comunidad Santa Rosa, río arriba por el Madre de Dios desde Riberalta, entrando por Valparaiso, 10°52′S, 66°10′W, 130 m, 8 Nov 2003, *I. Jiménez 2023* (LPB).

**Adiantum incertum** Lindm., Ark. für Bot. 1: 204, tab. 9, fig. 4. 1903.

This species belongs to the group of *Adiantum latifolium* Lam. and can be easily recognized by long-creeping rhizomes, 2-pinnate laminae, and pinnules

abaxially bearing hairlike scales with a few basal processes. *Adiantum humile* Kunze and *A. terminatum* Kunze ex Miq. are similar to *A. incertum*, but they differ by the nodose, short-creeping rhizome, and the septate hairs on the abaxial lamina surface.

Distribution and Ecology.—Paraguay and Brazil (Lindman, 1903; Prado & Lellinger, 2002). Here recorded from Bolivia, where it grows inside forest along river margins, 150–250 m.

Specimens Studied.—BOLIVIA. **Santa Cruz:** Prov. Velasco, Parque Nacional Noel Kempff Mercado, Arroyo Las Lontras; bosque de sartenejal, Parcela permanente de estúdio, al S del Arroyo, 14°24′S, 61°8′W, 150 m, 26 Jul 1996, *L. Arroyo 1375b* (NY). **Beni:** Prov. Ballivián, Espíritu en la zona de influencia del Río Yacuma, Isla de Espíritu, 14°9′S, 64°23′W, 200 m, 13 Apr 1981, *S. G. Beck 5368* (LPB); Prov. Ballivián, km 35 on Yucumo-Rurrenabaque road Agric.-Tech High School at Río Colorado, 14°55′S, 67°5′W, 235 m, 13 Jul 1990, *A. Fay & L. Fay 2682* (MO); Prov. Yacuma, Estación Biológica del Beni, bosque de altura inundado a 400 m al N del Río Curiraba, 14°37′S,66°22′W, 250 m, 24 Dec 1987, *M. Moraes 954* (LPB). **Cochabamba:** Carrasco, Puerto Villarroel, propiedad de Bernardino Rodríguez, al lado de la carretera Ivirgarzama-Puerto Villarroel, Transecto 38, 16°50′S, 64°48′W, 190 m, 6 Oct 2000, *R. C. Paz 788* (LPB).

**Adiantum ornithopodum** C. Presl ex Kuhn, Linnaea 36: 74–75. 1869.

The color of the stalks passing into the segment bases, quadrangulate to trapeziform median segments with rounded apices, glabrous rachises, as well as glabrous segments on both surfaces distinguish this species.

Distribution and Ecology.—Brazil (Prado, 2004); reported here for the first time from Bolivia, where it grows in drough-deciduous Chiquitano forest.

Specimens Studied.—BOLIVIA. **Santa Cruz:** Prov. Velasco, San Juanito ca. 30 km al N de San Ignacio, 16°14′S, 60°58′W, 400 m, 05 Apr 1986, *S. G. Beck 12397* (LPB); Prov. Velasco, San Ignacio 4 km hacia el S, 16°22′S, 60°55′W, 400 m, 25 Jun 1986, *R. Seidel 689* (LPB).

**Adiantum patens** Willd., Sp. pl. ed. 4, 5: 439. 1810.

The blade architecture is a distinctive character of this species. The laminae are ovate to nearly circular in outline, with 2 recurved rachises, each rachis bearing pinnules on only the basiscopic side. Superficially resembles *Adiantum pedatum* L., a common species in North America.

Distribution and Ecology.—Mexico, Mesoamerica, Colombia, Venezuela, Ecuador, Peru, and Bolivia (Lellinger, 1989; Tryon & Stolze, 1989; Jermy, 1995; Mickel & Smith, 2004); here reported from Brazil, where it was found growing at 860 m, in dry forests.

SPECIMEN STUDIED.—BRAZIL. **Ceará:** Planalto da Ibiapaba, PNU, Jun 1978, *A. Fernandes & F. J. A. Matos s.n.* (EAC 4015).

**Adiantum poeppigianum** (Kuhn) Hieron., Hedwigia 48: 231. 1909. *Adiantum lucidum* (Cav.) Sw. var. *poeppigianum* Kuhn, Jahrb. Köngl. Bot. Gart. Berlin 1: 340. 1881.

This species can be distinguished by the dark color of the pinna stalks continuing into the bases of the pinnae and the distinct midveins of the pinnae.

DISTRIBUTION AND ECOLOGY.—Peru (Tryon & Stolze, 1989); reported here for the first time from Bolivia and Brazil; 100–500 m.

SPECIMENS STUDIED.—BOLIVIA. **Beni:** Prov. Ballivián, Río Colorado, Colegio Técnico Agropecuario de Río Colorado, 15°00′S, 67°10″W, 24 Jun 1989, *A. Fay & L. Fay 2127* (LPB). **La Paz:** Prov. Sud Yungas, Alto Beni, cerca de Sapecho, planicie aluvial del río, 15°30′S, 67°20′, 12 Jun 1994, R. *Seidel 7642* (LPB). BRAZIL. **Acre:** Mun. Sena Madureira, basin of Rio Purus, Rio Laco, right bank, Nova Olinda, between Igarapé Santo Antônio and Igarapé Boa Esperança, 10°07′S, 69°13′W, 29 Oct 1993, *D. C. Daly et al. 7972* (NY); Mun. Assis Brasil, basin of Rio Purus, Rio Acre, left bank, Seringal São Francisco, Colocação Derretida, 10°56′31″S, 69°45′33″W, 26 Mar 1998, *D. C. Daly et al. 9810* (HPZ, NY); Mun. Santa Rosa do Purus, Rio Purus, left bank, Seringal Santa Helena, 9°7′49″S, 70°10′37″, 24 Mar 1999, *D. C. Daly et al. 10034* (NY); Mun. Marechal Thaumaturgo, basin of Rio Juruá, Rio Arara (tributary of Rio Juruá), 25 m, 9°4′13″S, 72°46′40″W, 6 May 2001, *D. C. Daly et al. 10950* (NY).

**Adiantum ruizianum** Klotzsch, Linnaea 18: 551. 1845.

*Adiantum ruizianum* is distinct by its long-creeping rhizomes, long-stalked pinnae, suborbicular, glabrous segments that are not articulate, and the color of the stalks passing into the bases of the pinnae. Sterile pinna margins have veins ending in sinuses.

DISTRIBUTION AND ECOLOGY.—Peru and Bolivia (Tryon & Stolze, 1989; Smith *et al.*, 1999); reported here for the first time from Brazil, near the Bolivian border.

SPECIMEN STUDIED.—BRAZIL. **Mato Grosso:** Mun. Cáceres, Serra do Pitacanudo, 16°5′S, 57°40′W, 2 Nov 1987, *A. Salino 182* (GH, HB, UEC).

**Adiantum scalare** R. M. Tryon, Amer. Fern J. 47: 141–142, tab. 15. 1957.

This very distinct species has narrowly deltate pinnae that are pubescent abaxially (long brown hairs) with a small auricle on the acroscopic side. The indusia are linear and continuous on each side of the pinna.

DISTRIBUTION AND ECOLOGY.—Previously known only from Peru (Tryon & Stolze, 1989) and Ecuador (Jørgensen & León-Yánez, 1999); here for the first time

reported from Bolivia and Brazil, growing on Terra Firme with palms and bamboo (*Guadua*).

Specimens Studied.—BOLIVIA. **La Paz:** Prov. Iturralde, Cantón San José de Chupiamonas, Serranía de Eslabón, 14°15′S, 68°4′W, 19 Apr 1997, *S. G. Beck 24080* (LPB); F. Tamayo, Parque Nacional Madidi, Río Hondo, arroyo Negro, pica hacia la serranía de Toregua, Bosque amazónico Estaciónal húmedo, en planicie ondulada, 14°40′S, 67°49′W, 340 m, 25 Mar 2002, *Fuentes 4054* (SP). BRAZIL. **Acre:** Mun. Cruzeiro do Sul, Reserva Extrativista do Alto Juruá, 8°55′S, 72°31′W, 11 Mar 1992, *D. C. Daly et al. 7331* (NY); road Trauacá to Feijó, Km 17, 17 Sep 1968, *G. T. Prance et al. 7335* (GH, K, NY, US); Mun. Marechal Thaumaturgo, basin of Rio Juruá, Rio Tejo, right bank, 9°2′52″S, 72°16′24″W, 1 Dec 2000, *D. C. Daly et al. 10304* (NY).

**Adiantum senae** Baker, London J. Bot. 23: 217. 1885.

This species has a very small size (2.5–6 cm tall), flexuous rachises, and diminute, flabelate pinnules (2–3 mm long) each with only one sorus on the distal side.

Distribution and Ecology.—Previously known only from Brazil; reported here for the first time from Bolivia. It grows on calcareous rocks in open places.

Specimen Studied.—BOLIVIA. **Santa Cruz:** Prov. Velasco, campamento Las Torres, margen del Río Tienes, frontera con Matto Groso, lado NE de la Serranía de Huanchaca, 24 km al S de Flor de Oro, 50 km al N del Río Verde, 13°39′S, 60°48′W, 400 m, 26 May 1991, *M. Peña-Chocarro 316* (LPB).

**Adiantum sinuosum** Gardner in Hooker, Ic. pl. 6: tab. 504. 1843.

This is a very distinct species in having laminae up to 3-pinnate proximally, yellowish rhizome scales, flabellate to roundish pinnules, and oblong to strongly lunate indusia.

Distribution and Ecology.—Previously known only from Brazil (Prado, 2005b); reported here for the first time from Bolivia. It grows on open places, near the base of shrubs.

Specimens Studied.—BOLIVIA. **Santa Cruz:** Prov. Velasco, Parque Nacional Noel Kempff Mercado, Campamento Los Fierros, 10 km E y 1 km S, vegetación cerrada, 14°33′S, 60°56′W, 150 m, 1 May 1994, *B. Mostacedo 1498* (MO); Prov. Velasco, Parque Nacional Noel Kempff Mercado, 13°58′S, 60°50′W, 500 m, 5 Jan 1997, *A. Soto 478* (MO).

**Bolbitis oligarchica** (Baker) Hennipman, Amer. Fern J. 65: 30. 1975.
*Acrostichum oligarchicum* Baker in Hooker & Baker, Syn. Fil. 418. 1868.

The proliferous buds in the axils (or on stalks) of the pinnae and the usually greatly reduced single pair of proximal pinnae readily distinguish this species.

DISTRIBUTION AND ECOLOGY.—Mesoamerica, Colombia, Ecuador, Peru, and Bolivia (Murillo-Pullido & Harker-Usech, 1990; Tryon & Stolze, 1991; Hennipman & Moran, 1995); reported here for the first time from Brazil. Inside wet forests on steep slopes.

SPECIMEN STUDIED.—BRAZIL. **Acre:** Mun. Mâncio Lima, Bacia do Alto Juruá, Rio Moa, Parque Nacional da Serra do Divisor, fundo do vale do Igarapé do Amor, 7°26′55″S, 73°39′41″W, 16 Jun 1996, *M. Silveira et al. 1361* (NY).

**Lellingeria phlegmaria** (J. Sm.) A. R. Sm. & R. C. Moran, Amer. Fern. J. 81: 86. 1991. *Polypodium phlegmaria* J. Sm., London J. Bot. 1: 194. 1842.

This species can be recognized by its linear-deltate, dark, rigid, glabrous scales, linear laminae with short segments (up to 1 cm long), and superficial or slightly sunken sori. It resembles *Lellingeria depressa* (C. Chr.) A. R. Sm. & R. C. Moran, a species restricted to the Atlantic Forest in Brazil, which has broader laminae and deeply sunken sori.

DISTRIBUTION AND ECOLOGY.—Antilles, Honduras, Costa Rica, Colombia, Venezuela, Ecuador, Peru, and Bolivia (Moran *et al.*, 1995; Bishop & Smith, 1995; Kessler & Smith, pers. comm.), and now is known from Brazil.

SPECIMEN STUDIED.—BRAZIL. **Roraima:** Dec 1909, *E. Ule* 8523 (MG).

**Pleopeltis repanda** A. R. Sm., Ann. Missouri Bot. Gard. 77: 259. 1990.

The entire laminae with undulate or repand margins, and the discrete sori lacking paraphyses are good characters to distinguish this species from its closest relatives in the genus, such as *Pleopetis macrocarpa* (Bory ex Willd.) Kaulf. (Smith, 1990). It is also similar to *Neurodium lanceolatum* (L.) Fée, a monotypic genus that differs by having the sori confluent in marginal coenosori.

DISTRIBUTION AND ECOLOGY.—This species occurs in eastern Venezuela and Guyana (Smith, 1990; A. R. Smith, pers. comm.), and is here reported from northern Brazil. In addition to the collection cited below, there is a collection from "Ceará, Guaramiranga", Nov 1897, *J. Hueber g120* (MG), which we believe was mislabeled. Collector, locality, and collection number were each annotated with three distinct handwritings on the label, casting doubt on the label information. Occurrence of *P. repanda* in northeastern Brazil would considerably amplify the distribution of this species.

SPECIMEN STUDIED.—BRAZIL. **Roraima:** Proximidades da divisa com a Venezuela, Km 11-2 do marco BV-9, Cordilheira Pacaraima, 3440 ft, 24 Nov 1979, *N. A. Rosa & O. C. Nascimento 3538* (MG).

**Tectaria draconoptera** (D. C. Eaton) Copel., Philipp. J. Sci. 2C: 410. 1907. *Aspidium draconopterum* D. C. Eaton, Mem. Amer. Acad. Arts, n.s., 8: 211. 1860.

This species can be recognized by its erect rhizomes, proximal pinna pair connected to the pair above by the alate rachis, and small, exindusiate sori in four or more irregular rows between main lateral veins.

DISTRIBUTION AND ECOLOGY.—Mesoamerica, Colombia, Ecuador, Peru, and Bolivia (Murillo-Pullido & Harker-Usech, 1990; Tryon & Stolze, 1991; Moran, 1995; Navarrete, 2001; M. Kessler & A. R. Smith, pers. comm.), and now reported for Brazil. It grows in primary forests on Terra Firme in Acre State, steep hills dissected by many small streams.

SPECIMENS STUDIED.—BRAZIL. **Acre:** Mun. Brasiléia, basin of Rio Purus, upper Rio Acre, Colônia Santo Antônio, 10°56′29″S, 69°15′41″W, 29 Mar 1998, *D. C. Daly et al. 9882* (HPZ, NY); Mun. Bom Futuro, Associação Chico Mendes, Km 52 of Brasiléia-Assis Brasil road, 18 km on Ramal (side road) "Tocandeira", 10°44′41″S, 69°2′57″W, 18 May 2003, *D. C. Daly et al. 11891* (NY).

**Terpsichore asplenifolia** (L.) A. R. Sm., Novon 3: 479. 1993. *Polypodium asplenifolium* L., Sp. pl. 2: 1084. 1753.

This species can be recognized in having ciliate scales on the stems, up to 2 mm long, ciliate sporangia, and unforked veins. *Terpsichore chrysleri* (Copel.) A. R. Sm. is similar, differing by its longer scales on the stem (up to 6 mm), and forked veins.

DISTRIBUTION AND ECOLOGY.—Mexico, Central America, Antilles, Colombia, Venezuela, Trinidad, Ecuador, Peru, and Bolivia (Moran *et al.*, 1995; Bishop & Smith, 1995; M. Kessler & A. R. Smith, pers. comm.), and now reported from Brazil.

SPECIMEN STUDIED.—BRAZIL. **Bahia:** Mun. Camacan, RPPN Serra Bonita, 835 m, 15°23′30″S, 39°33′55″W, 3 Feb 2005, *F. B. Matos et al. 308* (CEPEC).

**Thelypteris membranacea** (Mett.) R. M. Tryon, Rhodora 69: 7. 1967. *Phegopteris membranacea* Mett., Fil. Lechl. 2: 22. 1859.

Within *Thelypteris* subgenus *Meniscium*, this species can be distinguished by the relatively thin laminae, appressed, thin costal hairs, buds in the axils of proximal pinnae, and straight secondary veins that unite to create very narrow areoles.

DISTRIBUTION AND ECOLOGY.—Colombia, Peru (Smith, 1992), and Bolivia (M. Kessler & A. R. Smith, pers. comm.), reported here for the first time from Brazil. In wet forests on steep slopes.

SPECIMEN STUDIED.—BRAZIL. **Acre:** Mun. Mâncio Lima, Bacia do Alto Juruá, Rio Moa, Parque Nacional da Serra do Divisor, Morro Queimado, ao longo do Igarapé do Amor, 7°28′0″S, 73°37′27″W, 6 May 1996, *M. Silveira et al. 1249* (NY).

**Thelypteris opulenta** (Kaulf.) Fosberg, Smithsonian Contr. Bot. 8: 3. 1972. *Aspidium opulentum* Kaulf., Enum. Fil.: 238. 1824.

This species belongs to the subgenus *Cyclosorus* and can be recognized by sessile or short-stalked, deeply pinnatifid pinnae, not reduced proximal pinnae, prolonged laminar apices, supramedial, round sori confined to the pinna lobes, and indusia with glands at margins and sometimes also with hairs.

DISTRIBUTION AND ECOLOGY.—Mesoamerica, Antilles, Colombia, Venezuela, Guyana, Suriname, Ecuador, Peru, and Bolivia (A. R. Smith, pers. comm.). It is also occurs in Africa and Asia (Smith, 1992). According to Smith (1992), it is an introduced and common species in Peru. Here it is reported from Brazil as widely distributed in Acre State. In open forests with palms and scattered bamboo (*Guada*).

SPECIMENS STUDIED.—BRAZIL. **Acre:** Mun. Porto Acre, Reserva Floretal de Humaitá, Beira do Rio Acre, 6°53′S, 66°32′W, 22 Mar 1995, *C. Figueiredo & I. Riveiro 751* (NY); Mun. Manoel Urbano, Rio Chandless (tributary of Rio Purus), right bank, "Canamari", 9°23′0.6″S, 69°56′41″W, 19 Mar 2002, *D. C. Daly et al. 11458* (NY); Mun. Manoel Urbano, Rio Chandless (tributary of Rio Purus), right bank, "Ananaí", 9°28′23″S, 70°01′03″W, 20 Mar 2002, *D. C. Daly et al. 11498* (NY); Cruzeiro do Sul, Projeto RADAM-Sub-base de Cruzeiro do Sul - Ponto 2-SB-18-ZB, 16 Feb 1968, *L. R. Marinho 204* (NY); vicinity of Aldeota, Rio Juruá-Mirim, 24 May 1971, *P. J. M. Maas et al. P13304* (NY); vicinity of Serra da Moa, 24 Apr 1971, *G. T. Prance et al. 12428* (NY); basin of Rio Juruá, Rio Juruá-Mirim, left bank, "Vista Alegre", 8°8′2″S, 72°49′45″W, 15 May 2003, *D. C. Daly et al. 11858* (NY); Comunidade Assis Brasil, Ramal do Pentecoste, km 10, Ramal sem nome à direita, divisa entre AC-AM, Campinarana e Floresta de Baixio, 7°31′17″S, 72°51′15″W, c. 0–200 m, 23 Oct 2001, *J. Prado et al. 1333* (HPZ, SP); Mun. Mâncio Lima, Volta da Aurora, mata na várzea do Rio Moa, 7°33′26″S, 72°55′30″W, c. 0–200 m alt., 14 Oct 2001, *J. Prado et al. 1144* (HPZ, SP); Mun. Xapuri, margem direita do Rio Xapuri, mata de varzea, 19 May 2001, *L. G. Lohmann & E. C. de Oliveira 598* (NY); Mun. Marechal Thaumaturgo, basin of Rio Juruá, Rio Tejo, right bank, 9°2′52″S, 72°15′59″W, 3 Dec 2000, *D. C. Daly et al. 10366* (NY); Mun. Santa Rosa, Rio Chandless, tributary of Rio Purus, right bank, "Canamari", 9°22′59″S, 69°56′38″W, 28 Mar 1999, *D. C. Daly et al. 10158* (NY); Rio Purus, left bank, Colocação Santa Helena, trail to Rio Envira, 9°7′48″S, 70°10′37″W, 26 Oct 2001, *D. C. Daly et al. 11098* (NY); Idem, Rio Chambuiacu, right-bank tributary of Rio Purus defining border with Peru, 9°34′13″S, 70°35′23″, 14 Mar 2002, *D. C. Daly et al. 11327* (NY); Mun. Sena Madureira, Vic. of km 7, road Sena Madureira to Rio Branco, 29 Sep 1968, *G. T. Prance et al. 7662* (NY); Rio Macaua, Seringal Riozinho, Colocação Provenir, 9°43′S, 69°7′W, 31 Mar 1994, *L. de Lima et al. 553* (HPZ, NY).

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American Fern Journal 97(2):124–126 (2007)

# Shorter Notes

**A New Flavonoid, Quercetin 3-*O*-(*X*″-acetyl-*X*″-cinnamoyl-glucoside) and a New Fern Constituent, Quercetin 3-*O*-(glucosylrhamnoside) from *Dryopteris villarii*.**—Previous investigations on the flavonoids of the genus *Dryopteris* have led to the identification of ten flavonol glycosides based on kaempferol and quercetin, two flavanone *O*-glycosides (based on naringenin and eriodictyol) and three *C*-glycosylflavones (vitexin, vitexin 7-*O*-glucoside and orientin) by Hiraoka (Biochem. Syst. Ecol. 6:171-175. 1978) in eighteen *Dryopteris* species and to the identification of 3-desoxyanthocianins (mainly as apigenidin and luteolinidin 5-*O*-glucosides) in red sori of *Dryopteris erythrosora* (Eat.) Kuntze by Harborne (Phytochemistry 5:589-600. 1966). In addition, kaempferol 7-*O*-(6″-succinylglucoside) was found in four *Dryopteris* species and an unusual flavonoid (3,7,3′,4′-tetrahydroxy-5-acetoxyflavan having the methyl group of acetoxyl further bounded to C-4 of C-ring) was isolated from *Dryopteris filix-mas* (L.) Schott as shown in a review by Markham (pp. 427-468, *in* J.B. Harborne ed., *The Flavonoids, Advances in Research since 1980*. Chapman and Hall, London and New York. 1988). Ten flavonoids (seven flavonol glycosides based on kaempferol and quercetin including a new compound identified as kaempferol 3-*O*-(acetylrutinoside) and three flavonoid aglycones (apigenin, kaempferol and quercetin)) have been found recently in *Dryopteris villarii* (Bell.) Woynar by Imperato (Amer. Fern J. 96:93-95. 2006).

This paper deals with the isolation of four flavonoids (I-IV) from aerial parts of *Dryopteris villarii* collected in the Botanic Garden of University of Naples (Italy). The fern was identified by Dr. R. Nazzaro (Università "Federico II", Naples); a voucher specimen (*NAPEA 3496*) has been deposited in Herbarium of Dipartimento di Biologia, Università "Federico II", Naples, Italy (NAP).

Flavonoids (I-IV) were isolated from an ethanolic extract of aerial parts of *Dryopteris villarii* by preparative paper chromatography in BAW (*n*-butanol-acetic acid-water 4:1.5, upper phase), 15% HOAc (acetic acid) and BEW (*n*-butanol-ethanol-water (4:1:2.2). Further purification was carried out by Sephadex-LH 20 column chromatography eluting with methanol.

Color reactions (brown to yellow in UV+$NH_3$), $R_f$ values (0.55 in BAW; 0.28 in 15% HOAc) and UV spectral analysis in the presence of the customary shift reagents $\lambda_{max}$ (nm) (MeOH) 258, 266 (sh), 320, 337; +NaOMe 275, 388; +$AlCl_3$ 275, 310, 431; +$AlCl_3$/HCl 274, 311, 390 (sh); +NaOAc 270, 318, 381 showed that flavonoid (I) may be a flavonoid glycoside acylated with a cinnamic acid with free hydroxyl groups at positions 5, 7, 3′ and 4′ of the flavonoid skeleton. Both total acid hydrolysis (2M HCl; 2 hr at 100°C) and controlled acid hydrolysis (10% HOAc; 3.5 hr under reflux) gave quercetin and D-glucose. Alkaline hydrolysis (2M NaOH; 2 hr at room temperature in a sealed tube) gave quercetin 3-*O*-glucoside and cinnamic acid. Electrospray mass spectrum (positive mode) showed a pseudomolecular ion at m/z 659 $[M+Na]^+$ and ions

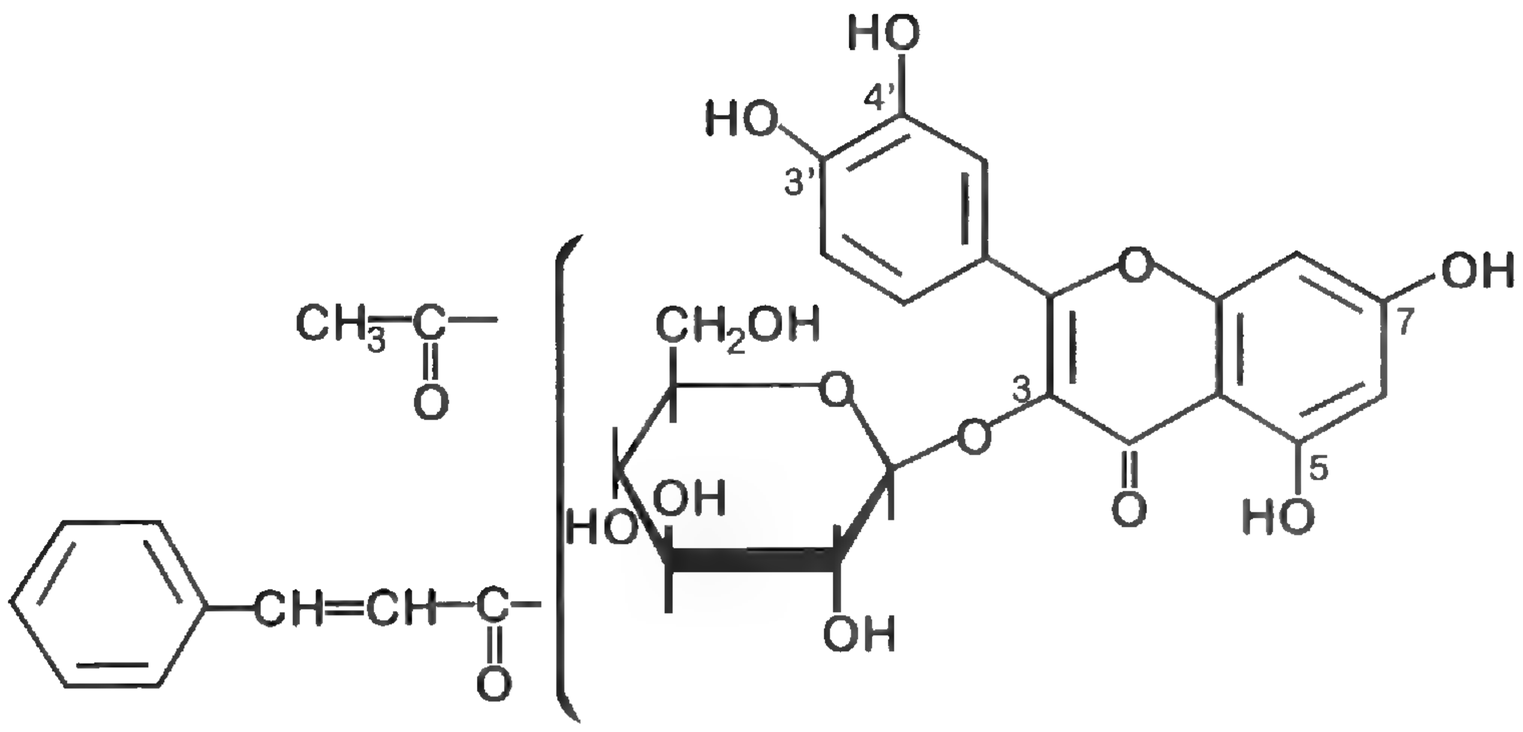


FIG. 1. The structure of flavonoid (I), quercetin 3-*O*-(*X*″-acetyl-*X*″-cinnamoyl-glucoside)

at m/z 617 $[(M+Na)\text{-acetyl}]^+$, m/z 507 $[(M+Na)\text{-(cinnamoyl)}]^+$, m/z 487 [quercetin glucoside+Na]$^+$, m/z 325 [quercetin+Na]$^+$ and m/z 130 [cinnamoy]$^+$. The above data show that flavonoid (I) is quercetin 3-*O*-(*X*″-acetyl-*X*″-cinnamoyl-glucoside), a new natural product (Fig. 1).

Flavonoid (II) was identified as quercetin 3-*O*-glucosylrhamnoside by UV spectral analysis with the customary shift reagents, total acid hydrolysis (which gave quercetin, D-glucose and L-rhamnose) and electrospray mass spectrum which showed a pseudomolecular ion at m/z 633 [M+Na]$^+$, an ion at m/z 448 [quercetin rhamnoside]$^+$ and an ion at m/z 325 [quercetin+Na]$^+$. Quercetin 3-*O*-(glucosyl-(1→4)-rhamnoside) was found for the first time in plants by Gautam and Mukharaya (Natl. Acad. Sci. Lett. 10:95-101. 1987) in leaves of *Euphorbia drancunculoides* Lam. (Euphorbiaceae); this flavonoid may or may not be the same as flavonoid (II) which is here reported for the first time from ferns.

Flavonoid (III) was identified as apigenin 4′-*O*-glucoside by UV spectral analysis in the presence of the customary shift reagents, total acid hydrolysis (which gave apigenin and D-glucose) and electrospray mass spectrum which showed an ion at m/z 887 [dimer+Na]$^+$, a pseudomolecular ion at m/z 445 [M+Na]$^+$ and an ion at m/z 185 [glucosyl+Na]$^+$. Apigenin 4′-*O*-glucoside has been found previously in the fern *Phegopteris polypodiodes* Fèe by Ueno as shown in the review by Markham and in the fern allies *Equisetum* x*litorale, E. fluviatile* L. and *E. arvense* L. as shown in a review by Imperato (pp. 39-75, in *Current Topics in Phytochemistry*. Research Trends, Trivandrum, 2000).

Flavonoid (IV) was identified as quercetin 3-*O*-rhamnoside by UV spectral analysis in the presence of the usual shift reagents, total acid hydrolysis (which gave quercetin and L-rhamnose) and electrospray mass spectrum (negative mode) which showed a quasimolecular ion at m/z 447 $[M-H]^-$ and an ion at m/z 301 [quercetin]$^+$. Quercetin 3-*O*-rhamnoside is here reported for the first time in the genus *Dryopteris*. This flavonoid has previously been found in *Onoclea sensibilis* L., *Woodsia polystichoides* Eat. and *W. manchuriensis* Hk. by Hiraoka (above reference) and in *Marsilea mucronata* A. Br., *M. vestita* Hk and Grev., *Pilularia americana* A. Br., *Glaphyropteridopsis erubescens* (Wall

ex Hook) Ching as shown in the review by Markham; more recently this flavonoid has been found in *Pilularia globulifera* L. and in *Equsetum fluviatile* and *Equisetum arvense* as shown in the review by Imperato (2000).

It is of interest that flavonoid (I) and three flavonoids (kaempferol 3-*O*-(acetylrutinoside), quercetin 3-*O*-(acetylglucoside) and quercetin 3-*O*-(acetylrutinoside)) previously found in *Dryopteris villarii* by Imperato (Amer. Fern J., 2006) show that this fern have acylated flavonoids which are reported for the first time from the genus *Dryopteris*.

The author thanks Mr Antonio Lo Vecchio for technical assistance and Università della Basilicata for financial support. Mass spectral data were provided by CNR/ISA (Avellino, Italy).—Filippo Imperato, Dipartmento di Chimica, Università della Basilicata, 85100 Potenza, Italy.

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# *Hyalotrichopteris* is Indeed a *Campyloneurum* (Polypodiaceae)

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ABSTRACT.—The relationships of the rare Mesoamerican fern *Campyloneurum anetioides* are inferred by comparing sequences of $trnL_{UAA}$-$trnF_{GAA}$ intergenic spacer of the plastid genome. In the past, this taxon was either treated as the single member of the genus *Hyalotrichopteris* or as part of the diverse Neotropical genus *Campyloneurum*. Analyses of the cpDNA give unambiguous support to the taxonomic placement of this species within *Campyloneurum*. The closest relatives within the genus *Campyloneurum* are currently unknown because limited taxon sampling and variation of the cpDNA sequences do not allow to elucidate this question. However, we can conclude that *C. anetioides* is unlikely the derivative of an early separation within *Campyloneurum*.

Relationships of *Hyalotrichopteris anetioides* (H. Christ) W. H. Wagner have been controversial. The species was first described in *Polypodium* by Christ (1909), and placed in its own genus, *Hyalotricha* (*H. anetioides* (H. Christ) Copel.), by Copeland (1953). *Hyalotricha* Copel. is a later homonym of *Hyalotricha* Dennis, and so the former has now been renamed *Hyalotrichopteris* W. H. Wagner (Wagner, 1978). Copeland (1953) indicated possible relationships of *Hyalotricha* to grammitid ferns, Polypodiaceae *s.s.*, Vittariaceae, and to "Aspidiaceae" (*Tectaria* Cav. and *Ctenitis* (C. Chr.) C. Chr.), but favored the last suggestion. On the basis of stomatal structure, van Cotthem (1970) suggested an alliance of *Hyalotricha* to Vittariaceae. Crabbe *et al.* (1975) placed *Hyalotricha* in the Grammitidaceae, near *Loxogramme* (Blume) C. Presl, without further comment. Pichi Sermolli (1977) postulated closest affinities of *Hyalotricha* to *Goniophlebium* C. Presl (= *Serpocaulon*; Smith *et al.*, 2006) and a largely American group of polypod genera (his Polypodiaceae *s.s.*) that also included *Campyloneurum* C. Presl. Wagner and Farrar (1976), in the most careful examination of the morphology of *Hyalotricha* to date, accepted its

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generic distinctness, and adduced strong evidence for its inclusion in subfamily Polypodioideae of the Polypodiaceae (rather than with the grammitid ferns, or Vittariaceae, or "Aspidiaceae"); however, Wagner and Farrar did not speculate on the generic level affinities of *Hyalotricha*, nor did they mention *Campyloneurum* as a possible relative. The later genus includes about 50 species occurring throughout South America, Mesoamerica, the Caribbean, to Florida in the North (Tryon and Tryon, 1982a, b; León, 1992, 1995).

Most recently, American authors have regarded *Hyalotrichopteris anetioides* (= *Campyloneurum anetioides* (H. Christ) R. M. Tryon & A. F. Tryon) either as a member of *Campyloneurum* (Tryon and Tryon, 1982a, b; León, 1992, 1995), or have accepted its generic distinctness as *Hyalotrichopteris* (Wagner, 1978; Lellinger, 1988, 1989). Lellinger (1988, 1989) considered *Hyalotrichopteris* a satellite of *Campyloneurum*, based on characters believed to be rare in the latter genus, namely, the small (not exceeding 10 cm) spathulate leaves, a single excurrent free veinlet in each areole, marginal free veinlets, and multicellular branched hairs *Hyalotrichopteris* (Lellinger, 1988). In her revision of *Campyloneurum*, León (1992, 1995) noted that these supposed unique features in *Hyalotrichopteris* are shared by species of *Campyloneurum*. Relatively small leaves (but still considerably larger than in *Hyalotrichopteris*) are found in *C. chrysopodum* (Klotzsch) Fée and *C. falcoideum* (Kuhn *ex* Hieron.) M. Meyer *ex* Lellinger, whereas branched multicellular hairs occur also in *C. aphanophlebium* (Kunze) T. Moore and *C. repens* (Aubl.) C. Presl. León considered the presence of *Campyloneurum*-like venation in *Hyalotrichopteris anetioides* as a critical character supporting close relationships of this species to *Campyloneurum*. Excurrent free veinlets along the blade margins occur in several species of *Campyloneurum* such as *C. brevifolium* (Lodd. *ex* Link) Link, as well as in juvenile fronds of larger species, and excurrent veinlets within non-costal areoles occur in *C. aphanophlebium* and *C. falcoideum*. Based on the shared morphological features, León (1992) considered *C. aphanophlebium* as the putative closest extant species to *Hyalotrichopteris/Campyloneurum anetioides*.

The introduction of DNA sequence data as a further marker to discover relationships has greatly improved our understanding of the phylogeny of polygrammoid ferns, as well as other groups of ferns (Schneider *et al.*, 2004a, b). These studies have resolved many uncertainties about the interpretation of relationships and the underlying morphological evidence. As an example, an extensive study on the grammitids found strong support for several genera that were recently proposed based on morphological evidence, which appears to be remarkable considering the high degree of homoplasy in these ferns (Ranker *et al.*, 2004). Other genera such as *Microsorum* Link and *Polypodium* L. were found to be polyphyletic as currently defined (Schneider *et al.* 2004a, b, 2006a, b; Smith *et al.*, 2006). Using nucleotide sequence variation of the chloroplast genome (cpDNA), relationships of several enigmatic polypod genera have recently been discovered, the Himalayan genus *Gymnogrammitis* Griff. (Schneider *et al.*, 2002), Malesian *Thylacopteris* Kunze *ex* J.Sm. (Schneider

*et al.*, 2004a), and southern South American *Synammia* C. Presl (Schneider *et al.*, 2006b). cpDNA data (Janssen and Schneider, 2005) have also confirmed the generic concept of *Aglaomorpha* Schott proposed by Roos (1985), who utilized exclusively morphological evidence.

Existing studies on polypod ferns (Polypodiaceae) have included one or more samples of *Campyloneurum*, and almost all other genera in the family, but sequence data has been unavailable for *Hyalotrichopteris anetioides*. In this study, we explore the relationships of this narrowly distributed Costa Rican and Panamanian species using cpDNA data.

## Materials and Methods

A sample of the rare *Hyalotrichopteris anetioides* was collected by one of us (A.F.R.A.) in Costa Rica. All samples representing other species of *Campyloneurum* or other genera included were collected either in Botanical Gardens or provided by colleagues. Some sequences were obtained from Genbank (see Table 1 for accession numbers). The sampling strategy follows the phylogenetic hypotheses outlined in previous studies (Schneider *et al.*, 2004a, b, 2006a, b). Besides *H. anetioides*, we collected sequences for eight species of *Campyloneurum*, one species of *Niphidium* (sister genus of *Campyloneurum*) and seven species of *Microgramma*, the sister to the clade comprising *Campyloneurum* and *Niphidium*. *Microgramma* was assigned as the outgroup clade. Table 1 gives information on vouchers and Genbank accession numbers. Sequences of the $trnL_{UAA}$-$trnF_{GAA}$ intergenic spacer (*trnL-F* IGS) region were obtained using primers and protocols described in previous studies (Haufler *et al.*, 2003; Schneider *et al.*, 2004a, b; Janssen and Schneider, 2005). Sequencing was carried out on a MegaBACE 1000 capillary sequencer using DYEnamic ET Primer DNA Sequencing Reagent (Amersham Biosciences, UK). All sequences were assembled and manually aligned using TreV (Staden Package, http://sourceforge.net/projects/staden). The final alignment was adjusted manually in MacClade 4.0 (Maddison and Maddison, 2000). Ambiguously aligned regions were excluded from all analyses. The few scattered indels did not include any phylogenetic information concerning the relationships of *H. anatioides*.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed with PAUP* version 4.0b10 (Swofford, 2000). Model and parameters were selected using the hierarchical likelihood ratio test and the Akaike information criterion as implemented in Modeltest (Posada and Crandall, 1998). Selected model and parameters were implemented in PAUP before conducting ML analysis. MP and ML analyses were calculated using the heuristic mode with 1000 (MP) and 100 (ML) random-addition-sequence replicates, TBR branch swapping, and MULTREES on. Bootstrap values were calculated for both (MP and ML) with 1000 bootstrap replicates, each with 10 random-addition-sequence replicates, TBR branch swapping and MULTREES on. ML analyses were also performed using PHYML (Guindon and Gascuel, 2003; Guindon *et al.*, 2005). In addition, we explored the robustness of the

TABLE 1. Taxon sampling: For each taxon the following information is given: voucher information including herbarium of deposition, accession number in the fern DNA database (http://www.pryerlab.net), and Genbank accession numbers

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***Campyloneurum*** C. Presl
*Campyloneurum anetioides* (H. Christ) R. M. Tryon & A. F. Tryon: Costa Rica; *Rojas 6281* (CR); **EF104510** – *Campyloneurum angustifolium* (Sw.) Fée: Costa Rica; *Chisaki & Carter 1004* (UC 1618523); **AY083647** – *Campyloneurum aphanophlebium* (Kunze) T. Moore: Bolivia; *Acebey 772* (UC 1735546); **EF104511** – *Campyloneurum asplundii* (C. Chr.) Ching: Mexico; *Kessler s.n.* (GOET); **EF104512** – *Campyloneurum brevifolium* (Lodd. *ex* Link) Link: cult. Göttingen, Old Bot. Garden; *Kreier s.n.* (GOET); **EF104513** – *Campyloneurum chlorolepis* Alston: Venezuela; *Smith 1159* (UC 1487590); **AY083648** – *Campyloneurum phyllitidis* (L.) C. Presl: cult. Berlin, Bot. Garden (Acc. 017-59-74-63); *Schuettpelz 612* (GOET); **EF104514** – *Campyloneurum sphenodes* (Kunze *ex* Klotzsch) Fée: Costa Rica; *Horich s.n.* (UC 1617915); **AY083649** – *Campyloneurum xalapense* Fée: (1) cult. Göttingen, Old Bot. Garden; *Kreier s.n.* (GOET); EF104515 & (2) Mexico; *Lautner L02-41* (GOET); **EF104516**.
***Niphidium*** J. Sm.
*Niphidium nidulare* (Rosenst.) Lellinger: Costa Rica; *Ranker 1831* (COLO); **EF104519**.
Microgramma *C. Presl*
*Microgramma bifrons* (Hook.) Lellinger: Peru; *van der Werff 18062* (MO); **DQ642224** – *Microgramma latevagans* (Maxon & C. Chr.) Lellinger: Bolivia; *Jimenez 1285* (LPB); **EF104517** – *Microgramma mauritiana* (Willd.) Tardieu:m (1) cult. Zürich Bot. Garden; *Kreier s.n.* (GOET); DQ642225 & (2) cult. Zürich Bot. Garden; *Kreier s.n.* (GOET); **DQ642226** – *Microgramma nitida* (J. Sm.) A. R. Sm.: Mexico; *Krömer 2678* (GOET); **EF104518** – *Microgramma squamulosa* (Kaulf.) de la Sota: cult. Zürich Bot. Garden; *Kreier s.n.* (GOET); **DQ642228** – *Microgramma tecta* (Kaulf.) Alston: cult. RBG Edinburgh (Acc. 19875234); *Schneider s.n.* (E); **DQ642230** – *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel.: cult. Charles Alford; *Kreier s.n.* (GOET); **DQ642231**

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results using the neighbor net algorithm as implemented in Splitstree 4 (Huson and Bryant, 2006), using different settings to evaluate the robustness of the analyses (e.g., different distance measures such as LogDets and number of dimensions calculated).

Analyses were performed in two steps. In the first step, we compared the *trnL-F* sequence of *Hyalotrichopteris anetioides* with all currently available sequences of this cpDNA region available in GenBank and with additional available data. This comparison was made using Blastn (Altschul *et al.*, 1990) and by performing maximum parsimony analyses and maximum likelihood analyses with all available data. In the second step, we inferred the relationships of this species with a data set including only *Campyloneurum*, *Microgramma*, and *Niphidium* species. The later dataset was inferred employing MP, ML and neighbor net analyses.

## RESULTS

A sequence of 342 base pairs was generated for *H. anetioides*, which was in the range of length variation (ca. 340 to 370 bp) currently known for the *trnL-F* IGS in *Campyloneuron* and relatives. The data set consisted of 230 included characters of which 37 were parsimonious informative and 35 were variable but non-parsimonious informative. The number of informative sites dropped

to 16 considering only *Campyloneurum* and *H. anetioides*. Both Blastn and the maximum parsimony analyses using all available *trnL-F* IGS sequences of Polypodiaceae indicated *Hyalotrichopteris anetioides* as a member of *Campyloneurum* (results not shown). Phylogenetic reconstructions using maximum parsimony, maximum likelihood, and network approaches found this species embedded in *Campyloneurum* (Figs. 1, 2). The maximum parsimony analysis resulted in 45 most parsimonious trees with a length of 96 steps (consistency index [CI] = 0.7288, homoplasy index [HI] = 0.2712, retention index [RI] = 0.8730, and rescaled consistency index [RC] = 0.7275). *Campyloneurum* was monophyletic in all 45 most parsimonious trees, and *Hyalotrichopteris anetioides* was sister to *Campyloneurum angustifolium* in 89% of 45 most parsimonious trees. A single unambiguous character state change separated *H. anetioides* from *C. angustifolium* and in turn one unambiguous character state change characterized this clade. The *Campyloneurum* clade had a bootstrap value of 98% (Fig. 1) and the clade was characterized by six unambiguous character state changes. Only two well supported clades were found in all most parsimonious trees: a clade comprising the two collections of *C. xalapense* and a clade consisting of *C. asplundii* and *C. chlorolepis*). Maximum likelihood analysis found a single tree (Fig. 1) with -ln = 834.16001, using the TVM model with freqA = 0.3331, freqC = 0.1636, freqG = 0.1842, freqT = 0.3190, R(a) = 1.9602, R(b) = 3.3907, R(c) = 0.0435, R(d) = 1.3489, R(e) = 3.3987, R(f) = 1.000. Based on the hierarchical likelihood ratio test, this model and parameters were best, whereas the Akaike information criterion preferred a slightly different model TVM + I. Analyses with the alternative model found the same topology.

The ML tree was fully resolved, but bootstrap values >75% were found only for clades present in the strict consensus tree of the most parsimonious trees obtained in the maximum parsimony analysis. *Hyalotrichopteris anetioides* was found to be sister to *C. angustifolium* (Fig. 1), but alternative relationships were found in the Splitgraph analysis (Fig. 2).

## Discussion

*Status of* Hyalotrichopteris anetioides.—Our data suggest that *Hyalotrichopteris anetioides* nests within *Campyloneurum*. To separate it generically renders *Campyloneurum* paraphyletic. *Hyalotrichopteris anetioides* shares with species of *Campyloneurum* the principal characters defining the genus, namely areolate venation with costal areoles containing one excurrent free veinlet and non-costal areoles containing (1–)2–5 excurrent free veinlets (León, 1992). Characters used by Lellinger (1988, 1989) to separate *Hyalotrichopteris* are either not restricted to *H. anetioides*, such as the branched multicellular hairs (which also occur in *Campyloneurum aphanophlebium*), or are unreliable, such as frond size and habitat. The unusually large number of marginal excurrent free veinlets, which is uncommon in *Campyloneurum* in general, may correlate with the reduction in leaf size.

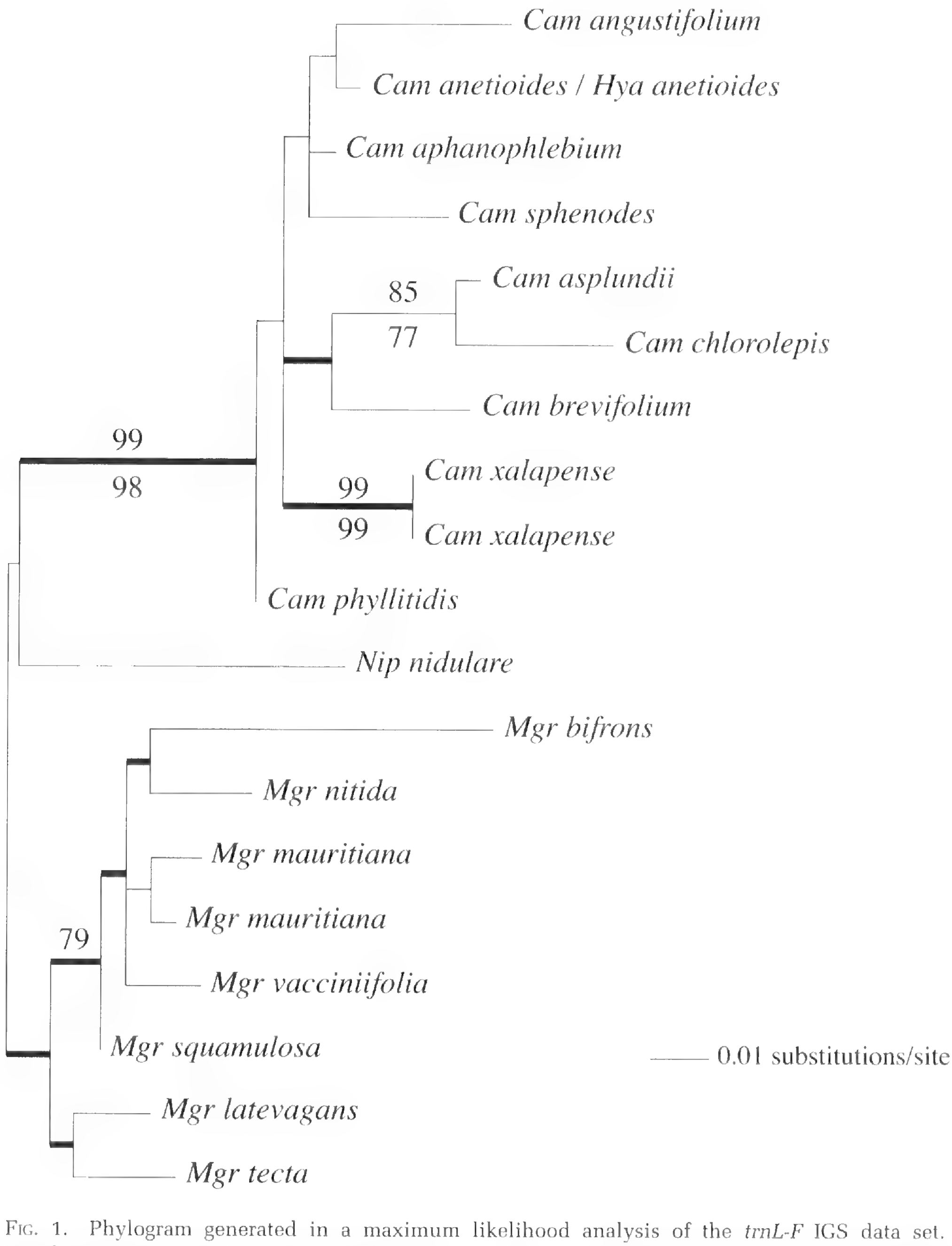


FIG. 1. Phylogram generated in a maximum likelihood analysis of the *trnL-F* IGS data set. Numbers above branches correspond to ML bootstrap values, and numbers below branches correspond to MP bootstrap values. BS values ≥ 75% are given. Thickened lines indicate branches present in the strict consensus of 45 most parsimonious trees. Abbreviations: *Cam* = *Campyloneurum*, *Hya* = *Hyalotrichopteris*, *Mgr* = *Microgramma*, *Nip* = *Niphidium*.

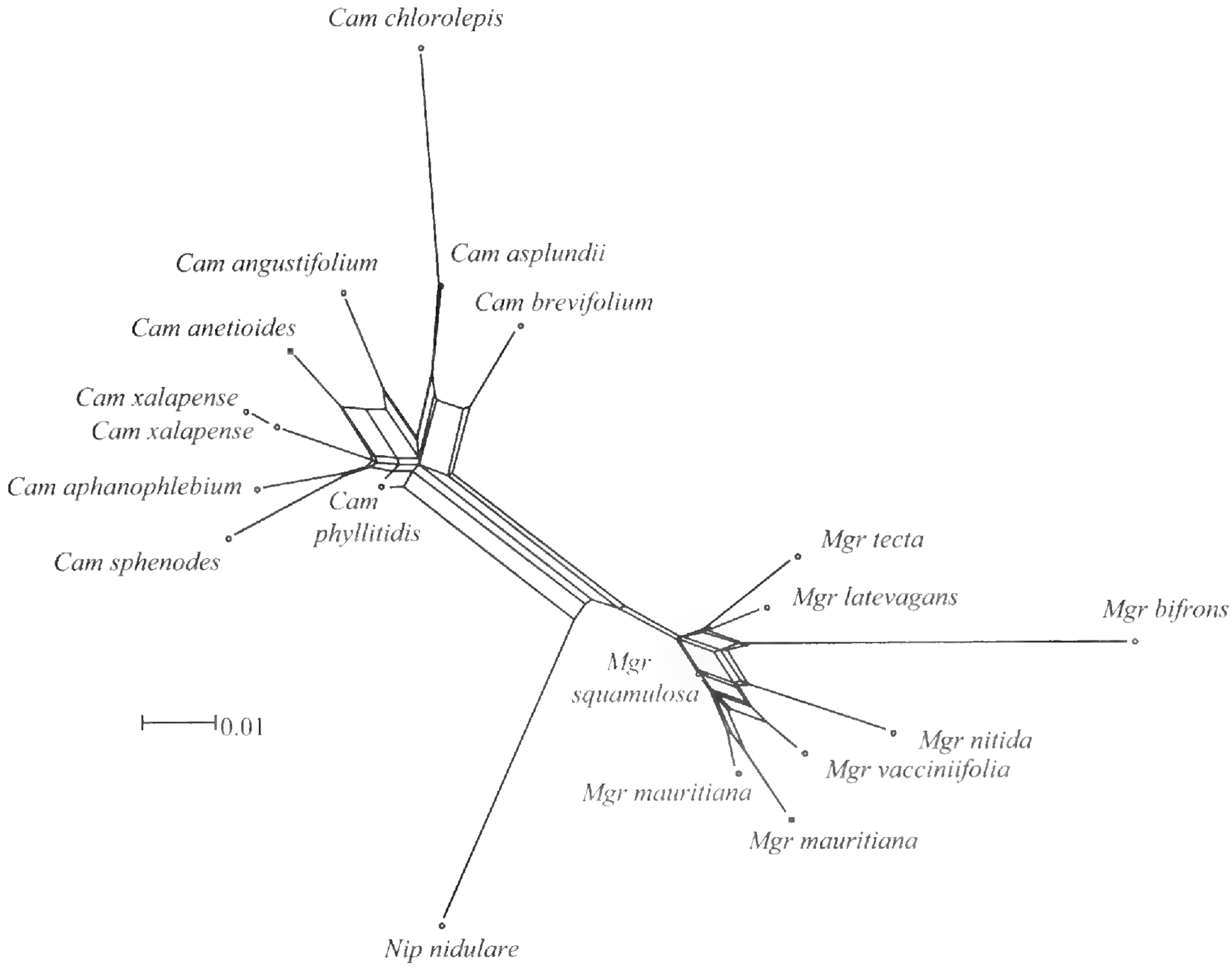


FIG. 2. Splitstree generated with the Neighbornet algorithm and uncorrected P-distances. Settings: weight modified as least squares, and maximum dimensions set to four. Statistics; nsSplits = 45, total weight = 0.40393516. Abbreviations as in Fig. 1.

*Relationships within* Campyloneurum.—León (1992) tentatively divided *Campyloneurum*, a genus of ca. 50 species, into ten groups, of which six are represented in our study. She placed *C. anetioides* in the *C. aphanophlebium* group, comprising only these two species. This group is characterized by having specialized branched, multicellular hairs. Further shared characteristics are the presence of undivided, primary, non-costal areoles, free veinlets along the margin, and medial sori.

Our results (Fig. 1) suggest that there is some affinity between *Campyloneurum anetioides* and *C. aphanophlebium*, but other species such as *C. angustifolium* may have closer relationships. Our current taxon sampling is insufficient to explore the relationships among species of the genus *Campyloneurum* as demonstrated in the low resolution in our maximum parsimony analyses and the many alternative topologies in neighbor net analyses. We cannot draw any conclusions about the monophyly of some proposed groups within the genus *Campyloneurum* and/or the species delimitation of some putative closely related species in the *C. angustifolium* group. These questions require a much denser taxonomic sampling. This restriction also applies to the position of taxa at the earliest split within the

genus, although *C. phyllitidis* is not only the sister to the remaining genus but also shows similarities in its morphology with the sister genus *Niphidium* such as prominent costa and veins on the abaxial leaf surface, coriaceous blades, and erect fronds. Our data set contains only 36 parsimony-informative sites from a total of 230 included characters. This low percentage (about 16%) of informative sites may indicate that *Campyloneurum* has diverged and radiated relatively recently. Similar low percentage of informative and/or variable sites were reported in other studies employing the *trnL-F* IGS region to elucidate relationships between closely related species of Polypodiaceae (Haufler *et al.*, 2003; Janssen and Schneider, 2005; Schneider *et al.*, 2006a, b; Smith *et al.*, 2006) and Aspleniaceae (Schneider *et al.*, 2005). In the Aspleniaceae study, divergence times were estimated for a lineage of asplenioid ferns (Schneider *et al.*, 2005) and the results indicated a diversification in the Miocene corresponding to a level of sequence divergence as observed in *Campyloneurum*. The exact timing of this genus diversification is currently unknown but the distribution of the genus suggests putative correlations with geological and climatic changes in the upper Tertiary shaping the extant plant diversity of South and Central America. Future studies require not only denser sampling of species within the genus and but also sampling of more variable markers.

## Acknowledgments

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# *Thelypteris tuxtlensis* (Thelypteridaceae), a New Species in Subgenus *Goniopteris* from Los Tuxtlas, Veracruz, Mexico

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ABSTRACT.—We describe and illustrate *Thelypteris tuxtlensis*, a new species in subgenus *Goniopteris* (Thelypteridaceae), from the biogeographic region of Los Tuxtlas, in the state of Veracruz, Mexico. This species appears to be most closely related to *T. hatchii* and *T. biolleyi*.

The genus *Thelypteris* (Thelypteridaceae) is distributed pantropically, and the family, as treated by Smith (1990), comprises about 1000 species, with about 350 in the Neotropics. Distinguishing characters of *Thelypteris* in its broadest sense (all species in the family) include the stipe vasculature of two bundles, acicular hairs on many parts of the fronds, usually bilateral spores with a prominent perispore, and chromosome base numbers from 27 to 36. *Thelypteris s.l.* has been subdivided by pteridologists into about 30 supposedly natural groups variously treated as genera, subgenera, or sections. In Mexico, *Thelypteris s.l.* includes the segregate genera *Amauropelta*, *Goniopteris*, *Meniscium*, *Stegnogramma*, and *Steiropteris* (Mickel and Smith, 2004). With only a few exceptions, subg. *Goniopteris*, with about 100 species, differs from all other subgenera of *Thelypteris* in the presence of forked or stellate hairs on some part of the blade or on the rhizome scales. *Goniopteris* is mainly restricted to low-and middle-elevation rain forests in the New World tropics and subtropics from Florida, the Antilles, and central Mexico to Bolivia and northeastern Argentina and Paraguay. Until now, 24 species have been found in Mexico, including 16 species in the state of Veracruz. Thirteen species of subg. *Goniopteris* are either entirely or preponderantly Mexican; thus, eastern and southern Mexico, along with the Greater Antilles, the Andes, and southern Brazil, can be considered principal centers of diversity within this subgenus.

During the field work for a current research project, T. Krömer and A. Acebey collected five specimens of *Thelypteris* on the slopes of the San Martín

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Tuxtla volcano, located in the northeastern region of Los Tuxtlas, in the state of Veracruz. This material could not be assigned to any known species reported from Mexico, Mesoamerica, or the Antilles.

***Thelypteris tuxtlensis*** T. Krömer, A. Acebey & A. R. Sm., ***sp. nov.*** TYPE.—MEXICO. **Veracruz:** Mpio. San Andrés Tuxtla, ejido Barrio Lerdo, faldas del volcán San Martín Tuxtla, 1000 m, 18°34′N, 95°10′W, 11 Aug 2005, *T. Krömer & A. Acebey 2475* (holotype: MEXU; Isotypes: UC, XAL). **Fig. 1.**

*Thelypteridi hatchii* affinis, a qua imprimis differt indusiis persistentibus grandioribus, 0.5–1.0 mm diam., et pilis interveniis acicularibus patentibus, vel aliquot furcatis, nec stellatis nec adpressis.

Terrestrial; rhizomes erect or suberect, caudices to 15 × 3 cm; fronds monomorphic, or nearly so; rhizome scales brown, to 7 × 1.5 mm, glabrous or with sparse acicular and furcate hairs to 0.1 mm long; stipes stramineous to brownish, ca. 28–50 cm × 2.5–5.5 mm, puberulous with dense stalked-stellate hairs, ca. 0.1–0.2 mm long; blades dark green, chartaceous, 1 pinnate-pinnatifid, 37–57 × 25–31(–50) cm; buds lacking; pinnae 12–16 lateral pairs and a subsimilar to nearly conform terminal one, 11–16 (–25) × 1.6–3.1 cm, incised ca. 0.4–0.6 the way to costae, sessile or the proximal ones stalked 1–2 mm, proximal 1–3 pairs deflexed and slightly to strongly narrowed towards their bases, also slightly shortened; segments oblique, subfalcate (3.5–)4–6(–7) mm wide, acutish to obtuse at tips, basal 1–3 pairs on proximal pinnae shortened; veins 8–13 pairs per segment, proximal 2 pairs from adjacent segments united below sinuses or connivent with a common vein leading to sinuses; indument abaxially of whitish furcate or stellate hairs mostly 0.1–0.2 mm long on costae and veins, densest along costae, also with some acicular hairs 0.3–0.7 mm, tissue between veins with scattered, mostly acicular hairs 0.1 mm, adaxially the blades similarly hairy but hairs less dense along costae and veins and with longer acicular hairs 0.8–1 mm, rachises with dense furcate or stellate hairs; sori supramedial, with whitish, persistent, round-reniform, marginally setose indusia, hairs mostly acicular, a few furcate, also with a few hairs on indusial surface; sporangia glabrous.

PARATYPES.—MEXICO. **Veracruz:** Mpio. San Andrés Tuxtla, ejido 1° de Mayo, faldas del volcán San Martín Tuxtla, 920 m, 18°33′N, 95°13′ W, 2 May 2005, *Krömer & Acebey 2063* (MEXU, UC); ejido Morelos, faldas del volcán San Martín Tuxtla, camino hacia la cruz, 1000 m, 18°33′N, 95°12′W, 4 May 2005, *Krömer & Acebey 2113* (MEXU, UC); faldas del volcán San Martín Tuxtla, arriba de la cruz, 1010 m, 18°33′N, 95°12′ W, 5 May 2005, *Krömer, Acebey & Velasco Sinaca 2135* (MEXU, UC); ejido Emiliano Zapata, terracería del ejido Ruiz Cortínez hacia El Diamante, faldas del volcán San Martín Tuxtla, 1100 m, 18°33′N, 95°09′W, 23 Jul 2005, *Krömer, Acebey & Pérez Peña 2320* (MEXU, UC, XAL).

*Thelypteris tuxtlensis* appears most closely related to *T. hatchii* A. R. Sm., known from southern Mexico, Guatemala, Honduras, and Costa Rica. The

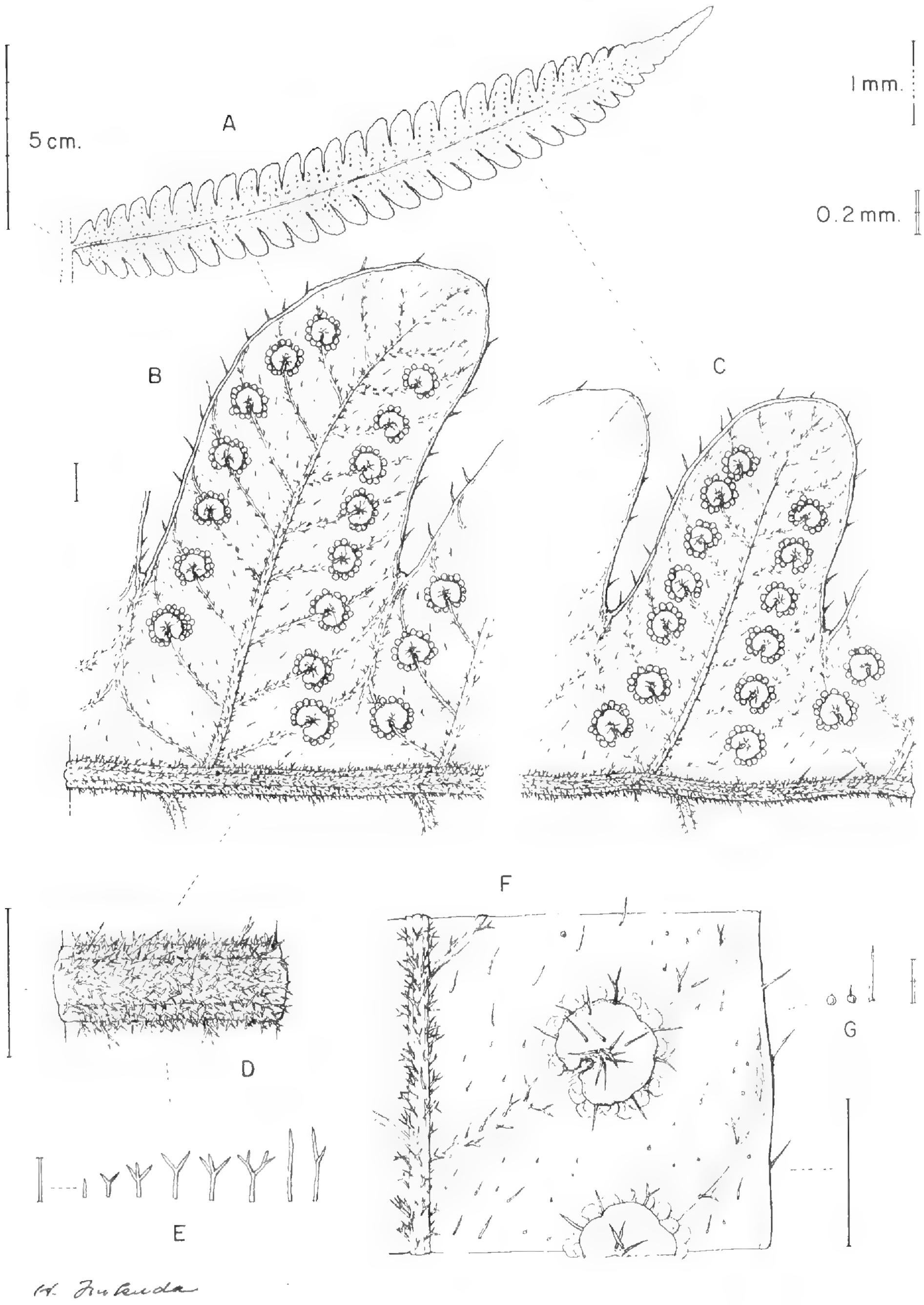


FIG. 1. *Thelypteris tuxtlensis*. **A.** Pinna, third from base of blade. **B.** Detail of abaxial surface, showing venation (anastomosing veins below sinuses), sori, and indument. **C.** Detail of abaxial surface, showing venation (veins connivent near the sinus), sori, and indument. **D.** Costa, detail showing stellate and furcate hairs. **E.** Simple, stellate, and furcate hairs, abaxial surface of costa. **F.** Detail of sori, showing indusial hairs (simple and a few furcate) and indument between veins, of simple hairs and a few pustules. **G.** Laminar pustules, some with reduced hairs (all from *Krömer & Acebey 2063*, UC).

latter species differs in having smaller indusia and appressed, sessile, stellate hairs on both sides of the laminae between the veins (such hairs lacking between the veins in *T. tuxtlensis*). Another similar species is *T. biolleyi* (Christ) Proctor, which bears sessile, stellate hairs on the laminae between veins (both sides of blade), as well as erect, anchor-shaped hairs on the costae and costules abaxially. The persistent indusia in *T. tuxtlensis* are larger than in any other species of subg. *Goniopteris* in Mexico except *T. paucipinnata* (Donn. Sm. & C. F. Reed) and *T. schaffneri* (Fée) C. F. Reed, both relatively narrow endemics in eastern and southern Mexico (the distribution of *T. paucipinnata* extends into Guatemala and Belize). From *T. schaffneri*, *T. tuxtlensis* differs in having sessile or short-stalked pinnae, 12–16 lateral pinna pairs (vs. 3–7 pairs), fewer pairs of veins per segment (8–13 vs. 15–18 pairs), a less distinct terminal pinna, and abaxially in having abundant furcate or stellate hairs on costae, costules, and veins (hairs mostly acicular in *T. schaffneri*). From *T. paucipinnata*, *T. tuxtlensis* differs in the greater number of pinna pairs, the lack of buds on the rachis, the much more pubescent blades (virtually glabrous in *T. paucipinnata*), the non-verrucose blades, the supramedial (vs. submarginal) sori, and the glabrous indusia.

*Thelypteris tuxtlensis* is endemic to the biogeographic region of Los Tuxtlas, in the state of Veracruz, Mexico. This species is a locally common terrestrial herb in the shady understory of the lower montane forest of the San Martín Tuxtla volcano between 920–1360 m, where it co-occurs with other ferns, such as *Asplenium auriculatum* Sw., *A. cuspidatum* Lam., *Dennstaedtia bipinnata* (Cav.) Maxon, and *Pteris orizabae* M. Martens & Galeotti. In this area, the forest in general is mostly undisturbed and somewhat protected as part of the core zone of the "Reserva de la Biosfera Los Tuxtlas"; thus, we believe that the populations of *T. tuxtlensis* do not suffer severe anthropogenic pressures. However, the additional discovery of three new fern records (*Hymenophyllum lanatum* Fée, *Selaginella guatemalensis* Baker, and *Trichomanes ovale* (E. Fourn.) Wess. Boer) for the state of Veracruz at the same general locality demonstrates that more inventories are needed since plant diversity in Los Tuxtlas is still threatened by the transformation of primary forest into pastures and plantations.

## Acknowledgments

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# Typification and Relationships of *Cheilanthes incisa* (Pteridaceae)

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Abstract.—*Cheilanthes incisa*, a small and rare endemic fern of Rio de Janeiro State (Brazil), was described in 1859. *Hypolepis serrata*, published ten years later, is usually considered a taxonomic synonym. Both names have typification problems, addressed here by designation of a neotype for *C. incisa* and selection of a lectotype of *H. serrata* from among Glaziou materials cited by Fée. Epidermal characteristics and spores of *C. incisa* are described for the first time and their diagnostic value assessed. Taxonomic relationships between *C. incisa* and putatively related cheilanthoid ferns are also discussed.

During preparation of the pteridophyte Catalog of the Southern Cone of South America we detected taxonomic and typification problems involving *Cheilanthes incisa* Kunze *ex* Mett. and *Hypolepis serrata* Fée. According to Mynssen & Windisch (2002), *Cheilanthes incisa* is a small fern endemic to Rio de Janeiro State, Brazil. *Hypolepis serrata*, described by Fée (1869) in the *Cryptogames vasculaire du Brésil v. 1*, is very similar morphologically and was considered a synonym of *C. incisa* by Baker (1872). Both names have typification problems. The protologue of *C. incisa* mentions one specimen (without collector and now lost) from Serra da Estrella, Rio de Janeiro. The original description of *H. serrata* cited two syntypes (*Glaziou 929* and *2336*) and requires selection of a lectotype to stabilize application of the name.

According to Mettenius (1859), *Cheilanthes incisa* is closely related to *C. californica* (Hook.) Mett. and *C. schimperi* Kunze because all three species have dentate segments. Most recent treatments (Lellinger, 1968; Smith, 1975; Mickel and Smith, 2004) have assigned *C. californica* to *Aspidotis* (Nutt *ex* Hook.) Copel., a small genus confined of the western USA and Mexico. *Aspidotis* can be recognized by dentate-mucronate segments and striate lamina. *Cheilanthes schimperi* has a paleotropical distribution (Africa and Madagascar) and it was included in *Aspidotis* by Pichi-Sermolli (1950) because of its similar morphology.

In this paper we review the typification of *Cheilanthes incisa* and *Hypolepis serrata* and discuss the similarities and differences between *C. incisa* and related species based on the morphology of the sporophyte and spores.

## Materials and Methods

To clarify the typification of *Cheilathes incisa* and *Hypolepis serrata* we examined materials from B, HBG, K, LE, R, RB, and P. The following specimens from K, MO, UC, RB, and SI were used for studies of spore and sporophyte morphology.

***Aspidotis californica*** (Hook.) Nutt. *ex* Copel.

Specimens studied.—UNITED STATES. **California:** Butte County, in mosses in rock crevices, embankment of Macabes Creek, ca. 1300 ft., 19 Feb 1980, *M. S. Taylor 2393* (MO); Tehama County, Paynes Creek Recreation Area located ca. 1.5 mi northeast of the Bend Bridge over the Sacramento River, ca. 6.5 miles north-northeast of Red Bluff, occasional in crevices in basalt outcrops in *Quercus wislizenii-Pinus sabiniana* woodland, 9 May 1991, *V. H. Oswald & L. Ahart 4564* (MO); Butte County, along trail to Feather Falls, ca. 5 mi N of the town of Feather Falls, yellow pine forest, granite rocks, 600 m, 28 May 1983, *L. Ahart, E. Ahart & M. Baer 4023* (MO); Devils Kitchen, Upper Bidell Park, 900 ft., dry crevices of rocks cliffs, basaltic cliffs, 14 Jan 1983, *M. S. Taylor 5213* (MO); Santa Catalina Island, Avalon, cañon, 1897, *B. Trask 5* (MO).

***Aspidotis densa*** (Brack.) Lellinger

Specimens studied.—UNITED STATES. **California:** 10 Apr 1934, *L. L. Brown 1* (MO); Tulare County, Sequoia National Park, between Franklin Pass and Kern River, 8400 ft., crevices among granite boulders, trail, 23 Jul 1942, *R. S. Ferris & L. Lorraine 10792* (MO); Del Norte County, Mill Creek, 27 Jun-1 Jul 1922, *L. R. Abrams 8423* (MO); Mts. about the head waters of Sacramento River, rocky places, 3Sep 1882, *C. G. Pringle 2778* (MO).

***Aspidotis meifolia*** (D. C. Eaton) Pic. Serm.

Specimens studied.—MEXICO. **San Luis Potosí**: near Los Canos, 15–21 Oct 1902, *E. Palmer 264* (MO); **Nuevo León**: Sierra Madre above Monterrey, 3000 ft., shaded banks, 8 Jun 1888, *C. G. Pringle 1987* (MO), 27 Aug 1903, *C. G. Pringle 11778* (MO, SI); **Coahuila**: Mun. Muzquiz, Rancho Agua Dulce, wooded canyon on the east slope of the Sierra de San Manuel, 28 Jun 1936, *F. Lyle Wynd & C. H. Mueller 350* (MO). Tamaulipas, oak forest on Jaumave road about 13 miles southwest of Ciudad Victoria, rough limestone mountainsides near summits of Sierra Madre, ca. 100 m, abundant, 13 May 1949, *R. McVaugh 10516* (MO).

***Cheilanthes incisa*** Kunze *ex* Mett.

Specimens studied.—BRAZIL. **Rio de Janeiro**: Mangaratiba, Reserva Ecológica Rio das Pedras, trilha Toca da Aranha, 350 m, local sombrio e úmido na mata densa de encosta, rupícola, ciófila, 4 Nov 1997, *J. M. A. Braga et al. 4427* (RB); Rio de Janeiro, Jan 1881, *A. Glaziou 12289* (K); Sierra dos Orgãos, Dec 1891, *Ule 17929* (UC); "Brasilia", *Riedel 1822* (K).

***Cheilanthes schimperi*** Kunze

SPECIMENS STUDIED.—MALAWI. Southern, Mt. Mulanje foot - Likabula Valley, 900 m, little fern, up to 30 cm tall, rhizome surrounded by a dense mass of roots, localized on shady road bank, nearby are *Actiniopteris dimorpha*, *Adiantum phillipense*, *Pellaea* spp., 20 Feb 1989, *J. D. & E. G. Chapman 9498* (MO); North Region, Rumphi District, Livingstonia escarpment, 1000 m, growing on shady bank, 25 Jan 1986, *I. F. La Croix 3594* (MO). DEMOCRATIC REPUBLIC OF CONGO (ZAIRE). Katanga (Shaba Meridional) Rivière Mabondo Route Mupala Kindalo entre Ifunda & Malashi, 1290 m, termitière en forêt galerie, 4 Apr 1987, *A. Bodenghien & F. Malaisse 2324* (MO); Katanga (Shaba Meridional) Kintu 27°28′E 11°49′S 1250 m Termitière 1Feb 1985 *A. Bodenghien & F. Malaisse 46* (MO). ZAMBIA. Southern Prov., Mazabuka Distr., Kafue Gorge below Kafue Dam, 15°48′S, 28°25′E, 950 m, humid, shaded slope in woodland, 30 Dec 1971, *J. Kornás 6741* (MO).

*Morphological studies.*—Several fronds were bleached with NaOH (5%) and sodium hypochlorite solution (5%), stained and mounted in glycerin jelly. The observations and illustrations were made using the light microscope Wild M2O and stereomicroscope Willd M5 both with camera lucida drawing.

*Spores.*—Spores obtained from dry herbarium specimens in the list were studied by light microscopy (LM) and scanning electron microscopy (SEM). For LM the spores were treated with 3% hot sodium carbonate for 2 min and acetolysed according to Erdtman (1960). For SEM the material was treated with 3% hot sodium carbonate for 2 min, washed with distilled water, dehydrated, suspended in 96% ethanol, and then transferred to acetate plates. After drying these were coated with gold. All of the observations were performed with a BH2 light microscope and a JEOL JSMT-100 scanning electron microscope at the Facultad de Ciencias Naturales y Museo de La Plata.

## RESULTS

*Typification.*—The syntypes of *Hypolepis serrata* were located at B, HBG, K, and P. At K, among the specimens identified and cited by Baker in the *Flora Brasiliensis*, we found one specimen (*Glaziou 2336*) with two labels: one containing the name *H. serrata* and another with *C. incisa*. For Baker, the latter epithet was the correct name for this material. There is an isosyntype, *Glaziou 929*, in HBG but we could not study it; we only located a photograph in BM. Based on this information, we chose the authentic specimen, *Glaziou 2336*, that we had seen and studied at K and P, as the lectotype of *Hypolepis serrata*.

No authentic type material of *Cheilanthes incisa* or any other old annotated specimen by Mettenius or Kunze were found in the European herbaria. According to the TL-2 (Stafleu and Cowan, 1981) the types of species described by Mettenius and Kunze housed at LZ were destroyed during the Second World War. Thus, for *Cheilanthes incisa* we selected a neotype, which is from the same area and district as the original Glaziou. The taxonomic summary is as follows.


FIG. 1. At top. Lectotype of *Hypolepis serrata* Fée, *Glaziou 2336* (K). At bottom. Neotype of *Cheilanthes incisa* Kunze *ex* Mett., *A. C. Brade 16288* (duplicate at SI).

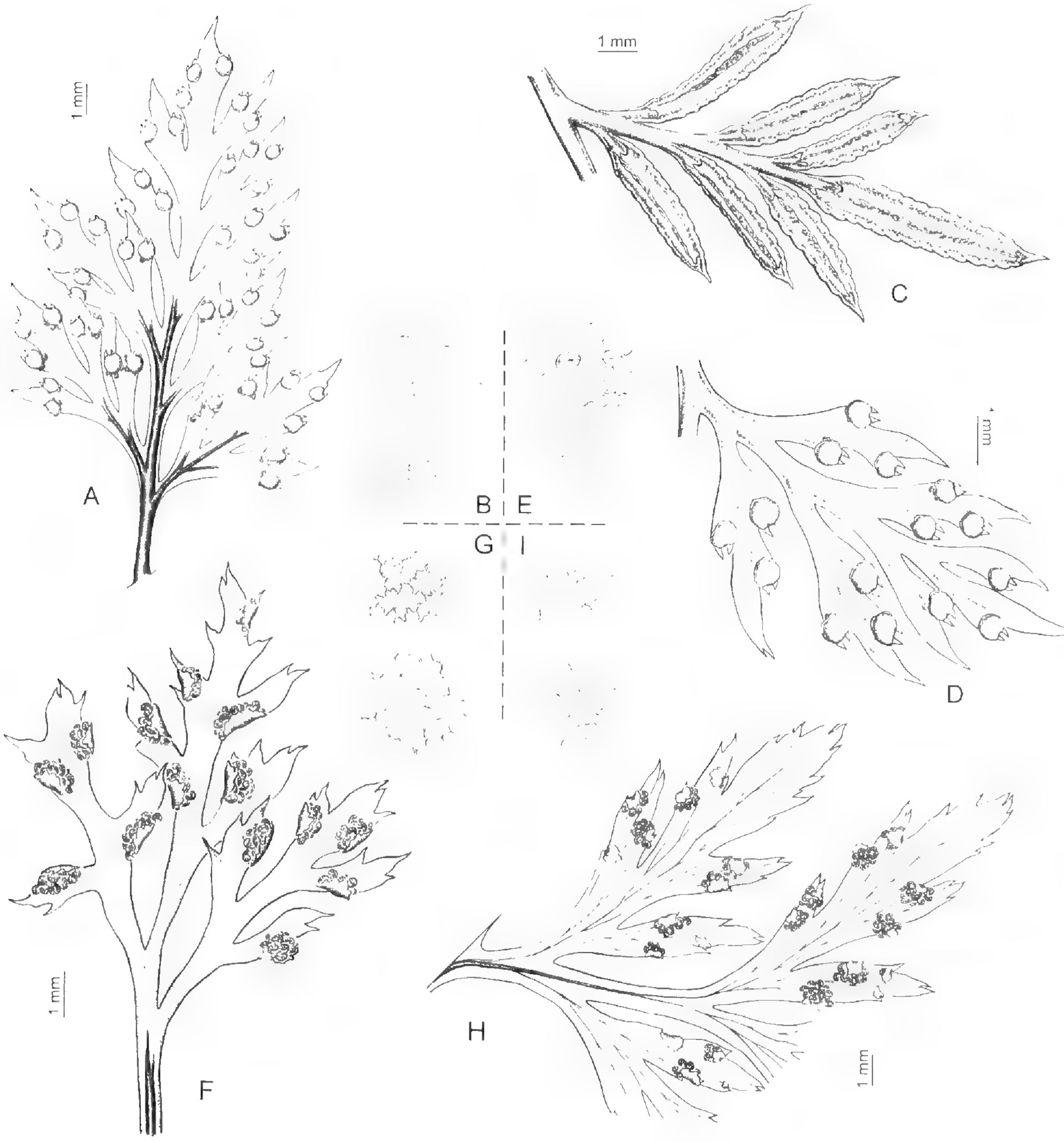


FIG. 2. Lamina and epidermal characteristics. A–B. *Aspidotis californica* (Hook.) Nutt. *ex* Copel. C. *Aspidotis densa* (Brack.) Lellinger. D–E. *Aspidotis meifolia* (D. C. Eaton) Pic. Serm. F–G. *Cheilanthes incisa* Kunze *ex* Mett. H–I. *Cheilanthes schimperi* Kunze.

***Cheilanthes incisa*** Kunze *ex* Mett., Cheil. 44 no. 65, tab. 3, fig. 28–31. 1859. Neotype (here designated): BRAZIL. **RIO DE JANEIRO:** Mun. Teresópolis, Grotão, 800 m, nas pedras, 13 Jun 1940, *A. C. Brade 16288* (RB, duplicate at SI).

***Hypolepis serrata*** Fée, Cr. vasc. Br. 1. 53, tab. 13, fig. 3. 1869. Lectotype (here designated): BRAZIL. **RIO DE JANEIRO:** *A. Glaziou 2336* (K; duplicates at B, P). (Fig. 1).

*Morphological characteristics and taxonomic affinities.—Aspidotis* (sensu Smith, 1975) is distinguished from most other cheilanthoid ferns by having striate lamina surfaces. This striation is formed by epidermal cells that are elongate and parallel with thickened walls. These aspects can be observed in

TABLE 1. Comparative spore characteristics of the studied taxa.

| | *Cheilanthes incisa* | *Aspidotis californica* | *Aspidotis densa* | *Aspidotis meifolia* | *Cheilanthes schimperi* |
|---|---|---|---|---|---|
| **Characteristics** | | | | | |
| Shape | subglobose, tetrahedral-globose | subglobose | globose | globose | globose |
| Color | light yellow | dark brown | brown, light brown | light brown | light brown |
| Equatorial diam. | 34–35 µm | 51–60 µm | 43–45 µm | 32–46 µm | 40–48 µm |
| Polar diam. | 27–34 µm | 45–48 µm | 40–50 µm | 34–39 µm | 36–58 µm |
| Perispore thickness | 1–2 µm | 2–3.5 µm | 1–3 µm | 2–3 µm | 3–4.5 µm |
| **Perispore Structure** | | | | | |
| Surface | rugulate, rugulate-cristate | rugulate-cristate | rugulate-cristate | reticulate, reticulate-cristate | cristate-reticulate |
| Cristae | 0.5 µm, isolated | 1.5 µm | 2 µm | 1–1.5 µm | 2–4 µm |
| Outer stratum | continuous | continuous | continuous | discontinuos | continuous |
| Middle stratum | interwoven threads | interwoven threads | interwoven threads | interwoven threads | interwoven trabeculae |
| Inner stratum | thin, continuous | thin, continuous | thin, continuous | thin, continuous | thin, continuous |

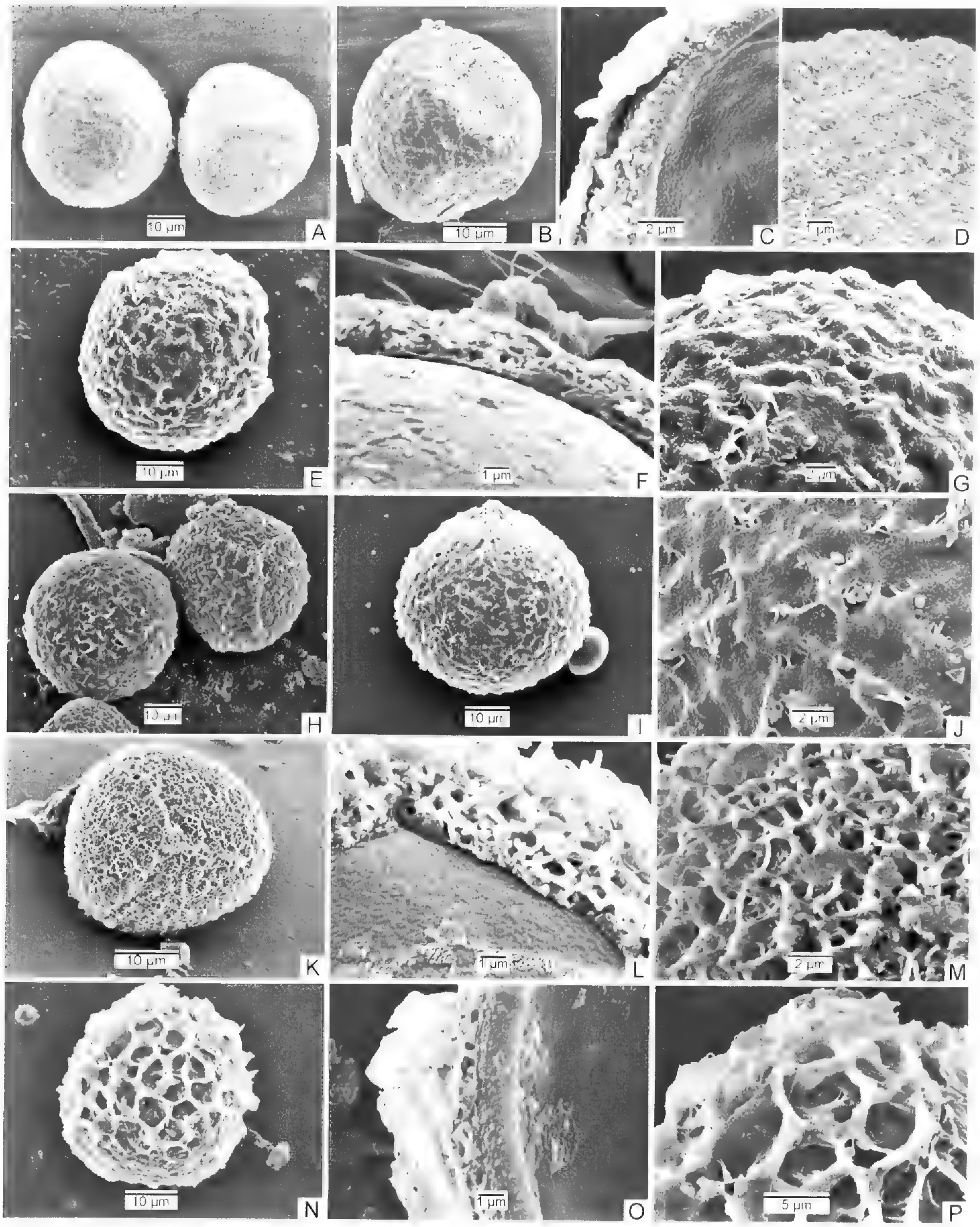


FIG. 3. Spores. A–D. *Cheilanthes incisa* Kunze *ex* Mett. A. Distal view. B. Equatorial view. C. Fracture of the sporodermis. D. Detail of the surface, *Braga et al.4427* (RB). E–G. *Aspidotis californica* (Hook.) Nutt. *ex* Copel. E. Distal view. F. Fracture of the sporodermis. G. Detail of the surface, *Oswald & Ahart 4564* (MO). H–J. *Aspidotis densa* (Brack.) Lellinger. H. Distal and proximal view. I. Equatorial view. J Detail of the surface, *Ferris & Lorraine 10792* (MO). K-M. *Aspidotis meifolia* (D. C. Eaton) Pic. Serm. K. Distal view. L. Fracture of the sporodermis. M Detail of the surface, *Wynd & Mueller 350* (MO). N–O. *Cheilanthes schimperi* Kunze. N. Distal view. O. Fracture of the sporodermis. P. Detail of the surface, *Bodenghieu & Mubala 2324* (MO).

*A. californica* (Hook.) Nutt. *ex* Copel., *A. densa* (Brack.) Lellinger, and *A. meifolia* (D. C. Eaton) Pic. Serm.(Fig. 2).

Although superficially similar to *Aspidotis* in the shape of the dentate-mucronate ultimate segments, *Cheilanthes incisa* and *C. schimperi* differ in having smooth (non-striate) laminae with sinuous, thin-walled epidermal cells and by the sori with lobate-laciniate pseudoindusia having erose or dentate margins (Fig. 2). Tryon and Tryon (1982) stated that the apparent morphological similarity of *C. schimperi* to the rest of their "*C. californica* Group" was due to morphological convergence, not to true relatedness, and they excluded *C. schimperi* from the group.

Other characteristics that distinguish *Cheilanthes incisa* from *Aspidotis* and *C. schimperi* are its erect rhizome and chartaceous or subcoriaceous lamina with hidden venation. *Cheilanthes incisa* occurs isolated in southeastern Brazil and no other species from Central and South America is morphologically similar to it.

*Cheilanthes schimperi* differs from *C. incisa* and *Aspidotis* species by its nodose rhizomes, membranaceous lamina with very thin mesophyll, and easily visible venation.

The spores of *Aspidotis* and *Cheilanthes* studied here are similar to the other cheilanthoid ferns such as *Argyrochosma* and *Pellaea*. The perispore with three layers is the main feature of the group (Morbelli and Michelena, 1989; Morbelli and Ponce, 1997; Morbelli *et al.*, 2001). The spore characteristics are summarized in Table 1 and SEMs of the spores are shown in Fig. 3. The studied material had some well-formed spores, but collapsed spores and spores intermediate between trilete and monolete were frequent.

## Discussion

Despite similarities between the dentate-mucronate lamina of *Cheilanthes incisa*, *C. schimperi* and *Aspidotis*, the former species lack the striate leaf tissue (formed by elongate epidermal cells with their thickened walls) and well-defined, scarious indusia characteristic of the latter genus. Our studies showed that the spores of *C. incisa* are slightly different from those of *Aspidotis* and *C. schimperi* as well. Although the palynological characteristics of the studied species are those of *Cheilanthes*, this genus is highly polymorphic (and polyphyletic; Smith *et al.*, 2006), and we are uncertain whether the observed spore features are indicative of a close relationship. Based on the morphological evidence here presented we retain *Cheilanthes incisa* and *C. schimperi* in *Cheilanthes*. However, the final decision regarding the correct generic placement of this species requires further study.

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# The Gametophyte of *Huperzia selago* in Culture

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ABSTRACT.—Cultured gametophytes of *Huperzia selago* are dorsiventral and strap-shaped. They are basically the same as cultured gametophytes of other terrestrial *Huperzia* species and also essentially the same as gametophytes of *H. selago* collected from loose soil. Besides shape, the cultured gametophytes have anatomical features found in gametophytes of *H. selago* from loose soil. These gametophytes are Type III gametophytes as characterized by Bruchmann in 1898. Although both gametangia occur on individual cultured gametophytes, the meristematic groove produces them at different times and they are not intermixed. The number of terrestrial *Huperzia* species with described gametophytes is still small (six), but that number has tripled as a result of the recent studies on these gametophytes in culture. Because all the terrestrial *Huperzia* species to date have Type III gametophytes, it would not be unexpected for the undescribed gametophytes of other species in this group to also be Type III gametophytes.

In early studies on gametophytes of the Lycopodiaceae, Bruchmann (1898) recognized five types for *Lycopodium s.l.*. The shapes and type of nutrition of the gametophyte types can be briefly characterized as follows: Type I carrot-shaped (mycorrhizal); Type II disk-shaped (mycorrhizal); Type III elongated uniaxial (mycorrhizal); Type IV pincushion-shaped (photosynthetic); and Type V branched cylindrical (mycorrhizal). These types are still recognized and they demonstrate the range of variation in the gametophytes of this family.

*Lycopodium selago* L. (=*Huperzia selago* (L.) Mart. & Schrank) gametophytes from soil were the basis for Type III gametophytes (Bruchmann, 1898). Prior to culturing gametophytes, the only other Type III gametophyte described was that of *Lycopodium lucidulum* Michx. (=*Huperzia lucidula* (Michx.) Trevisan) by Spessard (1922). To provide a better understanding of terrestrial *Huperzia* gametophytes, gametophytes of four tropical, terrestrial *Huperzia* species were recently grown in axenic culture (Whittier, 2006).

All of the cultured *Huperzia* gametophytes (Table 1) were dorsiventral and strap-shaped. Because this was one of the shapes that Bruchmann (1898) described for gametophytes of *Huperzia selago* from soil, these tropical terrestrial *Huperzia* gametophytes were considered Type III gametophytes. Additional support for this conclusion would be provided if cultured gametophytes of *H. selago* had a dorsiventral shape. To examine this possibility, gametophytes of *H. selago* were grown in culture.

## MATERIALS AND METHODS

Spores of *Huperzia selago* were obtained from plants collected in September and May in the Jeseniky Mts. of the Czech Republic. Vouchers are on deposit at

TABLE 1. Terrestrial *Huperzia* species with known gametophytes.

| Species | Source | Reference |
|---|---|---|
| *H. selago* | soil | Bruchmann, 1898 |
| *H. lucidula* | soil | Spessard, 1922 |
| *H. lucidula* | culture | Whittier & Webster, 1986 |
| *H. crassa* | culture | Whittier, 2006 |
| *H. cumingii* | culture | Whittier, 2006 |
| *H. hypogaea* | culture | Whittier, 2006 |
| *H. saururus* | culture | Whittier, 2006 |
| *H. selago* | culture | present study |

TENN. The spores were sown within one month of their collection. To reduce the incidence of contamination, the spores were wetted and stored in water for 24 hours and surface sterilized with 20% Clorox (1.1% sodium hypochlorite) by the method of Whittier (1964). The spores were then suspended in sterile water, and sown on 13 ml of nutrient medium in culture tubes (20 × 125 mm) with screw caps that were then tightened. The cultures were maintained in darkness or under a 12 hour photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from cool white fluorescent lamps at 22 ± 1°C.

The nutrient medium contained 100 mg $NH_4Cl$, 50 mg $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl_2$, and 50 mg $K_2HPO_4$ per liter. Minor elements and FeEDTA completed the composition of this medium (Whittier, 1998). The carbon source was provided by the addition of 2.5 g of glucose per liter for spore germination and early gametophyte growth or 5 g of glucose per liter for the growth of older gametophytes. The medium was solidified with 1.1% agar and was at pH 5.9 before autoclaving.

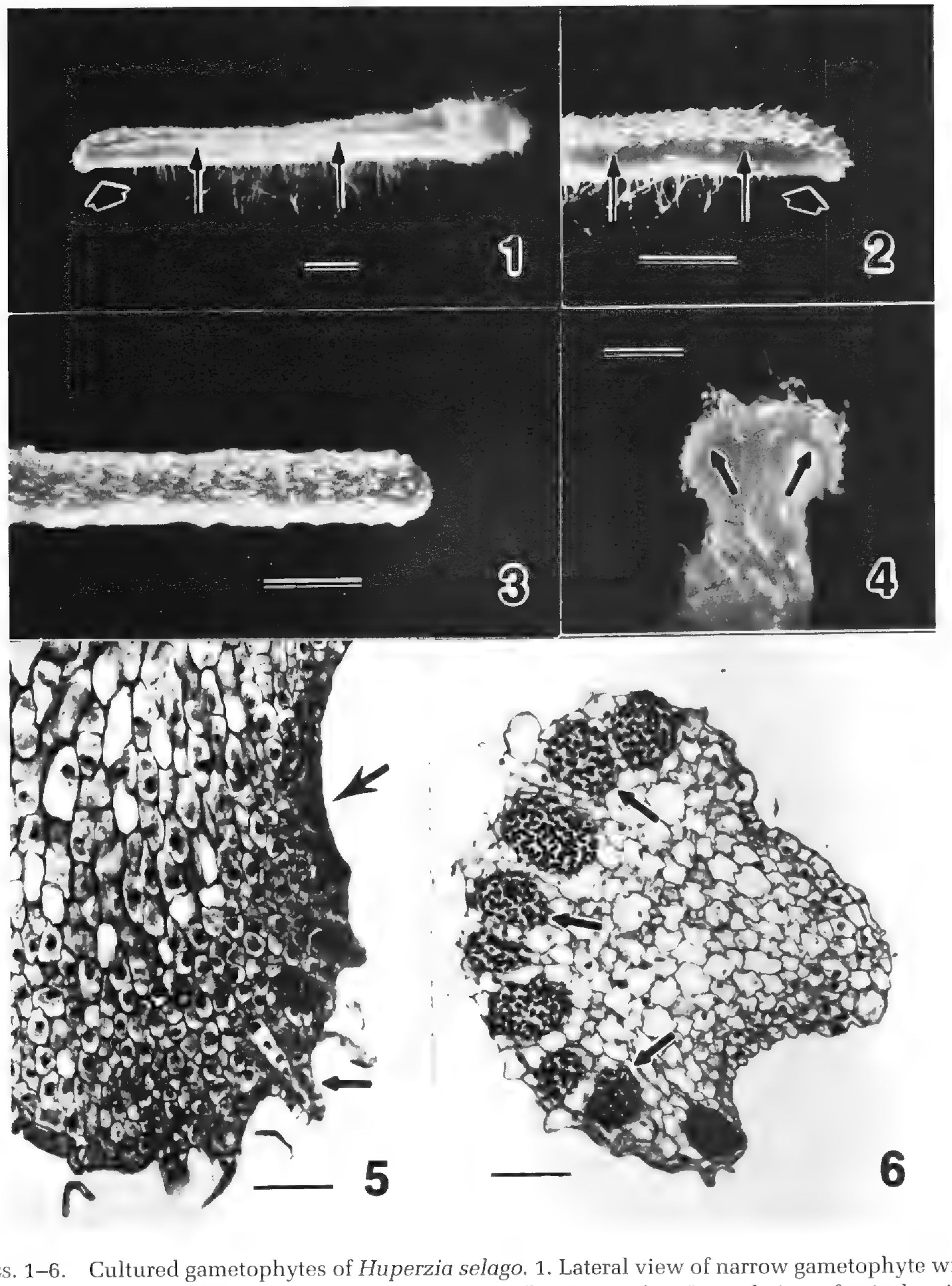
A total of 6000 spores were examined to determine the percentage of spore germination. The sample for calculating the average sizes of the gametangia and paraphyses was 30.

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

## RESULTS

About 0.1% of the spores from both the May and September collections germinated after 6 months in the dark and none germinated after 13 months in illuminated cultures. Gametophytes began to mature a year after sowing and a total of 42 young and mature gametophytes developed from the sown spores.

Mature gametophytes are long, narrow, axial structures with distinct dorsal and ventral surfaces (Figs. 1, 2, 3). Some are straight (Figs. 1, 2, 3) and others are more sinuous. Lateral grooves along the sides of the gametophytes separate the dorsal and ventral regions (Figs. 1, 2). Most gametophytes have an




FIGS. 1–6. Cultured gametophytes of *Huperzia selago*. 1. Lateral view of narrow gametophyte with lateral groove (small arrows) and meristematic region (large arrow). 2. Lateral view of apical region with lateral groove (small arrows) and meristematic region (large arrow). 3. Dorsal view of strap-shaped gametophyte. 4. Ventral view of apical region of wider gametophyte with meristematic groove (arrows). 5. Non-median sagittal section of apical region with mature archegonium (small arrow) and meristematic region (large arrow). 6. Cross section of narrow gametophyte with antheridia (arrows) at dorsal surface. Bars = 1 mm for Figs. 1–4 and 100 μm for Figs. 5–6.

hourglass shape in cross section but a smaller ventral region can cause narrower gametophytes to lose this shape.

The meristematic tissues are not terminal at the gametophyte apex (Figs. 4, 5). The immature dorsal tissues overarch the meristematic groove shifting it to the lower subterminal region of the apex. The derivatives to the upper side form the dorsal tissues including the gametangia and paraphyses. Derivatives to the lower side form the ventral region of the gametophyte with the rhizoids. The meristematic groove is continuous with the lateral groove on the sides of the gametophyte. However, there is essentially no meristematic activity in the lateral grooves of these cultured gametophytes.

The cells of the central region, which encompasses most of the internal tissue, are medium sized and somewhat elongated longitudinally (Fig. 5). The cells just above the ventral surface, which contain the mycorrhizal fungus in gametophytes from soil, are small and isodiametric. There are no vertically elongated cells near the ventral surface of the gametophytes.

The paraphyses are uniseriate filaments with an average length of 189.3 μm. They are composed of 2–3 cells with an average cell number of 2.9.

Each antheridium contains an ellipsoidal mass of gametes (Fig. 6) with an opercular cell in the jacket layer at the gametophyte surface. The average size of the gamete mass was 72.4 μm wide and 95.5 μm long.

The archegonia are normal for terrestrial *Huperzia* species (Fig. 5). The average length from egg base to neck tip is 136.8 μm and the neck protrudes from the gametophyte an average of 79.4 μm. The average number of tiers of four neck cells in the neck is 4.9 and there are 4 neck canal cells in the neck canal above the egg.

Antheridia and archegonia are in different regions of the same gametophytes and not intermixed with each other. It appears that the meristem forms either antheridia or archegonia (Figs. 5, 6). There is no evidence that antheridia and archegonia are produced simultaneously from the meristematic groove.

## Discussion

The mature gametophyte of *H. selago* from culture is dorsiventral and strap-shaped. This shape is one of the several shapes described for *H. selago* by Bruchmann (1898). Gametophytes of *H. selago* in culture have the same shape as those of *H. selago* from loose soil and as the other terrestrial *Huperzia* gametophytes grown in culture (Whittier and Webster, 1986; Whittier, 2006).

The gametophytes from culture are without mycorrhizal fungi. However, they are structurally the same as the dorsiventral gametophytes of *H. selago* described from soil (Bruchmann, 1898, 1910). The similarities are easily seen by examining Bruchmann's illustrations of cross and longitudinal sections in his 1898 (Fig. 38) and 1910 (Figs. 27, 28) publications. The cultured gametophytes have longitudinally elongated central cells and small isodiametric cells and no vertically elongated cells in the area that correlates with the mycorrhizal zone in gametophytes from soil. The apical meristem is on the lower side of the apex with overarching dorsal tissue. The medium-sized

antheridia and archegonia with medium-sized necks are initiated in the overarching dorsal tissue and as the meristem continues to form new cells the maturing gametangia and associated tissues are shifted to the dorsal surface. Besides the mature gametangia, short paraphyses are present on the dorsal surface. Along the lateral surfaces there are grooves that connect with the meristematic groove of the apex.

In addition to these features being in common with the gametophytes of *H. selago* from soil, they are found in the four other terrestrial *Huperzia* gametophytes grown in culture (Whittier and Webster, 1986; Whittier, 2006). The sizes of the paraphyses of *H. selago* in culture are within the range of sizes for the paraphyses of the other *Huperzia* gametophytes grown in culture (Whittier, 2006). The same is true for the gametangia.

The formation of archegonia and antheridia at different times on these gametophytes was observed in other terrestrial gametophytes of *Huperzia* grown in culture (Whittier, 2006; unpublished data). Although both types of gametangia can be on the same gametophyte, they are in different regions along the gametophyte. The gametangia do not intermix on these cultured gametophytes. If this is true for *Huperzia* gametophytes growing in natural habitats, it might help to explain why Soltis and Soltis (1998) found that *H. miyoshiana* (Makino) Ching does not exhibit high rates of intragametophytic self-fertilization.

Bruchmann (1898) described a considerable amount of variation with the gametophyte of *H. selago*. He found ninepin and compact roundish shapes, elongated variously curved axial forms with some being more or less cylindrical and a range of intermediates. The above forms were found in dense soil and another form occurred in loose soil. He showed that young immature conical gametophytes shifted to dorsiventral, axial gametophytes in loose soil. The dorsiventral gametophytes had paraphyses and gametangia on their dorsal surface and rhizoids ventrally.

The gametophytes of the terrestrial species of *Huperzia* grown in culture lack the variability found with gametophytes of *H. selago* growing in soil (Bruchmann, 1898; 1910). Less variability in the cultured gametophytes is probably related to the more uniform conditions in culture. Obstructions to gametophyte growth in soil are absent on an agar surface. This seems to explain the gametophyte shape in culture because the agar surface is more similar to loose soil than other soil conditions. The internal structure of these *Huperzia* gametophytes is not altered by the lack of a mycorrhizal fungus or by growth on a nutrient medium in culture.

The cultured gametophytes of *H. selago* and those of the other *Huperzia* species (Whittier and Webster, 1986; Whittier, 2006) are alike. Because gametophytes of *H. selago* are characterized as Type III gametophytes (Bruchmann, 1898), the gametophytes of these other *Huperzia* species are Type III gametophytes.

Terrestrial *Huperzia* gametophytes from culture have the same shape and internal structure as the gametophytes of *H. selago* from loose soil. This occurs even though the cultured gametophytes are growing without a mycorrhizal

fungus. The morphological stability of Type III gametophytes under these conditions indicates that this developmental pattern is distinctive from those of the other four types of gametophytes recognized by Bruchmann (1898). The structural stability of Type III gametophytes under different conditions is important to support the long held view that gametophyte characters of clubmosses have taxonomic value (Bruchmann, 1898; Bruce, 1976; Wagner & Beitel, 1992). Had the morphology of these cultured gametophytes been different from the previously described Type III gametophytes from soil, the validity of using gametophyte characters for taxonomic purposes would be questioned.

Acknowledgments

We thank James Hickey and Benjamin Øllgaard for encouraging the earlier study of tropical, terrestrial *Huperzia* gametophytes that led to this study. We gratefully acknowledge Hans-Willi Honegger for help translating sections of Bruchmann's publications.

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# *Diphasiastrum multispicatum* (J.H. Wilce) Holub (Lycopodiaceae) in Thailand

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Abstract.—Review of a herbarium specimen (QBG) and subsequent field studies have revealed that *Diphasiastrum multispicatum* occurs near the summit of the two highest mountains in Thailand. This species is restricted to SE Asia and grows exclusively at higher elevations in the submontane to montane zone. Previously, it has been reported from China, the Philippines, Taiwan, and Vietnam, but not from Thailand. A comparison of the plants from Thailand with those from the type locality of *D. multispicatum* in the Philippines (Mt. Santo Thomas near Baguio City, Province of Benguet, Luzon), where this species is still present, shows the plants from Thailand to be *D. multispicatum*. Like other *Diphasiastrum* species, *D. multispicatum* is a weak competitor and grows on (disturbed) immature soils on slopes with more or less open and low growing vegetation. We also present morphological evidence that *Diphasiastrum multispicatum* is distinct from *Diphasiastrum complanatum s.s.*, which is a north temperate, circumboreal species (in northern and central Europe, Greenland, northern North America, Japan and northern Asia, excluding the tropics).

The genus *Diphasiastrum* comprises a relatively small group of lycopods that differ morphologically from the genus *Lycopodium s.s.* in several traits, such as leaves (mostly) 4–5–ranked (and not spirally arranged), leaves (mostly) di- or even trimorphic (and not isomorphic), and upright shoots (mostly) quadrate to flattened (and not rounded). This group was formerly treated as section *Complanata* of genus *Lycopodium* (Wilce, 1965; Øllgaard, 1987, 1989, 1990), but Holub (1975a) proposed it as a separate genus *Diphasiastrum*, and this treatment has been accepted in many modern floras (e.g., Dostál, 1984; Wagner and Beitel, 1993; Jermy, 1993; Kukkonen, 2000). The genus includes about 25 species of mainly north temperate and subarctic distribution. Only a limited number of species occur in the tropics or subtropics, where they are restricted to mountainous areas. Species with a southeast Asian distribution include *Diphasiastrum angustiramosum* (Alderw.) Holub, *D. multispicatum* (Wilce) Holub, *D. platyrhizoma* (Wilce) Holub, *D. veitchii* (Christ) Holub, and *D. wightianum* (Wall. ex Hook. & Grev.) Holub (Wilce, 1961, 1965). Additionally, taxa of unresolved relationship occur in this area and were called the "Chinese plant" and the "New Guinea plant" in the monograph by Wilce (1965). The "Chinese plant" might be conspecific with the plants described as *D. yueshanense* C. M. Kuo and *D. wilceae* Ivanenko (Kuo, 1985; Ivanenko, 2003). To our knowledge, no recent comparative studies are available for these critical taxa.

In the last century, *Diphasiastrum* was still unknown in Thailand (Tagawa and Iwatsuki, 1979). Later, a single species was reported under the name *Lycopodium complanatum* L. (Boonkerd and Pollawatn, 2000). However, *Diphasiastrum* (= *Lycopodium*) *complanatum* is a north temperate, circumboreal species which occurs in northern and central Europe, Greenland, northern North America and extending to Japan and northern Asia. Wilce (1965, p. 103) states explicitly: "There is no true *L. complanatum* either in the tropics (even on mountain slopes), or in the southern hemisphere". We present evidence that *Diphasiastrum multispicatum* (Wilce) Holub occurs in Northern Thailand, where it occupies the peaks of the two highest mountains.

## Materials and Methods

Pteridophytes in the herbarium of Queen Sirikit Botanic Garden (Mae Rim, Chiang Mai, Thailand; QBG) were studied by two of the authors (W.B. and P.S.) in September 2005 and in February 2006. Field collection of plant specimens on Doi Inthanon were made in the same months (by W.B. and P.S.) and on Doi Pha Hom Pok (by P.S.) in October 2005. Additionally, *Diphasiastrum* specimens kept in the herbaria of Berlin (B) and Aarhus (AAU) were annotated by K.H. The type locality of *Diphasiastrum multispicatum* on Mt. Santo Thomas (Luzon, Philippines) was revisited in November 2005 (by P.S.) and February 2006 (by W.B.). A Global Positioning System (GPS) instrument (Garmin GPS 72) was used to determine altitude and coordinates of the Philippine and Doi Inthanon populations; for Doi Pha Hom Pok, these data were obtained from a topographical map. SEM images of spores were made using a scanning electron microscope (DSM 950, Zeiss).

## Results and Discussion

*Morphology.*—A herbarium specimen that was labeled as "*Lycopodium complanatum*" (collected on the peak of the highest mountain in Thailand, Doi Inthanon, *Nanakorn s.n.* QBG no. 6601) was discovered while studying pteridophytes in the herbarium of Queen Sirikit Botanic Garden (Mae Rim, Chiang Mai, Thailand; QBG). The plant is clearly different in its morphology from *Diphasiastrum complanatum* (= *Lycopodium complanatum* L.; the type specimen of this species is unknown (see Wilce 1995, p. 143 ff. and Holub 1975b)) and was identified as *Diphasiastrum multispicatum*. The site and the population on Doi Inthanon were subsequently studied on several field trips. Later, another population was discovered on the second highest mountain in Thailand, Doi Pha Hom Pok (by P.S.). Plants from the type locality on Mt. Santo Thomas (Luzon, Philippines) were studied for comparison.

Table 1 summarizes diagnostic characters that distinguish *D. complanatum* from *D. multispicatum*. The latter is the more robust species, which becomes evident especially from the dimensions of the rhizomes and peduncles. Its most distinguishing characters are the long branchlets with a very glaucous lower side and strongly incurving lateral leaves, moderately well-developed

TABLE 1. Morphological comparison of *Diphasiastrum complanatum* and *Diphasiastrum multispicatum* (mainly after Wilce, 1961 & 1965).

| Characters | *Diphasiastrum complanatum* | *Diphasiastrum multispicatum* |
|---|---|---|
| **Characters being different** | | |
| rhizome | terete, 1.7 (1.1–2.7) mm in diameter | terete to somewhat flattened, 2.2 (1.3–3.2) mm broad and 1.7 (1.3–2.3) mm thick |
| color of lower side | pale, lighter in color than upper surface, but not glaucous | strongly glaucous |
| ventral leaves (free blades) | 1.3 (0.7–2.1) mm | 1.8 (1.2–2.5) mm |
| lateral leaves (including bases) | 5.0 (2.6–7.3) mm | 4.6 (3.2–6.2) mm |
| dorsal leaves (including bases) | 4.8 (2.8–7.0) mm | 3.8 (2.8–5.2) mm |
| diameter of peduncles | 0.6 (0.4–0.9) mm | 1.0 (0.8–1.1) mm |
| number of strobili | strobili few, 3–4, occasionally 5 or 6 per peduncle | strobili numerous, generally 8 or more, though occasionally as few as 4 per peduncle |
| **Characters in common** | lateral branchlets of upright shoots distinctly flattened, leaves of ultimate branchlets 4-ranked, scale-like, trimorphic, ventral leaves of branchlet less conspicuous than dorsal ones, strobili pedunculate | |

lower leaves with evident decurrent leaf bases (Fig. 1), a sharply defined transition between fertile branch and peduncle, and the number of strobili per peduncle being significantly greater than in *D. complanatum* (Wilce, 1961, 1965). Not surprisingly, the two species share a number of traits that are typical for the genus *Diphasiastrum*, like lateral branchlets of upright shoots flattened, leaves of ultimate branchlets 4-ranked, scale-like and trimorphic (Table 1).

Fig. 2 shows photographs of the habitat, part of the population and morphological details of the plants. Number of strobili per peduncle and the bright, almost silvery lower surface are good field characters to identify the plants as *Diphasiastrum multispicatum*. At the type locality on Mt. Santo Thomas (near Baguio City, Province of Benguet, Luzon, Philippines), the occurrence of *Diphasiastrum multispicatum* was confirmed, and 4 populations were found at different altitudes (Table 2). The growth habit and the morphology of these plants completely agree with the Thai plants (Fig. 2).

Another difference was found for the spore micromorphology (Fig. 3). In all Lycopodiaceae the spores are trilete and subglobose to subtriangular in outline (Tryon and Lugardon, 1991). Those of *Lycopodium s.l.* show a very characteristic structure composed of reticulate elements or baculae arranged in a honey-combed like pattern. Wilce (1972) recognized 4 types of such reticulate spores with the most common *clavatum* type being present in section *Lycopodium* (*Lycopodium s.s.*) and section *Complanata* (*Diphasiastrum*). While the reticulum is always continuous on the distal face, it may be broken, reduced, or lacking on the proximal face (Wilce, 1972; Tryon and

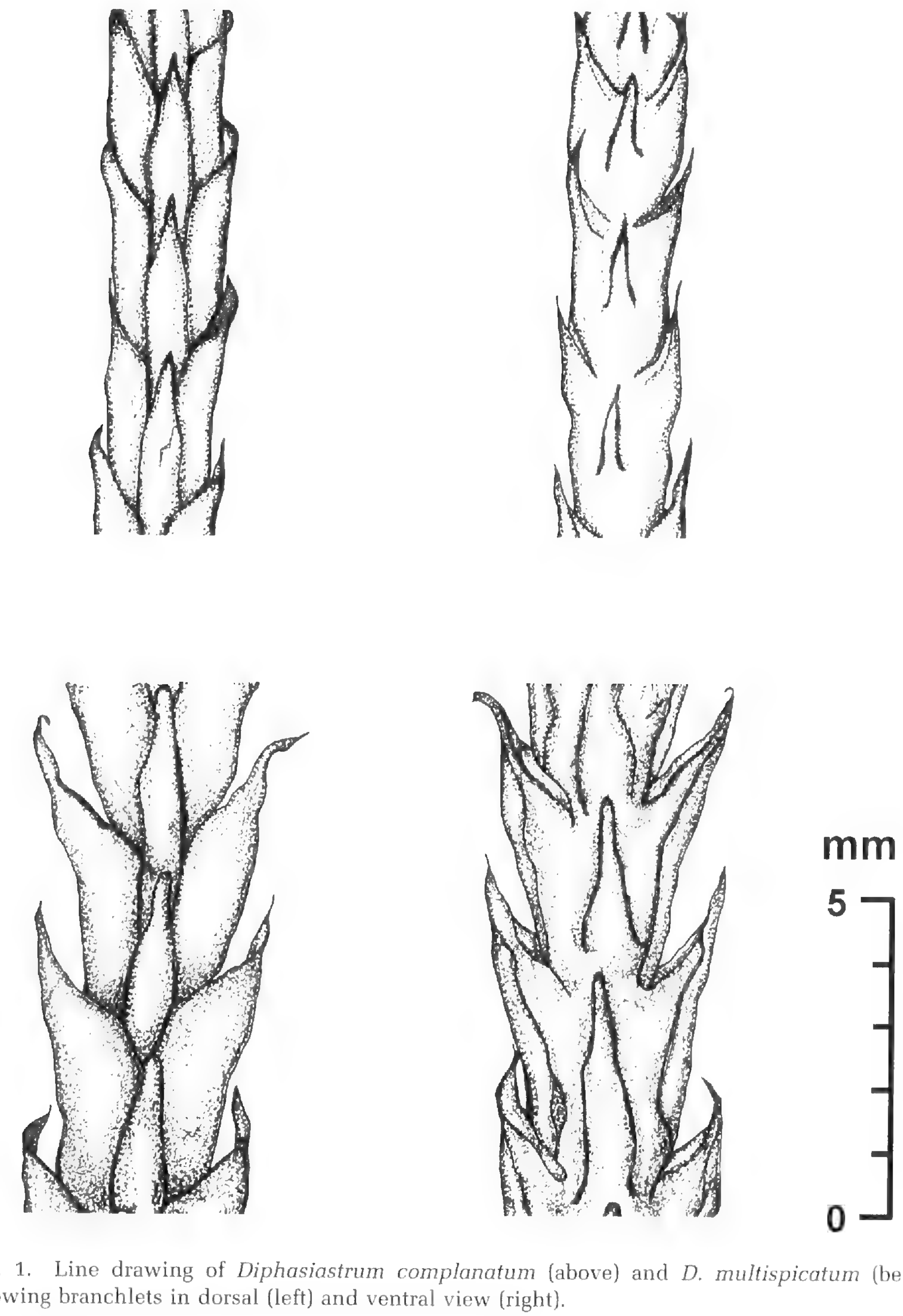


FIG. 1. Line drawing of *Diphasiastrum complanatum* (above) and *D. multispicatum* (below) showing branchlets in dorsal (left) and ventral view (right).

Lugardon, 1991). In *Diphasiastrum multispicatum*, the outer one-third of the proximal face is reticulate, but the meshwork is gradually reduced towards the aperture and is lacking between the triradiate arms. This is in contrast to *D. complanatum* and other European *Diphasiastrum* species, where the re-

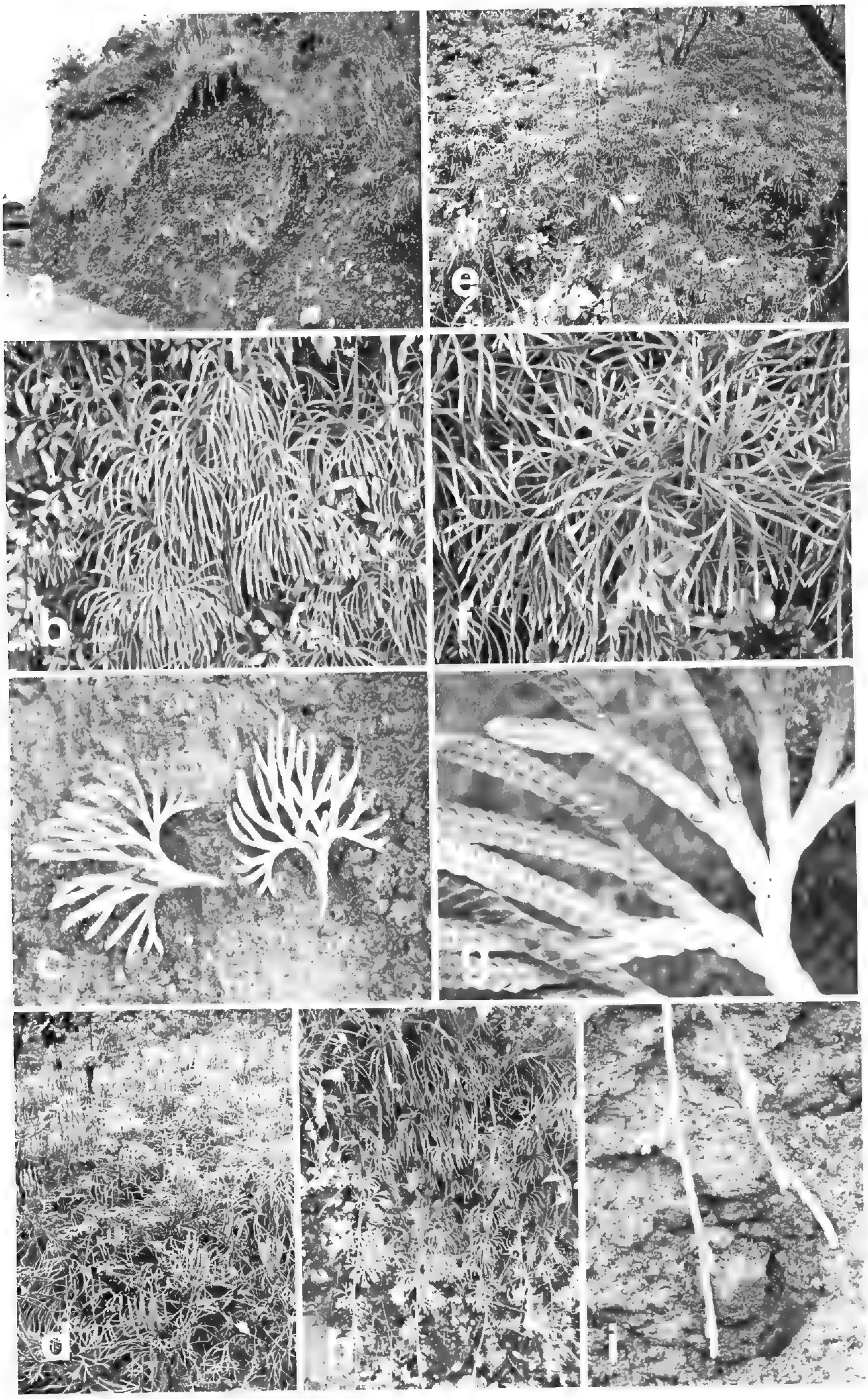


FIG. 2. *Diphasiastrum multispicatum* in the Philippines (a–d, h–i) on Mt. Santo Thomas (Luzon, type locality), and in Thailand on Doi Inthanon (e–g); open habitats along roadside (a, e), growth form of upright shoots (b, f), lower side of branches (c, g), part of colony with strobili (d), rhizome growth over blank soil on steep roadside slope (h–i).

TABLE 2. Geographical position of the two *Diphasiastrum multispicatum* localities in Thailand and of the type locality in the Philippines.

| Country | Altitude (m asl) | Coordinates |
|---|---|---|
| **Thailand** | | |
| Doi Inthanon | 2,243 | N 18° 33.754′ E 98° 29.046′ |
| Doi Pha Hom Pok | 1,950 | N 20° 06′ E 99° 07′ |
| **Philippines** | | |
| Mt. Santo Thomas | | |
| population 1 | 2,260 | N 16° 20.115′ E 120° 33.651′ |
| population 2 | 2,215 | N 16° 20.203′ E 120° 33.592′ |
| population 3 | 2,196 | N 16° 20.204′ E 120° 33.585′ |
| population 4 | 2,003 | N 16° 20.546′ E 120° 33.444′ |

ticulum is well-developed also on the proximal face and extends to the base of the raised arms of the aperture (Fig. 3; see also Ferrarini *et al.*, 1986). Another distinguishing feature of *D. multispicatum* is the frequent occurrence of perforations in the walls of the reticulum, visible on both faces (Fig. 2i–j).

*Diphasiastrum* is remarkable in North America and Europe for its ability to form homoploid, apparently fertile interspecific hybrids (Flora of North America, 1993; Stoor *et al.*, 1996), which are morphologically intermediate between the putative parents. Kuo (1985) describes *Lycopodium yueshanense* (= *Diphasiastrum yueshanense*) as a new endemic species from Taiwan and reports that it is intermediate between *L. veitchii* (= *D. veitchii*) and *L. multispicatum* (= *D. multispicatum*) both morphologically and ecologically. Thus, *D. yueshanense* may well represent the first example of a homoploid hybrid in the tropics.

*Ecology and Distribution.—Diphasiastrum multispicatum* is a weak competitor and colonizes (disturbed) immature soils on slope cuttings that have been created by man with more or less open and low growing vegetation. All checked sites in Thailand and in the Philippines are located on steep road cuts (Fig. 2). In most cases, two other lycopods, *Lycopodium clavatum* L. and *Lycopodiella cernua* (L.) Pic. Serm., were observed in the vicinity.

In other continents *Diphasiastrum* species often grow on secondary sites as well, both in the tropics and in the temperate zones. *Diphasiastrum thyoides* (Willd.) Holub from South and Central America, for example, is reported from way- and roadsides, clearings, scrub and fallow land (Øllgaard, 1988, 1995). Also *D. fawcettii* (F. Lloyd & L. Underw.) Holub from Jamaica and Hispaniola is known to occur on clearings, sunny embankments and open slopes with scattered shrubs (Proctor, 1985). In most parts of their North American and Central European range, the *Diphasiastrum* species display a preference for younger secondary habitats on immature soils with an only fragmentary plant cover. Examples are roadsides, slopes of forest roads, other cuttings, ski runs and their margins, abandoned pits, firebreaks along railways, forest aisles, clearings under power lines and younger afforestations (e.g., Ardelmann *et al.*, 1995; Horn, 1997; Bennert, 1999).

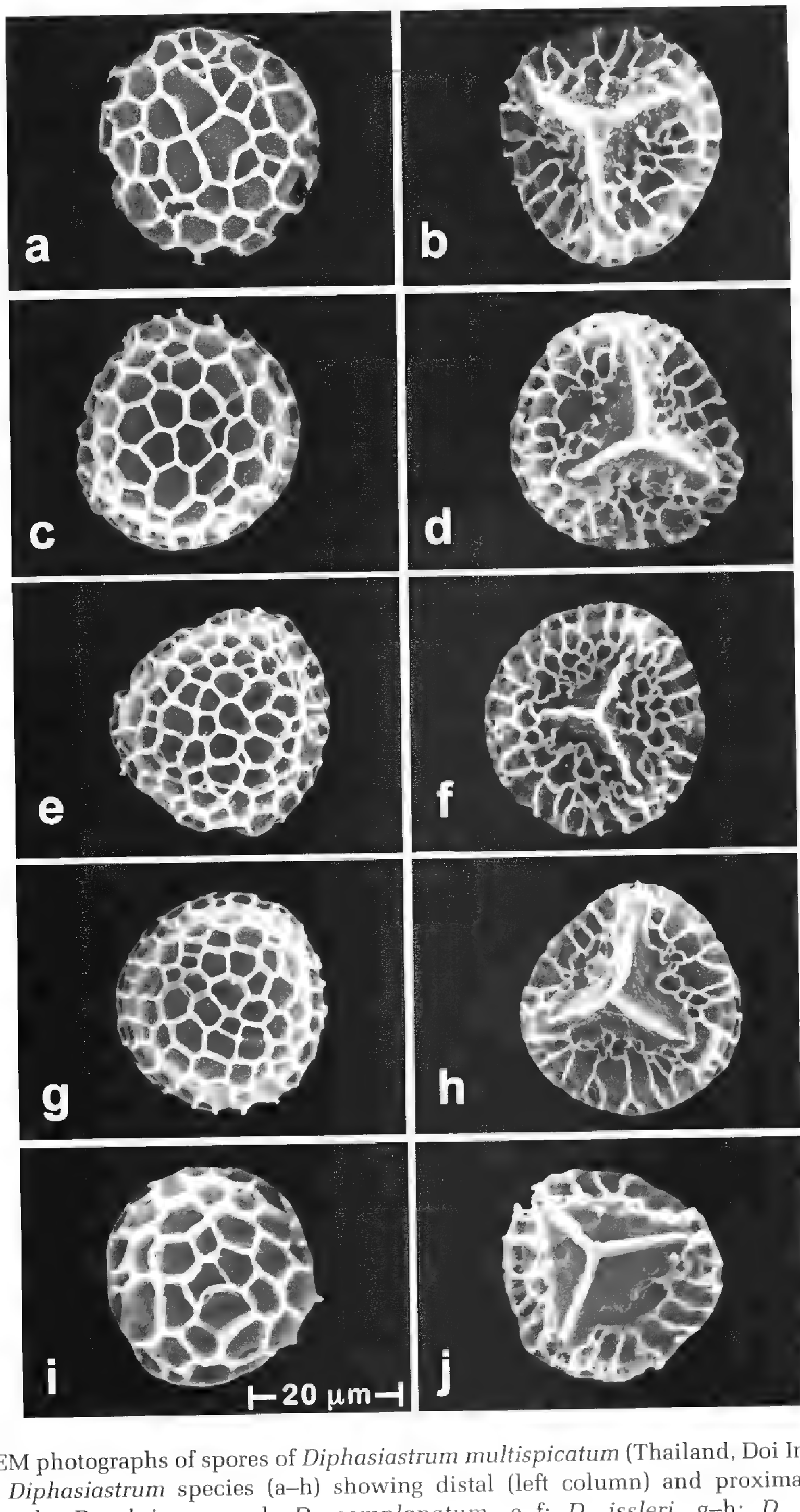


FIG. 3. SEM photographs of spores of *Diphasiastrum multispicatum* (Thailand, Doi Inthanon) and European *Diphasiastrum* species (a–h) showing distal (left column) and proximal face (right column); a–b: *D. alpinum*, c–d: *D. complanatum*, e–f: *D. issleri*, g–h: *D. zeilleri*, i–j: *D. multispicatum*.

The altitudinal range of *Diphasiastrum multispicatum* observed was 2,003–2,260 m on Mt. Santo Thomas in the Philippines, and in Thailand 1,950–2,243 m on Doi Inthanon and Doi Pha Hom Pok (Table 2); thus *Diphasiastrum multispicatum* represents a montane species in both countries.

Doi Inthanon National Park, approximately 80 km south of Chiang Mai, encompasses the highest mountain in Thailand, Doi (Mt.) Inthanon, as well as several lesser summits. The park covers an area of 48,240 ha. Its lowland areas (< 800 m) are covered by a dry dipterocarp forest which gives rise to an evergreen forest (above 1,000 m), where annual rainfall exceeds 2,500 mm. The slopes around the summit area (2,300–2,565 m) carry a moist hill evergreen forest ('cloud forest') with many epiphytes. The temperature may drop to −8°C and frosts are not unusual during the dry season. Doi Pha Hom Pok lies approximately 80 kilometers north of Chiang Mai is the second highest mountain in Thailand (2,285 m). Vegetation types are almost the same as on Doi Inthanon.

Mt. Santo Thomas lies above Baguio City (approximately 250 kilometers north of Manila) in the heart of the Province of Benguet. Baguio City itself is situated at an elevation of about 1,400–1,500 m and has an average annual temperature of 18.2°C and an annual precipitation of 4,179 mm (recording period 17 years; see: www.globalbioclimatics.org - climate diagram of Baguio City). If we assume the temperature to drop by ca. 0.5°C when the elevation increases by 100 m, an average annual temperature of about 14°C would result for the peak area of Mt. Santo Thomas. Annual precipitation is expected to be somewhat higher than in the city. The presently known area of distribution in Thailand comprises only two sites, and few additional localities are to be expected, as there are a limited number of higher mountains with an elevation exceeding 2,000 m. Other countries in SE Asia from which *Diphasiastrum multispicatum* has been reported are China (southern part), the Philippines, Taiwan, and Vietnam. In the recent treatment of *Lycopodiaceae* in the Flora of China (Xianchun and Libing, 2004), *Diphasiastrum multispicatum* is not recognized as a separate species, but united with *Diphasiastrum complanatum*. The latter has been split into two varieties, the typical var. *complanatum* and var. *glaucum*. From the short diagnosis given by Ching (1982) in his description of var. *glaucum* and from the appearance of the type specimen (a photo of which was supplied by Y. Ivanenko), we conclude that this variety refers to *Diphasiastrum multispicatum*. This already has been proposed by Ivanenko (2003). The total altitudinal distribution in these countries ranges from 1,165 m to 2,415 m corresponding to the submontane to montane zones (Wilce, 1961, 1965). Table 3 summarizes the recorded localities. Thus, the discovery of *Diphasiastrum multispicatum* in Thailand is a remarkable range extension, and the mountains in northern Thailand harbor the westernmost known populations of this species. *Diphasiastrum multispicatum* is likely to occur in the neighboring country of Laos as well, where several sufficiently high mountains are located with the tallest mountain, Phou Bia in the Annamese Cordillera, attaining 2,817 m above sea level.

TABLE 3. Records for *Diphasiastrum multispicatum*; data compiled from literature and obtained from herbarium revision (indicated by B [Botanischer Garten und Botanisches Museum Berlin-Dahlem, Germany] or AAU [University of Aarhus, Denmark]). The distribution given in the Chinese literature refers to *Diphasiastrum complanatum* var. *glaucum* which is a synonym of *Diphasiastrum multispicatum*.

| State | Region/province/district | Locality | Reference |
|---|---|---|---|
| **China** | Prov. Guangxi | | Xianchun & Libing, 2004 |
| | Prov. Yunnan | | |
| | Prov. Xizang (Tibet) | | |
| | Eastern Yunnan | Pingbian Xian | Ching, 1982 |
| **Taiwan** | Distr. Ilan | Taipingshan | DeVol & Kuo, 1979 |
| | Distr. Hsinchu | Tapachienshan | DeVol & Kuo, 1979 |
| | Distr. Nantou | Kuantaochi | DeVol & Kuo, 1979 |
| | Distr. Chiayi | Mt. Alishan | DeVol & Kuo, 1979 |
| | | | M.T. Kao (7486), 13.12.1968, det. C.M. Kuo as *L. complanatum*, rev. K. Horn (2003), AAU |
| | Distr. Kaohsiung | Kuanshanakou | DeVol & Kuo, 1979 |
| | Distr. Hualien | Mukuashan | DeVol & Kuo, 1979 |
| | | Mt. Taiheizan | Wilce, 1961 |
| | | Arisan | Wilce, 1961 |
| **Thailand** | Prov. Chiang Mai | Doi Inthanon | this paper |
| | Prov. Chiang Mai | Doi Pha Hom Pok | this paper |
| **North Vietnam** | Prov. Bac Phan (Tonkin) | Nam-kep, Massif du Pia-Quac | Wilce, 1961 |
| | | Chapu, Lo Qui Ho between Cao-Bang and Nguyen-Binh | Wilce, 1961 |
| | | Col de Lo Qui Ho, pres Chapa: Cha-pa et Cho-bo | E. Poilane (12641), 13.5.1927, as *L. complanatum*, rev. K. Horn (2003), *ex* P, AAU |
| **South Vietnam** | Prov. Kontum | no exact locality given | Averyanov *et al.* (VH 183), 17.3.1995, as *L. complanatum*, rev. K. Horn (2003), AAU |
| **Philippines** | Luzon, Prov. Benguet | Mt. Tabiao | Hb. E.B. Copeland, 25.10.1905, as *L. complanatum* var. *thyoides*, rev. K. Horn (2005), B |
| | Luzon, Prov. Benguet | Mt. Santo Thomas; Baguio | Wilce; 1961, 1965 |
| | Luzon, Prov. Nueva Viscaya (?) | Mt. Tonglon | Wilce; 1961, 1965 |
| | Luzon, Prov. Laguna | Mt. Maquiling; Mt. Banahao | Wilce; 1961, 1965 |

ACKNOWLEDGMENTS

We thank Mrs. Ilse Wessel, Bochum, for preparing the drawings of *D. complanatum* and *D. multispicatum*, and Mr. Marcus Streckenbach, Bochum, for providing the SEM photos of the spores of *D. multispicatum*. Also, thanks are due to Prof. Dr. Brigitte Zimmer, Berlin, and Prof. Dr. Benjamin Øllgaard, Aarhus, for sending herbarium specimens on loan (from the herbaria B and AAU). Dr. Yury Ivanenko, St. Petersburg (Russia), sent us a photograph of the type specimen of *D. complanatum* var. *glaucum* kept in PE (Institute of Botany, Chinese Academy of Sciences, Beijing), and Prof. Dr. Zhixiang Zhang, Beijing (China) contributed information on the occurrence of *D. multispicatum* in China; both are gratefully acknowledged. Jeff Stauffer, Wrightwood (CA, USA) and Dr. Nicola Bennert, Riverside (CA, USA) contributed to improving our English.

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# Confirmation of Two Endemic *Athyrium* Species (Woodsiaceae) in Taiwan

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ABSTRACT.—Two endemic species, *Athyrium tripinnatum* Tagawa and *A. minimum* Ching are confirmed to exist in Taiwan. Their taxonomic descriptions, pictures of living plants, illustrations, and additional notes are provided

The most recent checklist of Taiwanese ferns contained 18 species and 2 hybrids in the genus *Athyrium* (Lu and Yang, 2005). Several scientific names recorded in Flora Reipublicae Popularis Sinicae (Wang, 1999) were not recognized by Lu and Yang. However, based on recently collected materials, we believed that at least two of them, *A. tripinnatum* Tagawa and *A. minimum* Ching, should be re-established. In the past, these two species were less-known and usually treated as taxonomic doubtful species in the local flora of Taiwan (Yang and Liu, 2002).

*Athyrium tripinnatum* was based on the materials from Alishan area, Central Taiwan, and Sziyuanyako area, NE Taiwan. Kuo (1985) synonymised it into *Athyrium foliolosum* T. Moore *ex* R. Sim., which is a misapplied name of *A. fimbriatum* (Hook.) T. Moore in Taiwan (Fraser-Jenkins, 1997; Wang, 1999; Liu and Fraser-Jenkins, 2006). Wang (1999) treated it as a distinct species under the section *Mackinnoniana* (Ching & Y. T. Hsieh) Z. R. Wang, but suggested that the relationship between it and *A. foliolosum* (section *Polystichoides* Ching & Y. T. Hsieh) needed further study. Liu *et al.* (2000) and Lu and Yang (2005) accepted Kuo's classification and regarded it as *A. foliolosum* (= *A. fimbriatum*). After a detailed observation of the morphological characters and spore ornamentation, we confirm *A. tripinnatum* as a valid species and distinct from *A. foliolosum* and *A. fimbriatum* which belong to section *Polystichoides*.

The second species, *Athyrium minimum*, was based on a single collection by Hancock from Tamshui, north Taiwan (Holotype: *Hancock s.n.*, PE!) in 1881. Three small plants were mounted on a specimen sheet with the largest one less

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than 8 cm long (Ching, 1986), and there was a suggestion that these plants might represent precociously fertile specimens of *A. iseanum* Rosenst. (Fraser-Jenkins notes in sched.). Yang and Liu (2002) regarded it as a doubtful species in Taiwan. With the new material, we have confirmed that this taxon is a valid species and Ching's description, which was only based on very few specimens, should be revised.

Following the subdivision scheme of *Athyrium* proposed by Wang (1999), but modified by Fraser-Jenkins (pers. comm.), we placed both *A. minimum* and *A. tripinnatum* into section *Echinoathyrium* Ching & Y.T. Hsieh. The section *Mackinnoniana* included the type of the section *Echinoathyrium* and is therefore a superfluous name of *Echinoathyrium*. The following diagnostic morphological characters belong to this section (Wang, 1997): spore without folded perispore; short spines on adaxial costae and costules; indusia reniform, elliptic, short-linear, J-shaped and hippocrepiform; scales at stipe base usually light brown to brown; we confirmed that *A. tripinnatum* belongs to this section and transferred *A. minimum* into this section as well.

Both species with taxonomic descriptions, illustrations, and notes are presented here.

***Athyrium tripinnatum*** Tagawa Acta Phytotax. Geobot. 6: 163. 1937. **Fig. 1, 3A–C.**

*Athyrium foliolosum auct non.* T. Moore *ex* R. Sim.: C. M. Kuo, Taiwania 30: 34, 65. 1985; C. M. Kuo, Manu. Taiwan Vasc. Pl. 1: 111, 1997; Yang and Liu. Manu. Taiwan Vasc. Pl. 6: 116; Lu and Yang, Taiwania 50: 150.

Evergreen, terrestrial fern. Rhizome short erect, thick. Stipe 40 (25–70) cm long, bearing numerous lanceolate, russet- or pale-brown scales in its lower half, becoming small and scattered above, absent from the rachis; stipe and rachis purple or rarely green in the living state, glandular on the adaxial side. Fronds membranaceous to herbaceous, lamina 50 (40–70) cm long, 40 (25– 60) cm wide. Lamina broadly lanceolate in small plants, but becoming deltate-lanceolate in larger ones; rachis glandular adaxially, glabrous abaxially; pinnae pinnatifid in small plants, usually pinnate in larger plants; the acroscopic pinnules symmetrical with the basiscopic ones on each pinna; pinnules anadromous, varying considerably in size from small to large, pinnule base ± narrowly attached to the costa, but with a short petiole in lower pinnae of large plants, pinnule-apices narrowly obtuse or somewhat acute. Sori ± large, subreniform, curved, hippocrepiform, or J-shaped, with prominent indusia. Spores somewhat pale-brown, perispore of rugate type, granulate on the surface.

DISTRIBUTION.—Endemic to Taiwan. Altitude 1600–2500 m.

SPECIMENS EXAMINED.—TAIWAN. **Chiayi**: *M. Tagawa 398.* (Isotype. L. photograph from website http://132.229.92.200/Pictures/L0051500/L0051074.jpg); *R. Knapp 544*; **Nantou**: *Y. C. Liu 3626, 5315, 5316, 5318, 5320* (SYSU, TAIF), *P.*

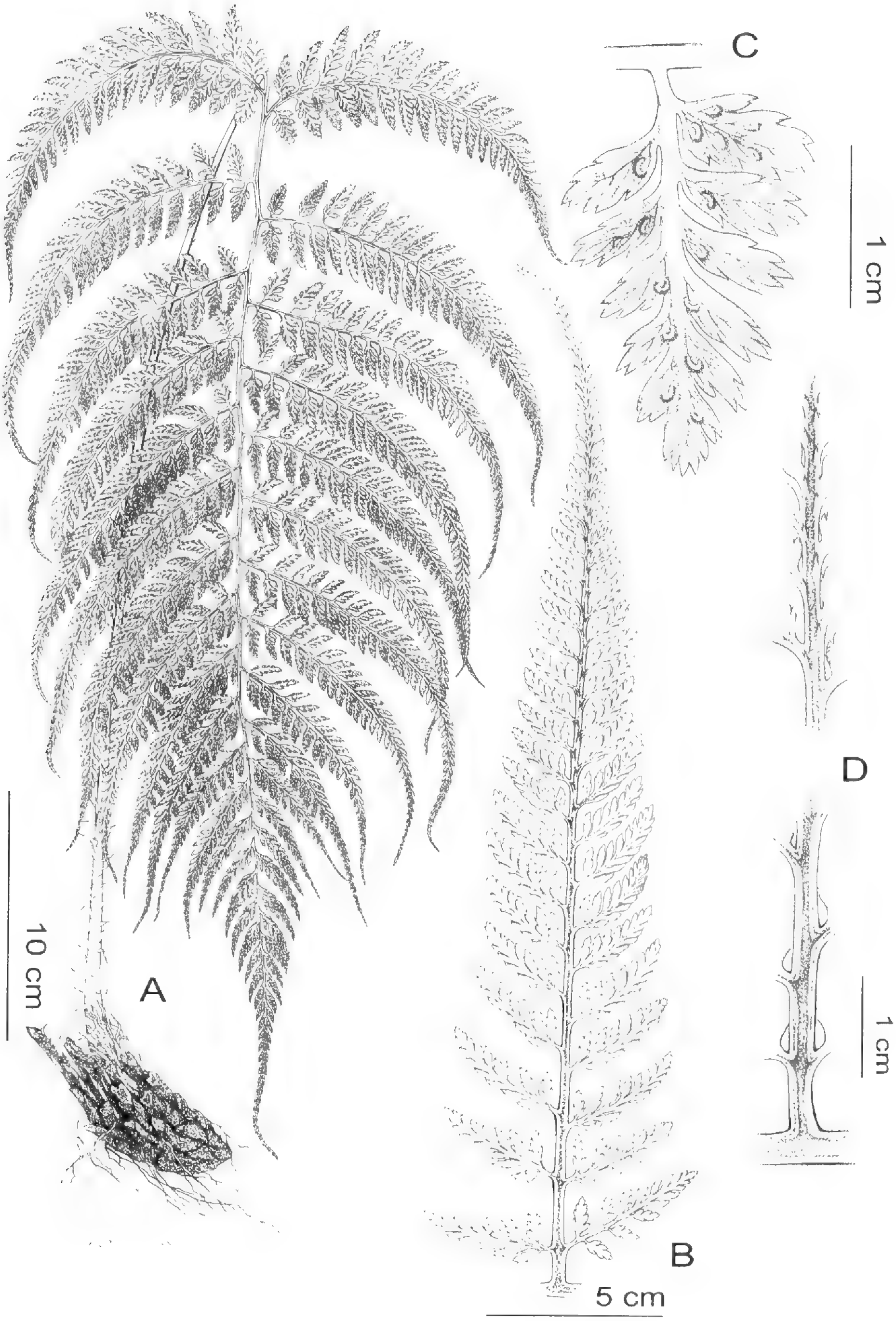


FIG. 1. Illustration of *A. tripinnatum*. **A**. Habit. **B**. Representative pinnae (adaxial surface) **C**. Representative sori position and venation (abaxial surfaces) D. Adaxial surface of costae, spines on the base of costule.

*H. Lee & J. M. Cheng 2121* (TAIF); **Kaohsiung**: *Y. C. Liu 3521, 3562* (SYSU); **Hsingchu**: *P. F. Lu 1531* (TAIF).

The section *Polystichoides* is a well known natural group under the genus *Athyrium* including *A. nakanoi* Makino, *A. anisopterum* Christ, *A. micropterum* Fras.-Jenk., *A. kumaonicum* Punetha, *A. puncticaule* (Bl.) T. Moore, *A. foliolosum* and *A. fimbriatum*. The group has significant morphological characters distinct from other species of *Athyrium*: an enlarged acroscopic pinnule in the basal pinnae, or the pinnule auricled on its acroscopic side; setae or spines absent on the adaxial surface of costae and costules and spores with cristate-retate perispore (Hsieh, 1986; Wang, 1999; Liu *et al.* 2000; Chang *et al.* 2001; Liu and Fraser-Jenkins, 2006). Although *A. tripinnatum* is superficially very similar to *A. fimbriatum*, it does not match the morphological characters of the section *Polystichoides* as it has spines on the costae and a rugose perispore. This species is unique in morphology compared to other taxa in Taiwan, where it is usually found in humid and shaded forest understory and is an endemic species. According to the morphological characters distinguishing sections, we suggest *A. tripinnatum* Tagawa must belong to the section *Echinoathyrium*, though more systematic study is needed to clarify the relationship to *A. fimbriatum* and *A. foliolosum* which are morphologically similar.

***Athyrium minimum*** Ching Acta Bot. Boreal.-Occid. Sin. 6:151. 1986.
**Fig. 2, 3D–F**.

Evergreen, terrestrial plant; rhizome short erect. Stipes up to 10 cm long, tufted, slender, usually shorter than the lamina, green, or sometimes turning red, glabrous, but densely scaly at the base; scales ovate or broadly lanceolate, light brown, 0.5–1 cm long, entire. Lamina ovate, 25 (5–40) cm long by 10 (2–15) cm wide, acuminate, bipinnate, glabrous, papyraceous; pinnae 7–10 on each side, narrowly lanceolate, often acuminate, 4–8 cm long, 2 (1–2.5) cm wide, slightly overlapping the adjacent ones, with short petioles (1–3 mm), costae narrowly canaliculated and with spines on the adaxial surface; pinnules anadromous slightly ascending, very shortly petiolulate, obtuse, with acute teeth at the apex, deeply lobed; rachis naked, canaliculate on the adaxial side as is the stipe, glabrous in the grooves. Sori 6–11 in a pinnule, dorsal in the middle of veinlets, J-shaped, 1–2.5 mm long; indusium membraneous, entire.

DISTRIBUTION.—Endemic to Taiwan, only occurring in the northern part, in Yangminshan National Park, from Lengshuikeng to Mt. Chihsing, Altitude ca. 800–950 m.

SPECIMENS EXAMINED.—TAIWAN. **Tamshui**: *Hancock, s.n.* Dec. 1881. Kew Fern List No. 134. (Holotype, PE!); **Taipei** alt. 600–800 m, *Y. C. Liu 3602* (SYSU); *P. F. Lu 1842, 1843, 2971* (TAIF).

During his taxonomic investigation of *Athyrium* in Taiwan, the first author, Yea-Chen Liu, found that some specimens of the genus collected from Yangmingshan National Park, north Taiwan, could not be determined as belonging to any known species. The plants varied in sizes from 5 to 40 cm in

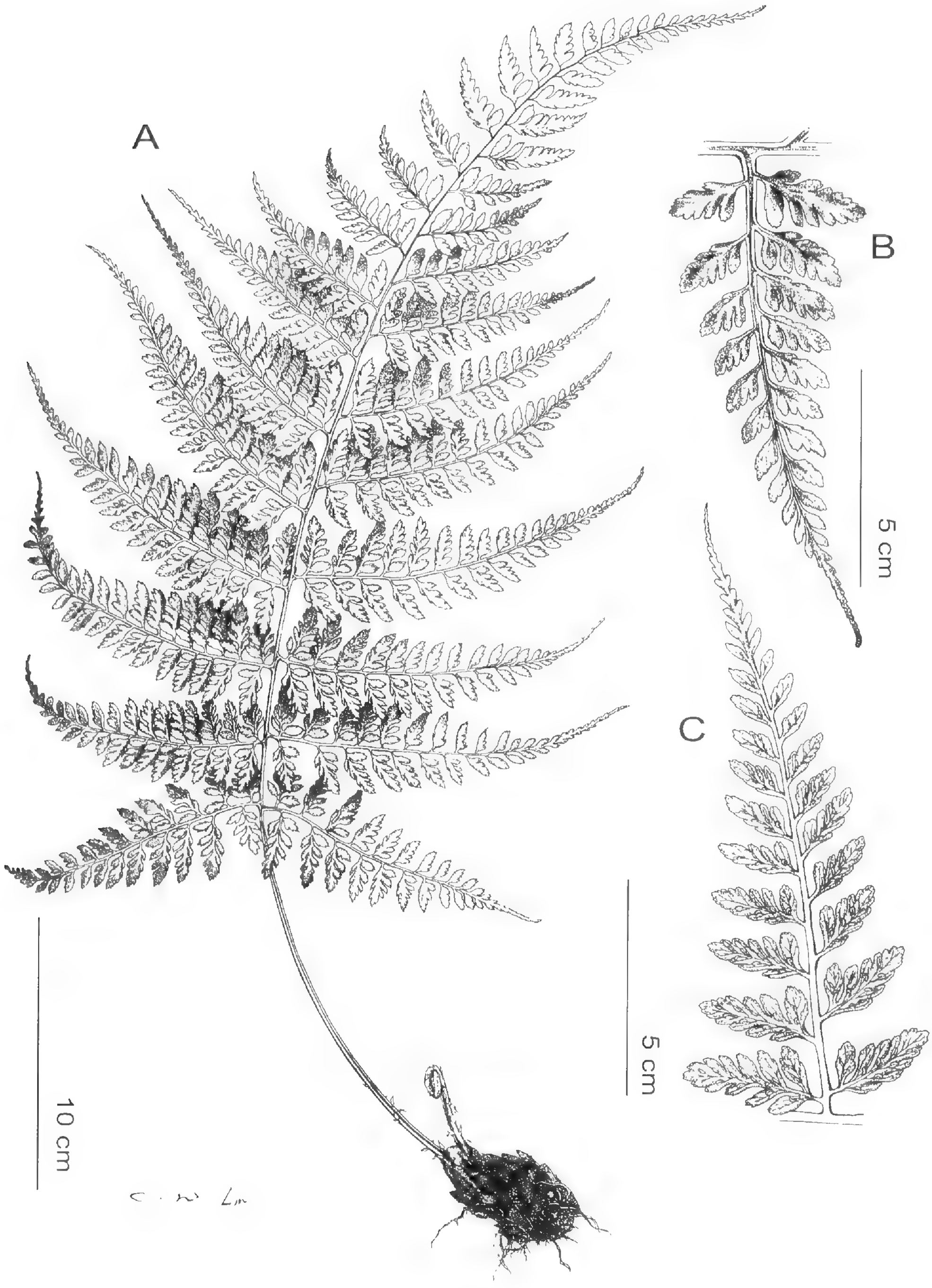


FIG. 2. Illustration of *Athyrium minimum*. **A**. Habit. **B**. Representative pinnae (adaxial surface) **C**. Representative sori position and venation (abaxial surfaces).

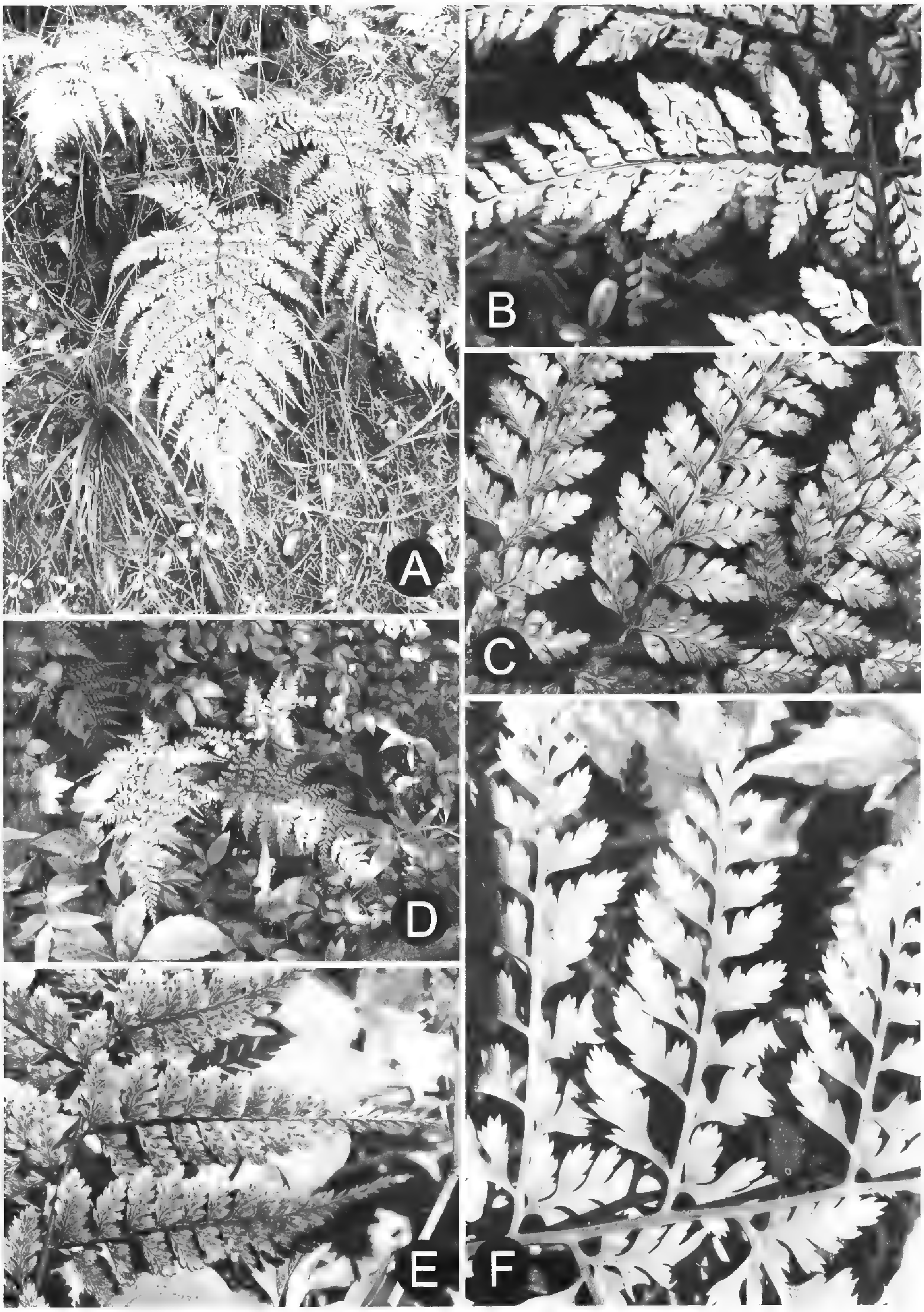


FIG. 3. *Athyrium tripinnatum* and *Athyrium minimum*. A–C *A. tripinnatum*. A. Habitat, B. Adaxial side view of lower pinnae base, C. Abaxial side view of pinnae; D–F *A. minimum*. D. Habitat, E. Adaxial side view of pinnae, F. Abaxial side view of pinnae.

frond length. When investigating native populations of it, the authors found that the quite small plants within the population were fertile and corresponded to the type specimen of *A. minimum*. Smaller individuals were usually found growing on huge moss-covered rocks and larger plants were growing on the slopes of a streamlet. Plant-size showed a cline of variation between the two types of habitat. Except for their variable size, they all resembled the type specimen of *A. minimum* in frond-morphology. Before the present report, *A. minimum* was only known from the type specimen, which has three small plants on the sheet. *Athyrium minimum* was difficult to recognize as, from the type specimen, it looked similar to small plants of different candidate species but did not appear to be, distinctly, any one of these species. Basing his identification on only one specimen led Wang (1997) to treat *A. minimum* as belonging to a distinct section *Minima* Z. R. Wang. However, based on more specimens and on observations of the native populations in the field in Taiwan, we believe it belongs to section *Echinoathyrium*, whereto we now transfer it.

*Athyrium minimum* looks similar to *A. iseanum*, but the latter has obvious long-soft spines on the adaxial side of costae and costules, and its chestnut-colored to dark brown lanceolate scales are different from those of the former. Furthermore, these two species have different habitats in Taiwan. *Athyrium iseanum* occurs mainly on the flat floor of shaded forests, at an altitude of 1700–2500 m, but *A. minimum* populations of ca. 100 individuals could only be found at lower altitudes, from 800–950 m in the Yangminshan area of north Taiwan.

## Key to sections and species that could be confused with *A. tripinnatum* and *A. minimum* in Taiwan

1. Upper surface of frond without spine-like processes along the costae and costules. . . . . 2
   2. Rhizome short creeping; pinnae sessile, not auricled at acroscopic pinnae bases . . . . . Sect. *Niponica*
   2. Rhizome erect, pinnae petiolate, auricled at acroscopic pinnae bases . . Sect. *Polystichoides*
1. Upper surface bearing spines/setae along the costae and/or costules, perispore without folds 3
   3. Indusia usually short-linear or oblong, straight, lateral along the veinlets, often close to the segment-midrib; scales at stipe-bases often black or dark-brown. . . . . Sect. *Otophora*
   3. Indusia J-shaped, horseshoe-shaped, reniform, elliptic, short-linear etc., lateral, crossing or dorsal on veinlets; scales at stipe bases often yellow-brown, brown or dark brown . . . . . Sect. *Echinoathyrium*, 4
      4. Pinnules or lobes of supra-medial pinnae catadromic or subopposite; rachis and costae usually pale purplish red, occasionally stramineous, pubescent abaxially. . . . . *A. vidalii* (Fr. *et* Sav.) Nakai
      4. Pinnules or lobes of supra-medial pinnae anadromous, occasionally catadromic or subopposite, rachis and costae stramineous, rarely pale purplish red, glabrous or sparsely pubescent abaxially . . . . . 5
         5. Fronds bipinnatifid, pinnules or lobes downwards-reflexed or not flat. . . . . *A. oppositipenna* Hayata
         5. Fronds tripinnatifid to tripinnate, pinnules or lobes flat . . . . . 6
            6. Adaxial surface of costae and costules pubescent; frond length usually more than 50 cm . . . . . *A. tripinnatum*
            6. Adaxial surface of costae and costules glabrous, or sparsely pubescent on grooves; frond length usually less than 40 cm. . . . . *A. minimum*

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## Erratum

**AFJ volume 97, issue 2, pp. 66-80 (April – June 2007)**

Figure 2 in the *American Fern Journal* article entitled **Ten New Species and Two New Combinations of *Blechnum* (Blechnaceae, Pteridophyta) from Bolivia** by Kessler et al. was originally published incorrectly. The habit of *Blechnum bruneum* has been corrected in the figure below (Fig. 2, J).

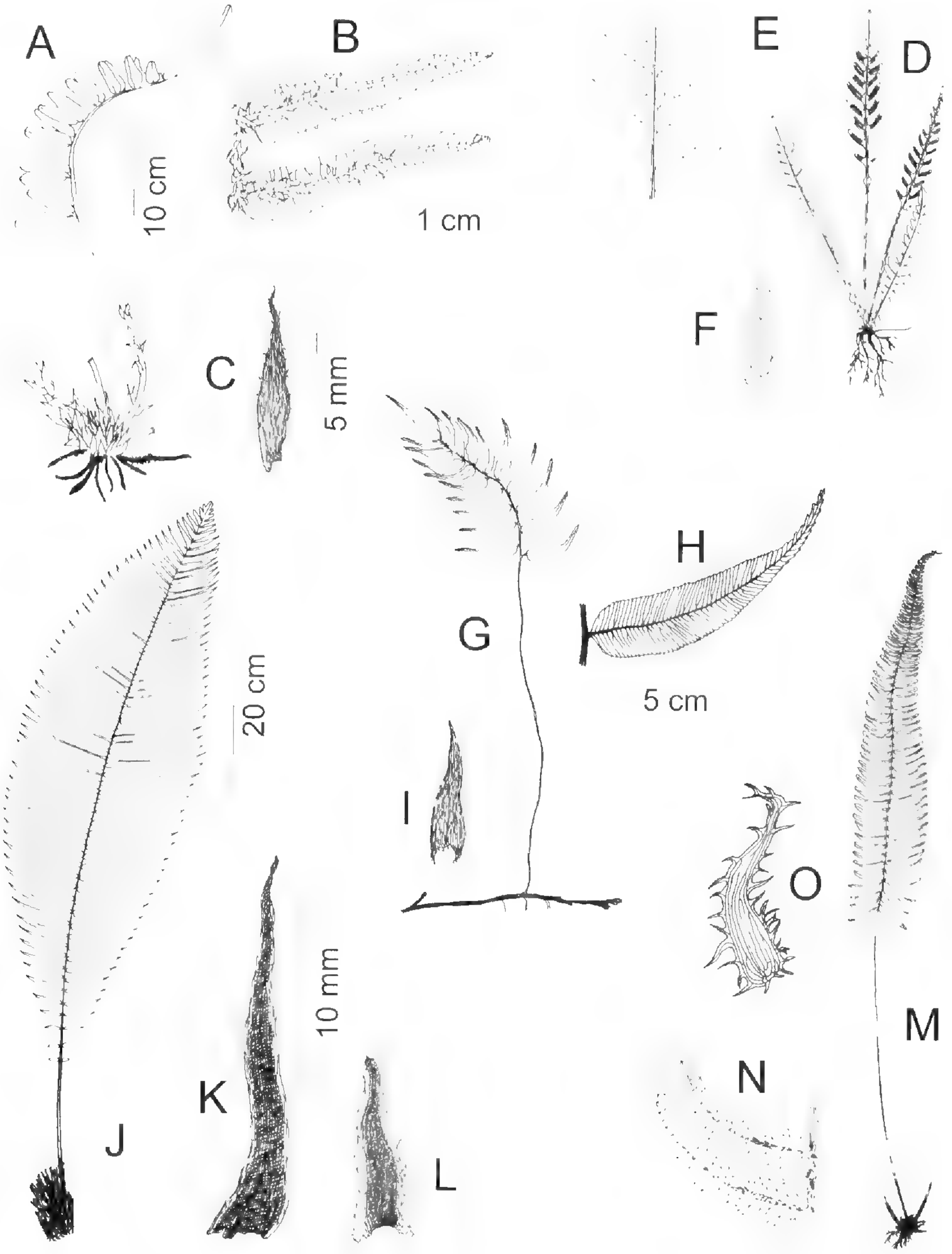


Fig. 2. *Blechnum reflexum*: A. Habit, B. Median pinnae, C. Proximal petiole scale. *Blechnum vallegrandense*: D. Habit, E. Median pinnae, F. Rhizome scale. *Blechnum repens*: G. Habit, H. Median pinna, I. Rhizome scale. *Blechnum bruneum*: J. Habit, K. Rhizome scale, L. Petiole scale. *Blechnum smilodon*: M. Habit, N. Median pinnae, O. Rachis scale. The drawings of habit, pinnae, and scales, respectively, are to the same scale for the upper two and lower three species.

# INFORMATION FOR AUTHORS

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# Desiccation and Rehydration Experiments on Leaves of 43 Pteridophyte Species

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ABSTRACT.—We conducted desiccation and rehydration experiments on the detached leaves of 43 pteridophyte species to assess the variability of drought adaptation strategies among pteridophytes. We found complete grades of desiccation responses from poikilohydric to homoiohydric strategies and within the latter category from mesomorphism to xeromorphism. These results suggest that pteridophytes have repeatedly evolved a wide variety of drought adaptation strategies (poikilohydry and xeromorphism at least 12 and 6 times, respectively) that are not adequately described by a simple distinction between homoiohydry and poikilohydry.

KEY WORDS.—drought adaptation strategies, homoiohydry, mesomorphism, poikilohydry, xeromorphism, desiccation, rehydration

Ferns and lycophytes are most abundant and species-rich in humid habitats (e.g., Kessler, 2001), but a considerable number of species have also become adapted to arid conditions (Pickett, 1931; Gaff, 1977; Page, 2002). Most pteridophytes can be characterized as mesomorphic and without any special adaptations to drought stress. However, numerous taxa show a variety of adaptive strategies to drought stress and desiccation (Table 1). These adaptive strategies have long attracted the attention of plant physiologists, and detailed ecophysiological studies have been performed on a number of taxa, including a variety of cheilanthoid ferns (Pickett and Manuel, 1926; Iljin, 1931; Hevly, 1963; Quirk and Chamber, 1981), *Hymenophyllum* (Härtel, 1940 a, b; Hietz and Briomes, 1998; Proctor, 2003), *Asplenium ceterach* L. (Rouschal, 1938), *Polypodium vulgare* L. (Kappen, 1964), and in particular *Pleopeltis polypodioides* (L.) E.G. Andrews & Windh. and its relatives (Pessin, 1924, 1925; Stuart, 1968; Müller *et al.*, 1981). These studies focussed on taxa with particularly conspicuous water drought adaptations, especially poikilohydry, and covered only a small range of the phylogenetic and physiological variability within ferns and lycophytes. Accordingly, there is currently no taxonomically representative sampling of drought adaptation strategies for pteridophytes. For example, poikilohydry has been documented in 19 pteridophyte genera (Proctor and Pence, 2002), belonging to at least 12 independent evolutionary lineages as recognized in the phylogenies of Schneider *et al.* (2004) and Smith *et al.* (2006). The anatomical, morphological, and physiological mechanisms involved in poikilohydry differ among several of these lineages. A conspicuous example of contrasting mechanisms is presented by the filmy-ferns with their one-cell-thick leaves that rehydrate by direct water contact (Härtel, 1940 a, b) and *Pleopeltis polypodioides* and its

TABLE 1. Several plant adaptive strategies to counter drought stress, and their representation among pteridophytes (modified from Levitt, 1958; Benzing, 1990; Lösch, 2001). The five lower categories together form the group of homoiohydric strategies.

| Strategy | Morphological and phenological characters | Reaction of leaves to desiccation | Reaction of leaves to rehydration | Fern taxa (examples) |
|---|---|---|---|---|
| Poikilohydric | leaves dry out under water stress and rehydrate when water is available, often densely scaly | rapid; leaves roll up; up to 95% water loss possible without lethal effects | rapid full recovery; water uptake through leaf surfaces | cheilanthoids, *Hymenophyllum*, *Pleopeltis* and allies, some *Selaginella* |
| Mesomorphic | leaves of intermediate thickness, soft to hard, sensitive to desiccation; no special adaptations to drought stress | rapid; leaves wither strongly and develop necroses after slight water loss | slow; water content does not or very slowly recover to original levels | most ferns |
| Xeromorphic | leaves thick, hard, narrow, often longitudinally folded or rolled, with abundant sclerenchyma; leaf surfaces with a waxy cover; stomata abundant, sunken into the leaf surfaces; venation density high | slow; leaves maintain their shape and thickness; survive moderate water loss | slow; water uptake mostly through roots | some *Asplenium*, *Campyloneurum*, some *Elaphoglossum*, *Niphidium*, some *Polypodium* |
| Succulent | leaves or rhizomes thick and spongy, strongly change their thickness in response to hydration status | slow; leaves become thinner as they dry out and survive high water loss | slow through leaves; water uptake mostly through roots | *Lemmaphyllum*, *Pyrrosia* |
| Drought-deciduous | leaves, pinnae, or pinnules are shed in the dry season, thin to medium thickness | rapid to intermediate; leaves, pinnae, or pinnules are shed under water stress | unknown | some *Adiantum*, *Nephrolepis*, *Phlebodium* |
| Impounding | leaves arranged in a funnel; water is not impounded directly as in bromeliads, but mostly stored in accumulated organic material | unknown | unknown | some *Asplenium* |

relatives that absorb water through specially adapted epidermal scales (Müller *et al.*, 1981). In other cases, a full transition from poikilohydric to homoiohydric behavior has been documented within a single species. For example, European *Polypodium vulgare* has homoiohydric responses in winter when water stress is negligible, and poikilohydric responses in summer when dry spells are frequent (Kappen, 1964). Thus, the simple distinction between these contrasting drought stress adaptations (Table 1) hide a wide spectrum of different, intermediate, or mixed strategies (Proctor and Tuba, 2002).

Given the taxonomically and physiologically biased sample of studies of drought adapation strategies among pteridophytes, the aim of the present study was to assess the variability of drought stress strategies among pteridophytes by experimentally desiccating and rehydrating excised leaves (leafy stem sections in the case of *Selaginella*) from 43 pteridophytes belonging to a wide range of taxonomic groups and morphological types. Our basic assumption was that the different adaptive strategies could be distinguished by the reaction of leaves to desiccation and rehydration (Table 1). This applies to water loss, since most transpiration takes places through the leaves. Water uptake in ferns mostly takes place through the roots, and our experiments with excised leaves can only distinguish between poikilohydric taxa capable of water absorption through leaves and homoiohydric taxa incapable of doing so. Historically, the study of detached leaves has provided reliable insights into plant physiology in general (Leprince and Golovina, 2002) and due to the difficulty of drying and weighing whole plants, has been the method of choice for desiccation experiments with ferns (Pessin, 1924, 1925; Pickett and Manuel, 1926; Iljin, 1931; Rouschal, 1938; Härtel, 1940 a, b; Kappen, 1964; Stuart, 1968; Müller *et al.*, 1981; Quirk and Chamber, 1981; Proctor, 2003).

## Material and Methods

Leaves or stem sections of 43 pteridophyte species were obtained from the living collection of the Botanical Garden of the University of Göttingen, Germany (Table 2). Species were selected to cover a wide range of taxa and life forms, with a focus on groups exhibiting an obvious variety of adaptations to drought stress (Table 1) and included 13 mesomorphic, 11 poikilohydric, four xeromorphic, two drought-deciduous, one water-impounding, and 12 intermediate species. An intermediate category was established for species that could not be unambiguously placed in either the mesomorphic or xeromorphic categories. No succulent species were available for study.

Experiments were conducted with excised leaves, pinnae (in the case of large fronds), or stem section (*Selaginella*). Total water content of fully hydrated leaves was determined prior to the desiccation experiment. Freshly cut leaves from well-watered plants were fully hydrated by wetting their surfaces and placing them overnight in closed plastic bags. Leaf surfaces were then dried with tissue paper and briefly dried at ambient temperature, and the leaves weighed to the closest 0.001 g on a precision scale. Total dry mass was determined by placing the leaves in a drying oven for two to three days at

TABLE 2. Desiccation resistance, desiccation delay, and recuperation capacity of excised leaves from 43 species of pteridophytes. Letters in brackets after the names indicate wether whole leaves (L), pinnae (P), or stem sections (S) were studied.

| Species | Morphological category | Desiccation resistance (% water contents) | Desiccation delay (hours) | Recuperation capacity (% water contents) |
|---|---|---|---|---|
| *Adiantum capillus-veneris* L. (L) | deciduous | 81 | 2 | 87 |
| *Adiantum macrophyllum* Sw. (L) | intermediate | 99 | 4 | 59 |
| *Adiantum trapeziforme* L. (L) | mesophytic | 51 | 2 | 84 |
| *Adiantum villosum* L. (L) | intermediate | 86 | 3 | 100 |
| *Anemia mexicana* Klotzsch (L) | poikilohydric | 74 | 2 | 75 |
| *Anemia rotundifolia* Schrad. (L) | poikilohydric | 86 | 3 | 90 |
| *Anemia tomentosa* (Sav.) Sw. (L) | poikilohydric | 99 | 4 | 67 |
| *Asplenium bulbiferum* G. Forst. (L) | xerophytic | 73 | 6 | 84 |
| *Asplenium daucifolium* Lam. (L) | intermediate | 86 | 6 | 54 |
| *Asplenium dimorphum* Kunze (L) | intermediate | 65 | 6 | 78 |
| *Asplenium mannii* Hook. (L) | mesophytic | 30 | 1 | 82 |
| *Asplenium marinum* L. (L) | intermediate | 81 | 3 | 76 |
| *Asplenium nidus* L. (L) | impounding | 58 | 7 | 57 |
| *Asplenium rutaefolium* Kunze (L) | intermediate | 75 | 5 | 80 |
| *Asplenium salicifolium* L. (L) | intermediate | 80 | 7 | 68 |
| *Blechnum brasiliense* Desv. (P) | mesophytic | 47 | 2 | 86 |
| *Blechnum cordatum* (Desv.) Hieron. (P) | mesophytic | 59 | 1 | 90 |
| *Blechnum occidentale* L. (L) | mesophytic | 68 | 1 | 96 |
| *Blechnum spicant* (L.) Roth (L) | intermediate | 81 | 4 | 55 |
| *Campyloneurum xalapense* Fée (L) | xerophytic | 81 | 7 | 74 |
| *Cheilanthes myriophylla* Desv. (L) | poikilohydric | 99 | 6 | 38 |
| *Cheilanthes notholaenoides* (Desv.) Maxon ex Weath. (L) | poikilohydric | 85 | 5 | 96 |
| *Elaphoglossum apodum* (Kaulf.) Schott ex J. Sm. (L) | mesophytic | 60 | 4 | 59 |
| *Elaphoglossum crinitum* (L.) Christ (L) | mesophytic | 63 | 2 | 49 |
| *Elaphoglossum engelii* (Karst.) Christ (L) | xerophytic | 44 | 7 | 75 |
| *Elaphoglossum erinaceum* (Fée) T. Moore (L) | mesophytic | 74 | 2 | 66 |
| *Elaphoglossum latifolium* (Sw.) J. Sm. (L) | intermediate | 74 | 6 | 49 |
| *Hemionitis arifolia* (Burm.) T. Moore (L) | poikilohydric | 96 | 4 | 75 |

TABLE 2. Continued.

| Species | Morphological category | Desiccation resistance (% water contents) | Desiccation delay (hours) | Recuperation capacity (% water contents) |
|---|---|---|---|---|
| *Hemionitis palmata* L. (L) | poikilohydric | 96 | 13 | 91 |
| *Microgramma piloselloides* (L.) Copel. (L) | poikilohydric | 95 | 6 | 35 |
| *Pecluma eurybasis* (C. Chr.) M.G. Price (L) | poikilohydric | 91 | 4 | 80 |
| *Pellaea sagittata* (Cav.) Link (L) | deciduous | 72 | 1 | 64 |
| *Polypodium australe* Fée (L) | intermediate | 91 | 6 | 47 |
| *Polypodium scouleri* Hook & Grev. (L) | xerophytic | 70 | 36 | 49 |
| *Polystichum lachenense* (Hook.) Bedd. (P) | intermediate | 89 | 2 | 100 |
| *Polystichum platyphyllum* (Willd.) C. Presl (P) | mesophytic | 74 | 4 | 72 |
| *Pteris quadriaurita* Retz. (P) | mesophytic | 46 | 2 | 99 |
| *Selaginella geniculata* (C. Presl) Spring (S) | mesophytic | 86 | 3 | 83 |
| *Selaginella helvetica* (L.) Link (S) | poikilohydric | 99 | 2 | 63 |
| *Selaginella martinensii* Spring. (S) | mesophytic | 67 | 2 | 93 |
| *Selaginella rotundifolia* Spring. (S) | mesophytic | 88 | 2 | 56 |
| *Selaginella trisulcata* Aspl. (S) | poikilohydric | 90 | 4 | 58 |
| *Selaginella vogelii* Spring. (S) | mesophytic | 69 | 1 | 86 |

105°C until weight constancy was achieved. Total water content was then calculated by subtracting its dry mass from the fully hydrated mass. In the actual desiccation and rehydration experiments, different fully hydrated leaves (see above) were placed in a drying oven at 40°C for 20 different time periods (0.5, 1, 2, 3, ..., 14, 15, 18, 24, 30, and 36 hours) and weighed afterwards. These leaves were then wetted and placed in closed plastic bags for one to three days until reaching weight constancy. Water loss and absorption were expressed as the percent value of the total water content of leaves for each species. Leaves were considered to be lethally damaged when at least 50% of the lamina tissue showed necroses. Replicates (five each) were made of only three species (*Adiantum capillus-veneris* L., *Blechnum brasiliense* Desv., and *Pteris quadriaurita* Retz.) due to the limited material available of most species.

We calculated the following parameters: *desiccation resistance*, the percentage of the total water contents that could be lost before leaves were lethally damaged; *desiccation delay*, the time until the leaves became lethally damaged; *recuperation capacity* (resaturation), the percentage of the original water contents that was regained at the point of desiccation resistance.

## Results

The studied species showed a wide variation of all measured parameters (Fig. 1, Table 2). For example, lethal desiccation levels were reached in different species after 1 to 36 hours and at water losses of 30–99%, as determined from the desiccation delay and desiccation resistance parameters, respectively (Table 2). It was not possible to discern distinct groups of species that could be assigned to specific drought adaptation strategies (Fig. 2). Because excised pinnae or stem sections were only used for a few, usually related species (five and six species, respectively), no formal test was possible of whether these samples differed systematically from species studied with entire leaves, but visual inspection of the results show no apparent trends.

The three species with five replicate measurements each showed reproducible patterns, with measurements having on average a coefficient of variation of 12%, which is much lower than the variation observed between species (54%).

## Discussion

To our knowledge, this is the first study to compare the desiccation and rehydration behavior of a large number of pteridophyte species with a consistent method. In the interpretation of the results it should be borne in mind that the experiment was conducted on detached leaves, and that the absolute values of the dehydration behaviors therefore do not correspond to the values of entire and intact living plants. This is especially relevant for the ability of the plants to rehydrate, because of the difficulty of accurately determining the health status of leaves based only on external visual examination. As a result of using excised leaves, some leaves were certainly

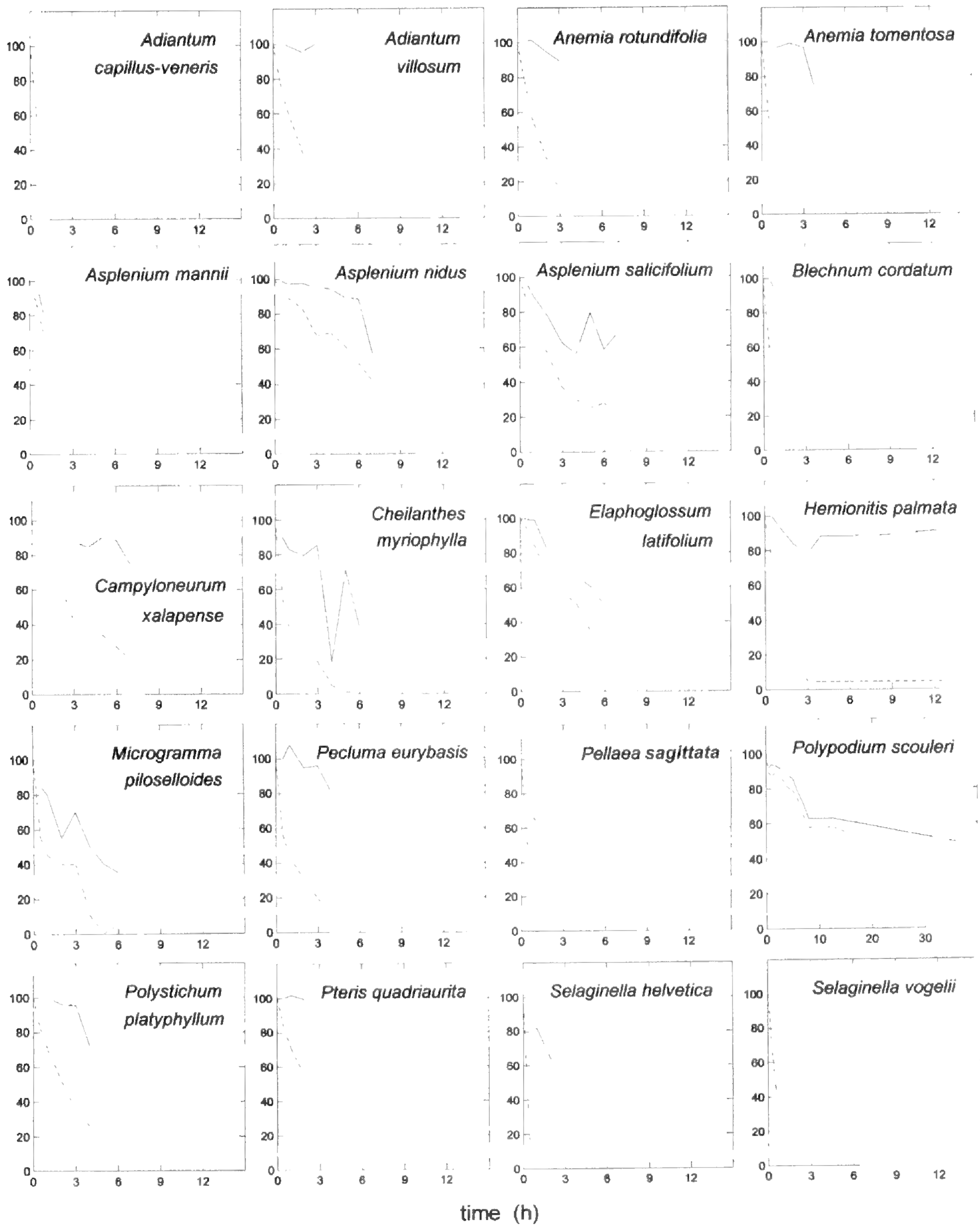


FIG. 1. Desiccation and rehydration responses of detached leaves, pinnae or leafy stem sections (in *Selaginella*) of 20 selected pteridophyte species. Species were chosen to represent the range of observed responses to the desiccation and rehydration experiments. Dashed lines show the water content when desiccated, continuous lines represent the water content after redydration.

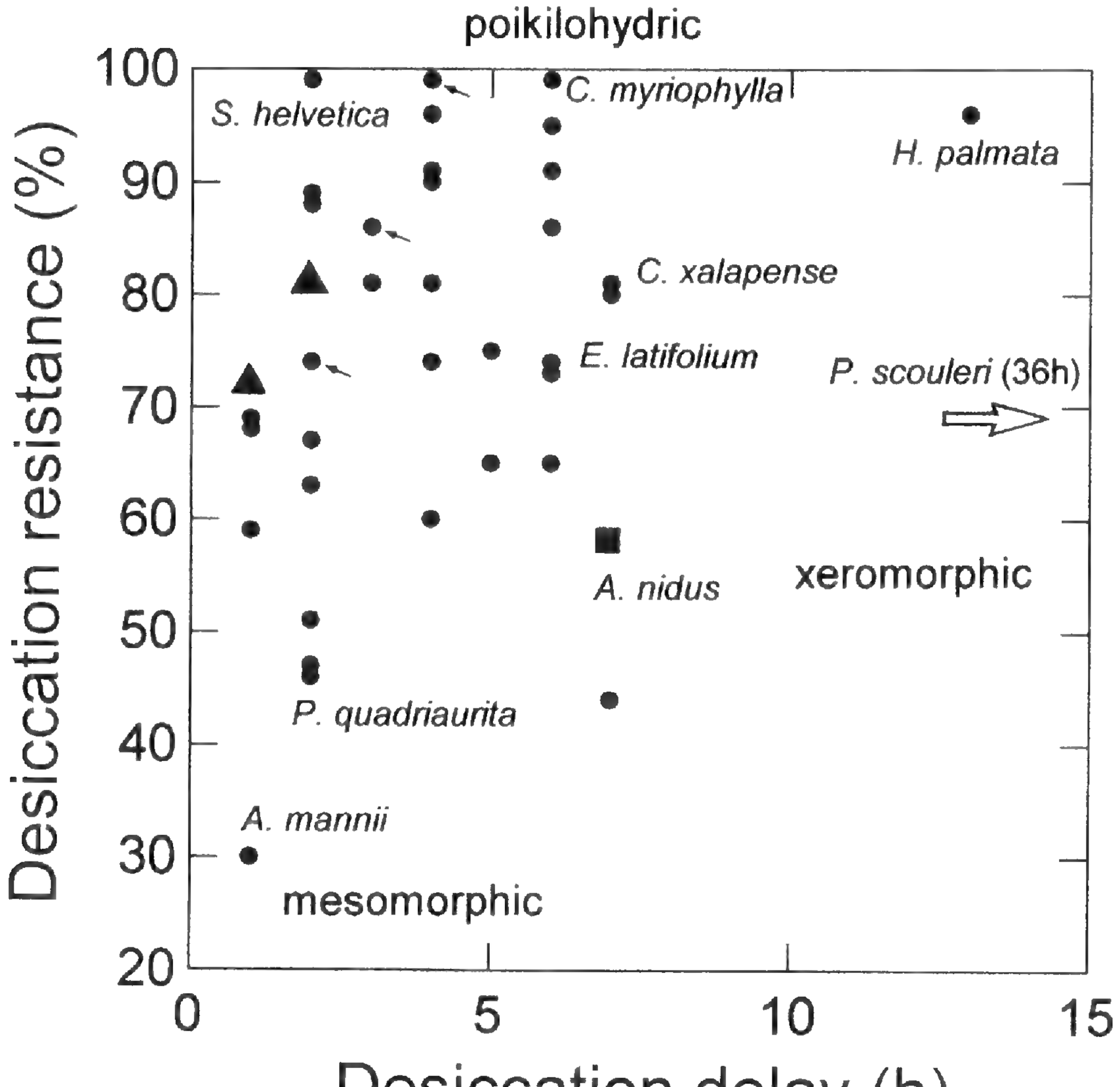


FIG. 2. Desiccation resistance versus desiccation delay of 43 pteridophyte species. Deciduous species are indicated by triangles, the water-impounding species by a squares, and species of *Anemia* by small arrows. All remaining species are indicated by circles. For the sake of clarity, only some distinctive species mentioned in the text are labelled with names.

more severely damaged by the handling than others, affecting their recuperation capacity. This is evident in Fig. 1, where, e.g., *Cheilanthes myriophylla* showed a wide variation of measurements, suggesting that some individual leaves might have been more strongly damaged than others. A general pattern observed for most species was the gradual decline in desiccation resistance and recuperation capacity with increasing desiccation time. The first of these declines clearly reflects the gradual loss of water over time, whereas the second one presumably reflects the physiological or anatomical damage due to longer and stronger desiccation that reduces the ability of the leaves to rehydrate. Despite these unavoidable methodological shortcomings, the present study is comparable to previous desiccation experiments with ferns that also used detached leaves (Pessin, 1924, 1925; Pickett and Manuel, 1926; Iljin, 1931; Rouschal, 1938; Härtel, 1940 a, b; Kappen, 1964; Stuart, 1968; Müller *et al.*, 1981; Quirk and Chamber, 1981; Proctor, 2003).

Perhaps the most important result of our study is the high and gradual variability in the responses to desiccation and rehydration among the species examined. Some species showed patterns that could easily be attributed to a "classic" drought adaptation strategy. Examples included *Asplenium mannii* (mesomorphic) that died quickly after relatively limited loss of water (30%), *Hemionitis palmata* (poikilohydric) that survived loss of most (96%) of its water and tolerated desiccation for a long period of time (13 hours), and *Polypodium scouleri* (xeromorphic) that dried out most slowly (desiccation delay = 36 hours). However, most species showed intermediate patterns and considering all species together there were complete grades of desiccation and rehydration responses from poikilohydric to homoiohydric species and within the latter category from mesomorphic to xeromorphic species. The cline of mesomorphic and xeromorphic behaviors has also been documented within single species by Kappen (1964).

As an example of variability within a given drought adaptation strategy we can compare the poikilohydric taxa *Anemia* and the cheilanthoid ferns (*Cheilanthes*, *Hemionitis*). One species of *Anemia* has been shown experimentally to be poikilohydric (Proctor and Pence, 2002), and our field experience with a number of other species in Bolivia also shows that these behave as poikilohydric species: their leaves roll up under drought stress and become brittle and brownish, but the plants can be readily rehydrated by placing them in plastic bags with water (M. Kessler, pers. obs.). However, in the experiments conducted here, two of three species of *Anemia* (marked by black arrows in Fig. 2) had a much lower desiccation resistance and desiccation delay than cheilanthoid ferns which are well-documented to be poikilohydric (Pickett and Manuel, 1926; Iljin, 1931; Hevly, 1963; Quirk and Chamber, 1981). Among the unambiguously poikilohydric species we measured water losses (desiccation resistance) of 74–99% (Table 2). This corresponds well with previous studies which have documented values of up to 94% water loss in *Notholaena marantae* R. Br. (Iljin, 1931), 95% in *Polypodium vulgare* (Lösch, 2001), 97% in *Pleopeltis polypodioides* and relatives (Müller *et al.*, 1981), and 96–98% in *Asplenium ceterach* (Rouschal, 1938).

Looking at taxa with unusual strategies, the desiccation behavior of the two deciduous species (*Adiantum capillus-veneris*, *Pellaea sagittata*; triangles in Fig. 2) was intermediate between poikilohydric and mesomorphic species. The difference between these groups is therefore found in their response to low leaf water content, with deciduous species shedding their leaves or pinnae when they are too dry. There are also some species with mixed poikilohydric/deciduous strategies, such as *Argyrochosma nivea* (Poir.) Windham. Our field experience with this species in the Bolivian Andes shows that at the beginning of the dry season dried-out plants can be readily rehydrated in wet plastic bags. Several months later at the end of the dry season, leaves had apparently died and pinnules had been shed, leaving only the naked rachises (M. Kessler, pers. obs.). It would be interesting to know if deciduousness in pteridophytes is associated with an active recovery of nutrients or assimilates from the

leaves, which would represent a distinct advantage over mesomorphic species that loose all tissue once the leaves are too dry. Considering the other unusual strategy, the only impounding species, *A. nidus*, behaved like xeromorphic taxa.

In conclusion, our study confirms the notion of Proctor and Tuba (2002) that plants have evolved a wide variety of drought adaptive strategies that are not adequately described by a simple distinction between homoiohydry and poikilohydry. The large number of cases in which poikilohydry has independently evolved in pteridophytes, at least 12 times and likely more often, suggests that different physiological mechanisms may have been developed to achieve the same adaptive response. A similar case can be made for xeromorphic adaptations, which appear to have evolved independently at least half a dozen times (M. Kessler, unpubl. data). A comparative study of the adaptive efficiency of these different evolutionary cases might yield interesting insights into the physiological constraints and possibilities present among pteridophytes.

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# Northward Invasion and Range Expansion of the Invasive Fern *Thelypteris dentata* (Forssk.) St. John into the Urban Matrix of Three Prefectures in Kinki District, Japan

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ABSTRACT.—This study investigated the current distribution of an invasive tropical fern, *Thelypteris dentata*, and its habitat type in three Japanese prefectures (Osaka, Kyoto, and Shiga) in the Kinki District. The results showed that *T. dentata* has expanded its distribution into highly urbanized areas in Osaka Prefecture and has reached southern Kyoto Prefecture and central Shiga Prefecture. The distribution of *T. dentata* populations thus seems to have expanded northward based on comparisons with the distribution that was determined in the 1980s. Because the fern's habitat types were mainly the side walls or bottoms of drainage channels, crevices in stone walls and roadsides, the urban matrix has not served as a barrier to the expansion of the range of *T. dentata*; on the contrary, it may be serving as a type of heat island corridor that is facilitating the spread of this species.

KEY WORDS.—*Thelypteris dentata*, range expansion, urban heat island, global warming, greenhouse weed

The downy maiden fern (*Thelypteris dentata* (Forssk.) St. John) is considered to be an alien fern that invaded Japan from tropical or subtropical countries (Yamazumi, 1988; Hotta, 2001). In the Honshu area, the first occurrence of *T. dentata* was documented in 1951 in southern Wakayama Prefecture of Kinki District (Yamazumi, 1988). Although the fern was initially treated as a rare species (Yamazumi, 1988), by the 1970s it had been reported in several additional places, mainly in southern coastal areas of Honshu such as southern Wakayama and Mie prefectures (Manago, 1986). The Japanese distribution of *T. dentata* was described along with that of native ferns and fern allies by Kurata and Nakaike (1983). According to their survey, in the Kinki District, the fern was found at 25 locations in Wakayama Prefecture, four in Mie Prefecture, and one in Hyogo Prefecture. The ages of voucher specimens used to create this distribution map dated mainly from the 1960s to the

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beginning of the 1980s. Detailed reports of the distribution of *T. dentata* were not found in the research literature after 1983, apart from a distribution map for Wakayama Prefecture by Manago (1986). Nakajima (1998), however, predicted that the distribution of *T. dentata* might have expanded since 1983 as a result of global warming.

We investigated potential changes in the distribution of *Thelypteris dentata* since the release of its distribution map in 1983. Because we were unable to investigate changes in the national distribution of *T. dentata*, we focused on its trends in 3 prefectures (Osaka, southern Kyoto, and Shiga prefectures) in the Kinki District of western Japan. These study areas were selected because the urban heat island effect (which can facilitate the survival and growth of invasive tropical species) is remarkable in Osaka and Kyoto cities (Ohashi and Kida, 2002). The term "urban heat island" is generally used for a city or industrial site having consistently higher temperatures than the surrounding rural areas because of the greater retention of heat by buildings, concrete, and asphalt. These cities and their outskirts are thus considered to be important areas in which the relationship between increasing temperature and the expanding distribution of plant species can be investigated.

## Materials and Methods

Field investigations were carried out by one or two investigators at each site from July 2004 to October 2006. The study focused on a 500-m radius around railway stations. The main reason for selecting railway stations as the center of each study site was that it has been reported that the primary habitats of the fern are human-made habitats such as walls (Manago, 1986; Reis *et al.*, 2006) and that these habitats are common features around railway stations. In total, we surveyed 79 railway stations in central Shiga Prefecture, southern Kyoto Prefecture, and Osaka Prefecture; these included 11 stations between Yamashina and Omi-Maiko on the Kosei Line of the Japanese Railways (J. R.), 35 stations between Nagahama and Osaka on the Hokuriku Line or Tokaido Line of the J. R., 18 stations on the Osaka Loop Line of the J. R., and 15 stations between Nanba and Misaki Koen on the Nankai Honsen Line of the Nankai Railways. Downy maiden fern was not found in the additional investigations conducted around Fukui and Tsuruga Stations (which are to the north of Shiga Prefecture) in July 2005 and August 2006.

In order to investigate each site at an equivalent intensity, the maximum investigation time was set at 2 h per site. The number of individuals at each site was classified into two categories (1–10 and 10–100 individuals). The microhabitat type for each fern was classified into several categories: crevices in stone walls, side walls or the bottom of drainage channels, alleys between buildings, roadside habitats, or in planters. The populations identified by the autumn of 2005 were investigated in the following spring in order to determine whether each individual had survived the winter. The meteorological data for our study areas were collected from nearby meteorological stations by using the Japan Meteorological Agency website (Japan Meteorological Agency, 2006).

In order to investigate the increase in temperature at each site, the temperatures were compared between the two study periods (1970–1982 and 1983–2005). Two-tailed t-tests were performed for statistical analysis, with significance set at $P < 0.05$.

## Results

The meteorological data for our study areas are shown in Table 1. All temperatures were higher after 1983 than before; most temperatures were significantly higher, with the exception of those at Torahime and Otsu (Table 1). This was particularly true for the highly urbanized cities of Osaka and Kyoto, and for Hikone City in central Shiga Prefecture.

Individuals of the downy maiden fern were discovered at 34 of the 79 sites (43.0%; Fig. 1; Table 2). The number of individuals in each population category recorded for each site is shown in Table 2; the total count was more than 250 individuals. The main habitats of the newly discovered populations were hard-surfaced human-made habitats such as side walls or the bottom of drainage channels or stone walls. The frequency of occurrence was higher in southern Osaka Prefecture than in the other areas. The northernmost population was discovered in Hikone City, in central Shiga Prefecture. The size of this population and of individual ferns were small, but the population was discovered in the winter of 2004, and had survived for at least two years until the spring of 2006. The distance of this population from the population in Wakayama Prefecture reported by Kurata and Nakaike (1983) and Manago (1986) was approximately 100 km, and the distance from southern Hyogo Prefecture, which was the northernmost location of the fern in Kinki District in the distribution map of Kurata and Nakaike (1983), was approximately 60 km. If we assume that the fern's distribution expanded northward for a total distance of at least 60 km during a period of about 20 years, the expansion distance averages approximately 3 km per year.

## Discussion

*Distribution of* Thelypteris dentata *in Japan.*—We recorded 34 new populations of *Thelypteris dentata* in three prefectures of Japan's Kinki District. *Thelypteris dentata* was recorded previously in local flora lists and publications (Nakaike, 1996; Kohata, 1997; Hiratsuka City Museum, 2001; Hotta, 2001; Mitsuta, 2002; Matsui *et al.*, 2003; Murakami *et al.*, 2003, 2004; Kita-Kawachi Nature Club, 2004), and some of these reports were in Kinki District (Mitsuta, 2002; Matsui *et al.*, 2003; Murakami *et al.*, 2003, 2004; Kita-Kawachi Nature Club, 2004). Downy maiden fern was also recorded from 2000 to 2004 in forests in Osaka and Kyoto Prefectures, including fragmented forests, wildlife habitat, and reclaimed forest in urban or suburban areas (Matsui *et al.*, 2003; Murakami *et al.*, 2003, 2004). In an investigation of the fern's distribution by the Kita-Kawachi Nature Club (2004), some populations of *T. dentata* were found from central to northern Osaka Prefecture. Mitsuta

TABLE 1. Temperature data at eight meteorological stations in and near the study area. (The data were obtained from the Japan Meteorological Agency website, 2006.) The locations of each station are shown in Figure 1. The statistical significance was determined by comparing the temperatures during the two study periods (1970 to 1982 and 1983 to 2005).

| | *Average annual temperature* (°C) | | | *Average minimum temperature* (°C) | | | *Average maximum temperature* (°C) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1970–1982 | 1983–2005 | Significance of difference | 1970–1982 | 1983–2005 | Significance of difference | 1970–1982 | 1983–2005 | Significance of difference |
| Torahime (a) | 13.2 | 13.9 | n.s. | −6.3 | −5.6 | n.s. | 33.7 | 34.8 | n.s. |
| Hikone (b) | 14.0 | 14.7 | ** | −4.8 | −3.7 | * | 34.3 | 35.1 | * |
| Otsu (c) | 14.8 | 14.9 | n.s. | −3.6 | −3.4 | n.s. | 34.7 | 35.2 | n.s. |
| Kyoto (d) | 15.3 | 15.9 | ** | −4.2 | −3.2 | * | 36.4 | 37.0 | ** |
| Osaka (e) | 16.1 | 16.9 | ** | −2.5 | −1.6 | * | 35.9 | 36.7 | ** |
| Sakai (f) | 15.3 | 15.8 | n.s. | −3.9 | 3.2 | n.s. | 34.5 | 36.4 | ** |
| Kumatori (g) | 14.9 | 15.6 | * | −2.6 | −2.1 | n.s. | 33.1 | 34.0 | * |
| Wakayama (h) | 16.0 | 16.7 | ** | −2.6 | −1.7 | ** | 35.3 | 35.7 | n.s. |

Differences between periods were determined using a two-tailed *t*-test: n.s., not significant; *, $p < 0.05$; **, $p < 0.01$

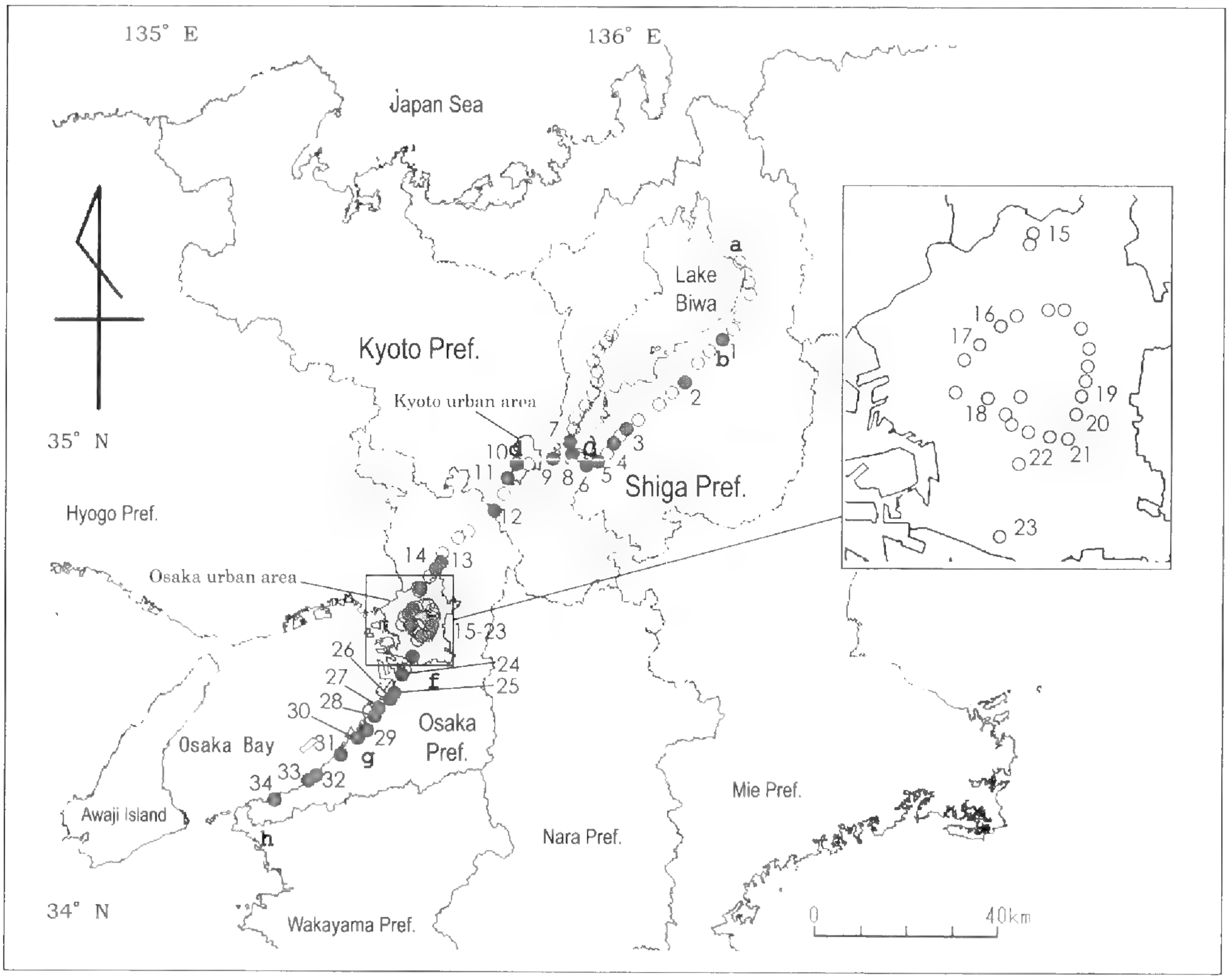


FIG. 1. Locations (the gray circles with numbers from 1 to 34) of newly discovered populations of *Thelypteris dentata* in Osaka, Kyoto, and Shiga prefectures of Japan's Kinki District. The open circles represent the study sites in which *T. dentata* was not discovered. Letters *a* through *h* represent the meteorological stations in Table 1.

(2002) also recorded *T. dentata* in a list of ferns and fern allies in Kyoto Prefecture, but did not report the locations of collected specimens or their distribution. The fern was not reported in Shiga Prefecture before the present investigation. Outside Kinki District, new populations have recently been reported from Funabashi City (Nakaike, 1996), which lies 500 km east of Osaka; in Isehara City (Hiratsuka City Museum, 2001), which lies 350 km east of Osaka; in Anjoh City (Hotta, 2001), which lies 150 km east of Osaka; and in Okayama City (Kohata, 1997), which lies 140 km west of Osaka. Because areas east or west of our study area were not investigated, it is unknown whether the fern's distribution is continuous from eastern Shiga Prefecture to Kanto Prefecture or from western Osaka Prefecture to the southern part of Chugoku Prefecture.

Some researchers noted that *T. dentata* is a species that invades new habitats by escaping from greenhouses and other artificial habitats (Uemura, 2000; Yamazumi, 1988), but it is unknown whether *T. dentata* followed this pattern in Japan. However, it is likely that many of the individuals we observed

originated in this manner because *T. dentata* is a greenhouse weed that has been reported from around the world (Wagner and Smith, 1993; Possley, 2004), and it has been observed in domestic greenhouses or accompanying potted plants in Japan (Yamazumi, 1988). We also found *T. dentata* in a greenhouse in Kyoto City in January 2006. Therefore, the probability of the fern's dispersal via greenhouses in each region seems to be high.

The current results do not clarify whether the Shiga and Kyoto populations genetically resemble the Osaka and Wakayama populations. *Thelypteris dentata* must have spread from greenhouses or potted plants purchased at floriculture stores in each area, but it is not clear whether northern individuals migrated from the southern Kinki District. However, the most important issue from the perspective of population genetics is not the dispersal routes of this species, but rather the fact that it has dispersed approximately 100 km from the Wakayama population, which was the primary location colonized by this species in the early 1980s (Fig. 2). The population identified in Moriyama City, in central Shiga Prefecture, included more than 20 full-grown individuals, thus it will not be difficult for this population to survive in the future.

The distribution of tropical species can usually be explained by the minimum winter temperature or by a cold index. The mean lowest temperature in Hikone City, which contained the northernmost population in our survey, was −3.7°C from 1983 to 2005 (Table 1). Although more detailed study is required to understand the ecology of *T. dentata*, this value provides a good preliminary indication of the threshold temperature for growth and survival of *T. dentata*.

Because of the limited area covered by our research, the populations that we discovered cannot be used to predict the northern limit of populations of this species in Kinki District. More information must be acquired in the future to confirm this limit. However, the climatic conditions in central Shiga Prefecture may be close to the northern distribution limit of the fern because the frequency of occurrence (25% of survey sites) is remarkably lower there than that in southern Osaka Prefecture (91.6% of survey sites).

*Habitats of* Thelypteris dentata.—During our investigation, a large population of *Thelypteris dentata* was discovered in the urban areas of Kinki District. Some individuals were also identified in central Osaka City, which is highly urban. The occurrence ratio was lower in central Osaka City (37.5%) than in the northern and southern Osaka areas (50% and 91.6%, respectively). This may have resulted from the lack of crevices at the edges of roads and the fact that fern may not be found in highly developed areas. However, some individuals were nonetheless observed in locations with large expanses of broken pavements and in walls with deep crevices, from where it was difficult to remove the fern's rhizome.

Most of the recorded microhabitat types were similar to those in previous reports (Manago, 1986; Hotta, 2001; Matsui *et al.*, 2003; Reis *et al.*, 2006): the side walls of drain channels, crevices in stone walls, roadsides, and alleys between buildings. Some individuals were discovered in pots or planters along with potted plants such as *Aloe* or *Cymbidium*. These ferns may have

TABLE 2. Locations of newly discovered populations of *Thelypteris dentata* and the associated number of individuals in each habitat type in Osaka, Kyoto and the Shiga prefectures of Kinki District, Japan. The open circles represent the *T. dentata* populations that survived from winter 2005 to spring 2006.

| | Name | Location | Number of sites at which *T. dentata* were recorded/ Number of sites surveyed | *T. dentata* populations that survived from winter 2005 to spring 2006 | Number of individuals in each microhabitat type | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | (Crevices of) stone walls | Sidewalls or bottom of drainage channels | Inner planted pots | Roadside | Alleys between buildings |
| *Shiga Prefecture* | | | 8 / 32 (25.0%) | | | | | | |
| 1 | Kawase | N35 13′ E136 13′ | | ○ | - | - | - | 1–10 | - |
| 2 | Aduchi | N35 08′ E136 08′ | | ○ | 1–10 | 1–10 | - | - | - |
| 3 | Moriyama | N35 03′ E135 59′ | | ○ | - | 10–100 | - | - | - |
| 4 | Kusatsu | N35 01′ E135 57′ | | ○ | 1–10 | 10–100 | - | - | - |
| 5 | Seta | N34 59′ E135 55′ | | | 10–100 | - | - | - | - |
| 6 | Ishiyama | N34 58′ E135 53′ | | | - | 10–100 | - | - | - |
| 7 | Nishi-Otsu | N35 01′ E135 51′ | | | 1–10 | - | - | - | - |
| 8 | Otsu | N35 00′ E135 51′ | | ○ | 1–10 | - | - | 1–10 | - |
| *Keihanshin area* (southern Kyoto Pref. and northern Osaka Pref.) | | | 6 / 12 (50.0%) | | | | | | |
| 9 | Yamashina | N34 59′ E135 49′ | | ○ | - | - | - | 1–10 | - |
| 10 | Nishi Oji | N34 58′ E135 43′ | | ○ | 1–10 | 1–10 | - | - | - |
| 11 | Mukoh-machi | N34 57′ E135 42′ | | ○ | - | 1–10 | - | - | - |
| 12 | Yamazaki | N34 53′ E135 40′ | | ○ | - | - | 1–10 | - | - |
| 13 | Senrioka | N34 47′ E135 33′ | | | - | - | 1–10 | - | - |
| 14 | Kishibe | N34 46′ E135 32′ | | | - | - | - | - | 1–10 |

TABLE 2. Continued.

| | Name | Location | Number of sites at which *T. dentata* were recorded/ Number of sites surveyed | *T. dentata* populations that survived from winter 2005 to spring 2006 | Number of individuals in each microhabitat type | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | (Crevices of) stone walls | Sidewalls or bottom of drainage channels | Inner planted pots | Roadside | Alleys between buildings |
| *Osaka City* (central Osaka Prefecture) | | | 9 / 23 (39.1%) | | | | | | |
| 15 | Higashiyodogawa | N34 40′ E135 30′ | | | - | - | - | 1–10 | - |
| 16 | Fukushima | N34 41′ E135 29′ | | | 10–100 | - | - | - | 1–10 |
| 17 | Noda | N34 41′ E135 28′ | | | - | - | 1–10 | 1–10 | 1–10 |
| 18 | Taisho | N34 39′ E135 29′ | | | - | 10–100 | 1–10 | - | 1–10 |
| 19 | Tsuruhashi | N34 40′ E135 31′ | | | - | - | 1–10 | 10–100 | 1–10 |
| 20 | Momodani | N34 39′ E135 31′ | | | - | - | - | 1–10 | - |
| 21 | Terada-cho | N34 38′ E135 31′ | | | - | - | 1–10 | 1–10 | - |
| 22 | Tennohji | N34 38′ E135 30′ | | | - | - | - | - | 1–10 |
| 23 | Suminoe | N34 36′ E135 29′ | | | - | - | - | - | 10–100 |
| *Southern Osaka Prefecture* | | | 11 / 12 (91.6%) | | | | | | |
| 24 | Minato | N34 34′ E135 27′ | | | - | - | - | 1–10 | 10–100 |
| 25 | Hagoromo | N34 32′ E135 26′ | | | - | 10–100 | - | 10–100 | - |
| 26 | Takaishi | N34 31′ E135 25′ | | | - | 10–100 | - | 10–100 | - |
| 27 | Izumiotsu | N34 30′ E135 24′ | | | - | 10–100 | - | 10–100 | - |
| 28 | Tadaoka | N34 29′ E135 23′ | | | - | 10–100 | - | - | - |
| 29 | Kishiwada | N34 27′ E135 22′ | | | 10–100 | 10–100 | - | - | - |
| 30 | Kaiduka | N34 26′ E135 21′ | | | - | 1–10 | - | - | - |
| 31 | Izumisano | N34 24′ E135 19′ | | | - | 1–10 | - | 1–10 | - |
| 32 | Tarui | N34 22′ E135 15′ | | | 1 -10 | 10–100 | - | - | - |
| 33 | Ozaki | N34 21′ E135 14′ | | | 1–10 | 1–10 | - | - | |
| 34 | Misaki-koen | N34 19′ E135 09′ | | | 1–10 | - | - | - | - |
| | *Total* | | | 34 | 11 | 16 | 6 | 13 | 8 |

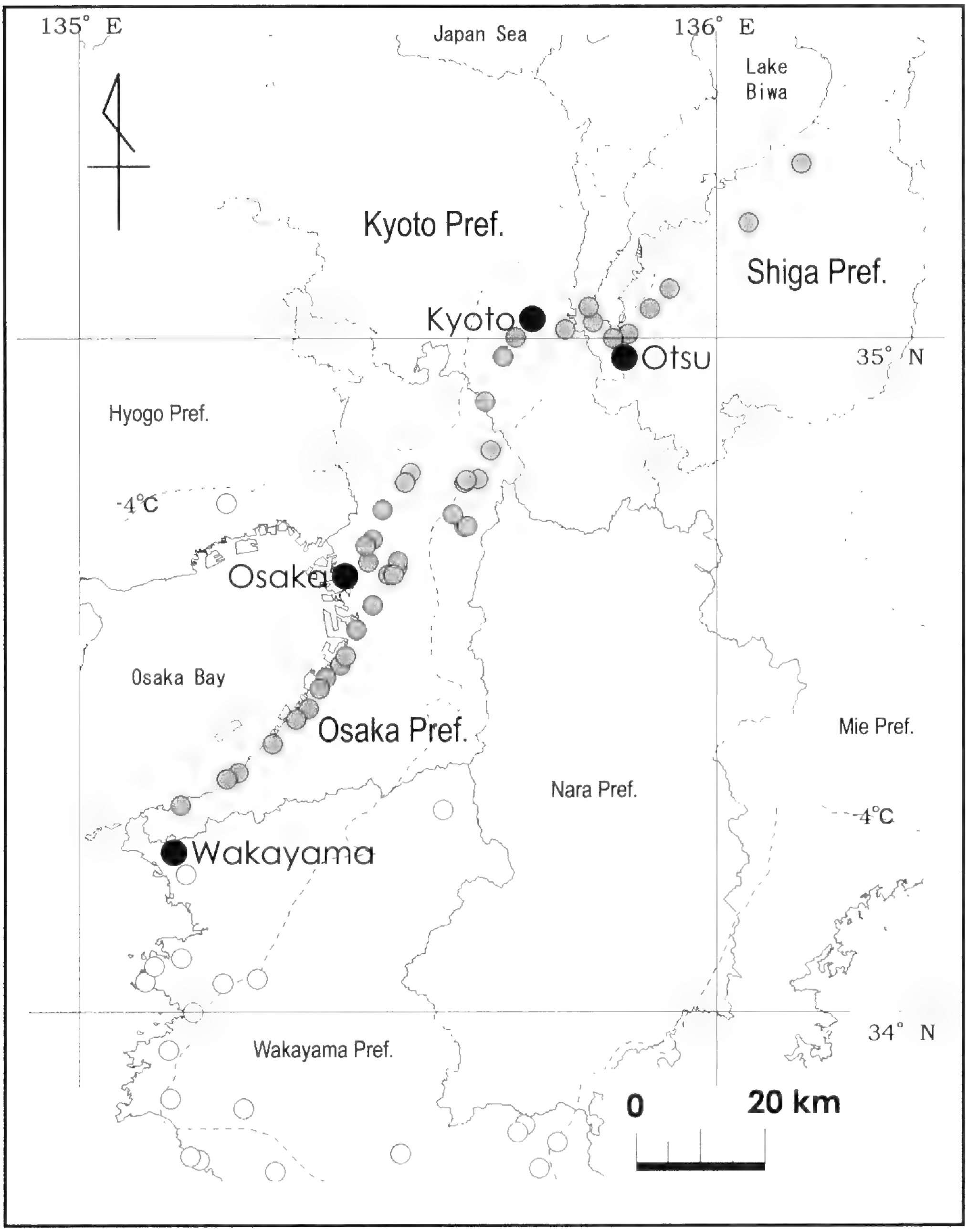


FIG. 2. Change in the distribution of *Thelypteris dentata* from 1983 to 2006. The open circles represent the distribution in 1983 (Kurata and Nakaike, 1983), and the gray circles represent the additional records of 2004–2006. The dashed line represents the −4 C isothermal line of the average minimum temperature (1990–2003) based on the data from the Japan Meteorological Agency website.

originated as spores in the potting soil of purchased plants, and the ferns may have survived due to a supply of water by humans. However, some ferns that invaded planters may have been removed if the owners did not like the invaders enough to retain them. Therefore, data from planters and gardens should not be used when rigorously evaluating habitat suitability for the downy maiden fern. Nonetheless, we have retained this information in Table 2 for the sake of completeness. Even if the absence of a fern in a planter or garden does not provide evidence of its absence in a region, the presence of a fern does provide evidence of its presence in that region.

*Can heat island corridors explain expansion of the fern's distribution?.*—Northward expansion of the range of the great Mormon butterfly (*Papilio memnon* L.) occurred at a rate of about 400 km over 22 years, from 1981 to 2003 (Yoshio, 2004; Yoshio and Ishii, 2004). The annual average expansion rate thus equals about 18 km. In contrast, the results of the present study indicate that the dispersal speed of the fern *Thelypteris dentata* is approximately 60 to 100 km over 20 years, for a rate of 3 to 5 km per year. Although the fern has expanded its distribution more slowly than the butterfly, this is likely because the butterfly can fly freely, whereas the fern sporophytes are sessile. Because the downy maiden fern grows in artificial urban habitats rather than in a natural environment such as that of forest-floor ferns, the city matrix may not function as a barrier to northward expansion of the distribution of this species. Our study focused on areas along railway lines, and almost all sites were in urban or suburban areas. In these areas, the city matrix is almost continuous and there are few geomorphic obstacles between the study sites. If this spatial structure represents a continuous warm corridor from Shiga to Wakayama, tropical or subtropical species that often grow in the Wakayama region may be capable of dispersal along these corridors to invade northern areas (Fig. 2). In contrast, fragmented forests surrounded by a hard-surfaced urban matrix generally prevent species of the forest interior from expanding easily, even if an urban heat island corridor connects two patches of forest. Urban weeds can expand their distribution more rapidly because the city matrix functions as a source of habitat, not as a barrier. If global warming continues, the distribution of such species will expand further and the species will become common across a wider range of urban areas.

*The Crinum Line and heat islands.*—In Japan, the Crinum Line and its effect on phytogeography is well known (Koshimizu, 1938; Nakanishi, 1980). This line represents a border formed by the line representing an average annual temperature of approximately 15°C and an annual lowest temperature of −3.5°C (Koshimizu, 1938), and is regarded as the standard distribution limit for subtropical or tropical species (Nakanishi, 1980). This line is still used as the distribution borderline for not only *Crinum asiaticum* L. var. *japonicum*, the species that gives the line its name, but also for various subtropical and tropical species, including *Hibiscus hamabo* Sieb. et Zucc, *Canavalia lineata* (Thunb.) DC., *Chloranthus glaber* (Thunb.) Makino, *Debregeasia edulis* (Siebold & Zucc.) Wedd. and many more (Taniguchi, 1956; Murata, 1968; Horikawa, 1976; Nakanishi, 1980). The annual average temperature and

annual lowest temperature (1983 to 2005; Table 1) at Hikone City are 14.7°C and −3.7°C, respectively, and these values approach those that define the Crinum Line. Moreover, the minimum temperatures of the Kyoto and Osaka hot spots and the heat island corridor that lies between them are higher than the minimum of −4 °C (Table 1; Fig. 2), thus temperatures in this region should be capable of supporting subtropical or tropical plants. When this corridor continues to northern Shiga Prefecture, the distribution expansion of this fern may be supported further. In Kyoto City, tropical plants such as the formerly rare species *Epipogium roseum* (D. Don) Lindl. and the subtropical species *Dioscorea bulbifera* L. have been discovered (Murata, 1998). In Kyoto City, the rapid increase in the escape of the woody plant species *Litsea cubeba* in recent years was reported by Nakamura and Kobayashi (2003). The original distribution of this species is in warmer southern areas of Japan close to subtropical areas (Nakanishi, 1996). It will likely be difficult to definitively conclude whether the expanded distribution of plant species has resulted from global warming without more examples, but the present research suggests that *T. dentata* will provide one such example.

*Conclusions.*—We investigated the range expansion of *Thelypteris dentata* in three prefectures of Japan's Kinki District since 1983, with a focus on the possible relationship between this change and warming of the environment. There is considerable evidence that *T. dentata* is an invasive alien species, and in almost all Japanese examples, *T. dentata* has been found growing on artificial structures such as stone walls; to date, it has never migrated into forest-floor environments. Therefore, this weedy fern appears unlikely to pose a serious risk of damage to natural Japanese ecosystems.

The current study leaves room for future research to resolve gaps in our knowledge. One such gap relates to elucidation of the life history of *T. dentata*. The nature of this species should be discussed, for example, using a phenological approach to determine its responses to seasonal or temperature changes, or using comparisons with other species, to more deeply understand its adaptations to the urban environment and its high rate of distribution expansion. Second, testing the heat island corridor hypothesis will require surveys of the genetic relationships among the different populations of *T. dentata*. Third, if the distribution of *T. dentata* continues to expand, we should attempt to predict its future distribution. Although this would be difficult based solely on local-scale observations, it may become possible by means of GIS analysis.

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# Tracheary Elements in Ferns: New Techniques, Observations, and Concepts

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ABSTRACT.—Longisections of xylem were studied with scanning electron microscopy (SEM) for roots of *Angiopteris*, upright axes of *Psilotum*, and rhizomes of eight species of leptosporangiate ferns of diverse habits and varied ecological preferences. In contrast to earlier studies using macerations, razor-blade sections of fixed material from living plants were prepared. All materials studied showed porose or reticulate pit membranes present on presumptive end walls of tracheids. Contrasting non-porose pits were observed on lateral walls of some tracheids. Tracheid to parenchyma pit pairs may have porose pit membranes on the tracheid side and nonporose pit membranes on the parenchyma side; thus degree of porosity in a section can represent the degree to which one primary wall or the other is pared away. Reticulate pit membranes on tracheary element end walls are evidently widespread in ferns. Such cells should not be considered vessel elements, although the reticulate pit membranes suggest a degree of transition toward the membrane-free perforations of typical vessels. True vessels (pit membranes absent in perforations) do occur in roots in a limited number of fern genera. The preparation methods of the present study produced results freer from artifacts than did macerations, and interpretations must be altered accordingly. Reports of lateral, multiple, and interrupted perforation plates in ferns are probably the result of loss of pit membranes due to the oxidative action of maceration and should be rejected. Likewise, "pit dimorphism" (alternately wide and narrow pits) and "striate" (corrugated) pit membranes in ferns represent artifacts. True vessel elements in ferns probably always have secondary wall architecture of end walls different from that of lateral walls.

KEY WORDS.—conductive tissue, scanning electron microscopy, tracheids, ultrastructure, xylem

Progress in study of the nature of tracheary elements in ferns has been gradual, limited by techniques and technology. With light microscopy and macerations, vessels were early reported in *Pteridium aquilinum* (L.) Kuhn (Russow, 1873; Bliss, 1939) and *Nephrodium filix-mas* (L.) Schott (Russow, 1873). Gwynne-Vaughan's (1908) reports that vessels occurred in a number of other ferns have been discounted (Bancroft, 1911). Light microscope studies of macerations revealed distinctive perforation plates in roots of *Marsilea* (White, 1961; Loyal and Singh, 1978). White (1962) reported possible vessels in *Astrolepis*, *Phlebodium*, *Polystichum*, and *Woodsia*. The report of possible vessels in these four genera by White (1962) was based on the presence of tracheary element end walls that were perceptibly different from lateral walls, an appearance which in turn is based on differences between end walls and lateral walls in secondary wall architecture.

Because we had access to a scanning electron microscope (SEM), we endeavored to confirm White's (1962) reports. In our studies of xylem of *Woodsia obtusa* (Spr.) Torr. (Carlquist *et al.*, 1997) and *Phlebodium* and *Polystichum* (Schneider and Carlquist, 1997), we obtained similar results. In the case of *Woodsia obtusa*, we used SEM study of sections prepared by paraffin sectioning techniques. Excessive fracturing of tracheid walls by

paraffin sectioning in *Pteridium* (Carlquist and Schneider, 1997a) discouraged us from relying on paraffin sectioning, however, and in subsequent studies, we employed macerations studied by SEM.

We confirmed with SEM that tracheary elements with end walls differing from lateral walls in secondary wall architecture also differ by lacking pit membranes in end walls but not in lateral wall pits. This was reported in *Pteridium* (Carlquist and Schneider, 1997a), *Astrolepis* (Carlquist and Schneider, 1997b), *Woodsia ilvensis* (L.) R. Br. (Carlquist and Schneider, 1998a), *W. scopulina* Eat. (Schneider and Carlquist, 1998a), and *Marsilea* (Schneider and Carlquist, 2000b). In these studies, as in those done during this period on vessels of Araceae (Carlquist and Schneider, 1998b; Schneider and Carlquist, 1998b), we thought that macerations provided reasonably reliable material for establishing pit membrane presence or absence. All of the four genera listed above were regarded as having vessels.

In subsequent studies on fern tracheary elements done with macerations, we therefore applied similar interpretive criteria (Carlquist and Schneider, 1998c, 1999, 2000a, 2000b, 2000c, 2001; Carlquist, Schneider *et al.*, 1999, 2000; Schneider and Carlquist, 1998c, 1998d, 1999a, 1999b, 1999c, 2000a); the fern groups studied in these respective papers lack differences between end walls and lateral walls in secondary wall architecture. We observed porose pit membranes, which at that time we considered indicative of vessel presence, on pits of tracheids of all of these ferns. We also observed absence of pit membranes. Since those studies, we became concerned that some artifacts may have resulted from the maceration process. We resolved to reinvestigate selected ferns using fixation with aqueous ethanol solutions, sectioning by hand with razor blades, and drying in air. These methods had provided reliable preservation of pit membranes in tracheary elements for such workers as Meylan and Butterfield (1978) and Sano (2005).

## Material and Methods

All materials studied were from rhizomes (stems) of ferns except for *Angiopteris*, in which roots were studied, and *Psilotum*, in which subaerial portions of upright axes were selected. Roots of the ferns available to us other than *Angiopteris* were too slender to be readily sectioned by hand. Suitable portions were fixed in aqueous 70% ethanol. Sections 1–2 mm thick were cut by hand using single-edged razor blades. These sections have the advantage of withstanding the sectioning process better than thinner sections, and our observations with SEM confirmed that delicate primary walls sectioned well and were not damaged. No more than six or eight sections were cut with a given razor blade, because sharpness deteriorates rapidly. Sections were placed between glass slides with gentle pressure applied in order to prevent curling during drying, and were air-dried on a warming table. Dried sections were mounted on aluminum stubs, sputter coated with gold, and examined with a Hitachi 2600N SEM.

Cultivated material from commercial sources was used for the study of *Blechnum brasiliense* Desv., *Cyathea cooperi* (F. Muell.) Domin, *Davallia*

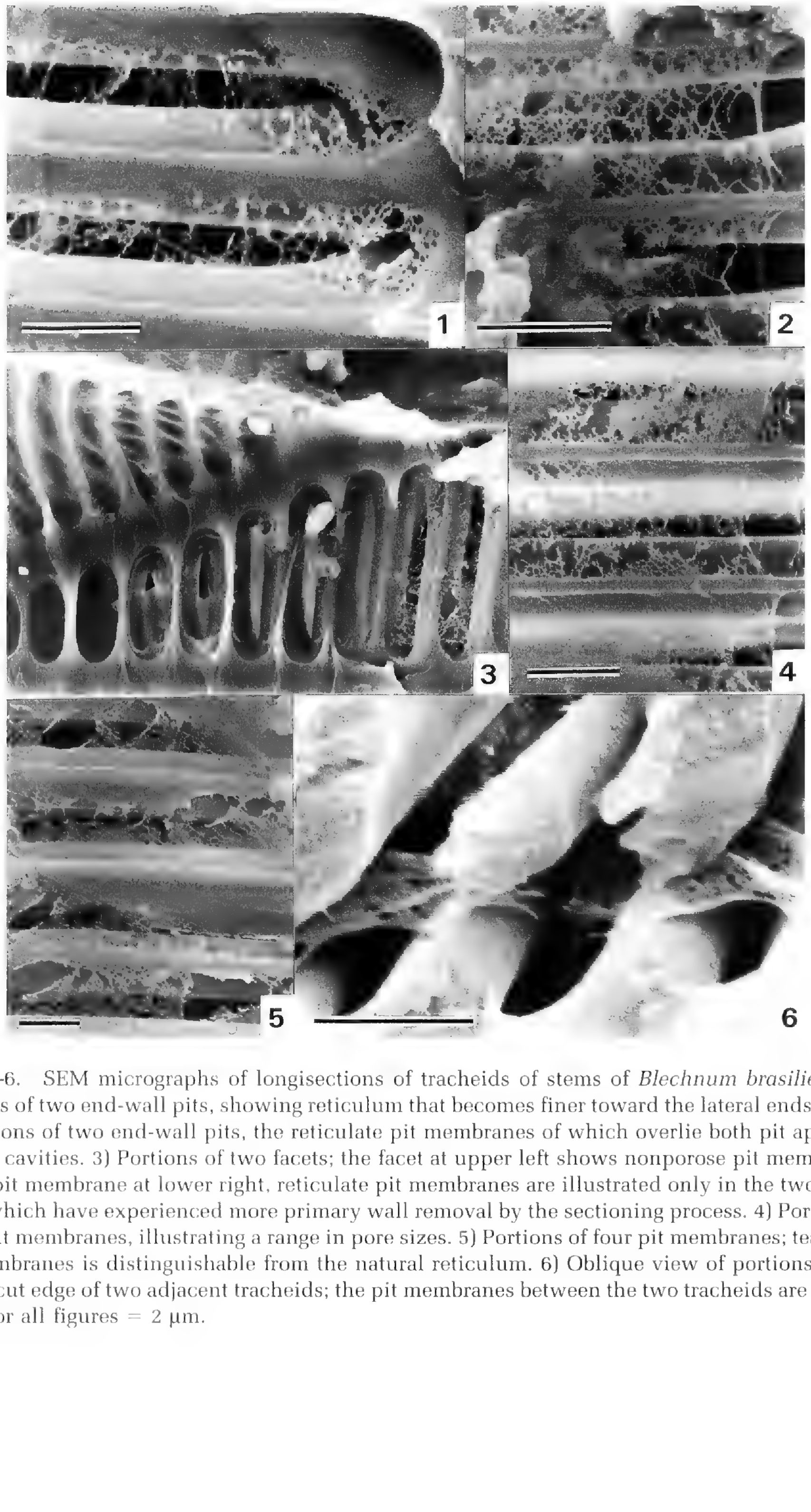
*fejeensis* Hook., *Pellaea falcata* (R. Br.) Fee, *Platycerium bifurcatum* (Cav.) C. Chr. The specimen of *Angiopteris evecta* (G. Forst.) Hoffm. was cultivated in the tropical greenhouse of the University of California, Santa Barbara. *Psilotum nudum* (L.) P. Beauv. is a common weed in the horticultural complex at that institution. *Polypodium californicum* Kaulf. has become naturalized in the Santa Barbara Botanic Garden. *Cyrtomium falcatum* Presl is adventive in gardens of Santa Barbara. The specimen of *Woodsia obtusa* (Spr.) Torr. was collected in the wild by George Yatskievich and George Taylor along a roadcut in Irons Co., Missouri (for details, see Carlquist *et al.*, 1997). Herbarium specimens (collection numbers by Sherwin Carlquist) of ferns used in this study have been deposited in the herbarium of the Santa Barbara Botanic Garden (SBBG).

## Results

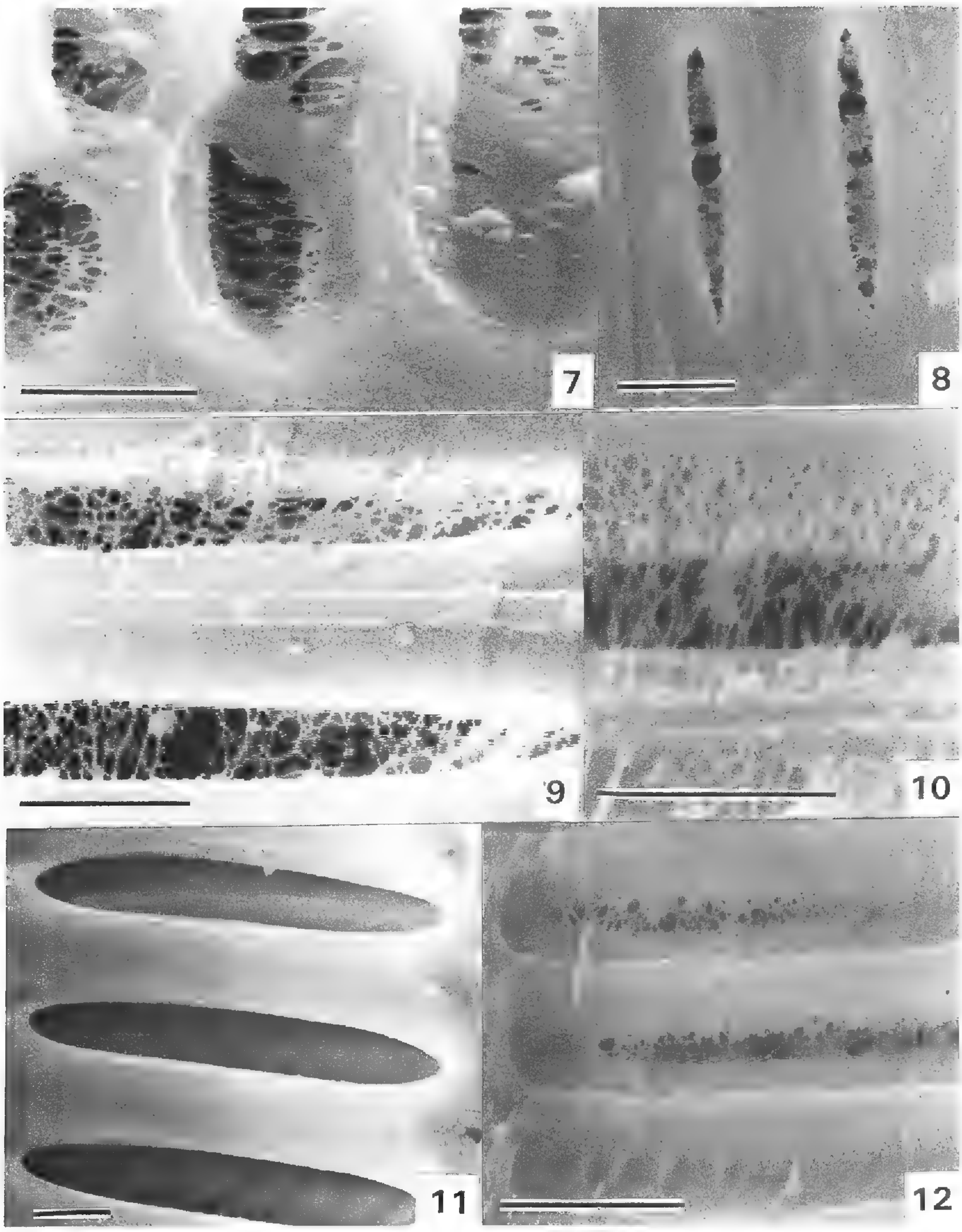
Illustrations and descriptions are presented for the "polypod" ferns (Pryer *et al.*, 2004) first, followed by Cyatheaceae, then the eusporangiate ferns.

*Blechnum brasiliense (*Figs. 1–6*).*—Longisections of tracheids show a reticulate pattern in some pit membranes (Fig. 1). Tearing of some of the strands in various patterns is readily apparent (Figs. 1, 4, 5), but the reticulate membranes are remarkably intact despite sectioning and handling procedures. Porosities or spaces in the pit membrane reticulum are most readily visible in areas superimposed above pit apertures, because of the greater contrast with a dark background at such locations (Figs. 1, 2, 5). However, the reticulate nature of the pit membrane can also be seen in front of the pit border, and is not limited to the central portion of the pit membrane (Figs. 1–6). Where pit membranes are less porose (e.g., Fig. 1, upper right; Fig. 4, top; Fig. 5, right), the double thickness of the pit membrane of the adjacent cells may be represented on account of the varied degrees to which pit membranes are shaved away in the sectioning process. The more porose pit membrane portion is that of a tracheid; the nonporose pit membrane may be that of a parenchyma cell. The more clearly reticulate patterns may be the primary wall of only one cell in face view. However, the oblique view of Fig. 6 is very valuable in showing that porose membranes occur between adjacent tracheids, where pit membranes of both of the adjacent tracheids must be present (the two seem fused together and not as separate entities in Fig. 6). The fact that the sectioning process is not highly destructive is revealed by the presence of portions of pit membranes so close to the cut surface (Fig. 6). In tracheid pit membranes where no porosities are visible (Fig. 3, upper left), the pit represented is very likely not from a tracheid end wall.

*Platycerium bifurcatum (*Figs. 7–11*).*—The longisections of stem tracheary elements show variously porose or reticulate pit membranes. The reticulate nature of pit membranes is clearly evident in Figs. 7, 9, and 10. In some pit membranes, small holes are present (Fig. 8). The reticulum, if present, characterizes the entire pit membrane, as shown in Figs. 9–10). Where the reticulate nature of a pit membrane is not visible over a pit border, as in Fig. 8, reticules in the pit membrane may not be observable because of lack of contrast

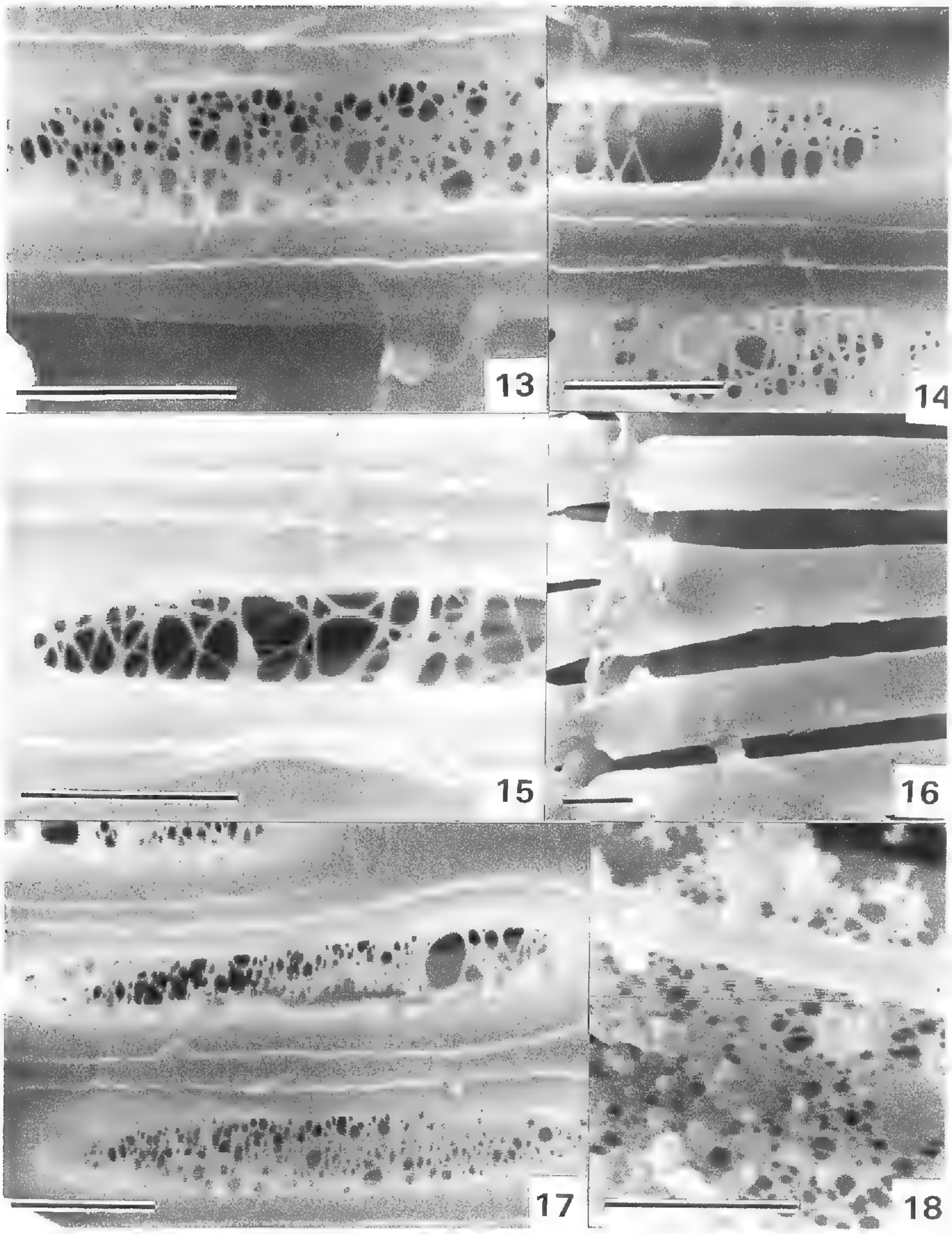



FIGS. 1–6. SEM micrographs of longisections of tracheids of stems of *Blechnum brasiliense*. 1) Portions of two end-wall pits, showing reticulum that becomes finer toward the lateral ends (right). 2) Portions of two end-wall pits, the reticulate pit membranes of which overlie both pit apertures and pit cavities. 3) Portions of two facets; the facet at upper left shows nonporose pit membranes; in the pit membrane at lower right, reticulate pit membranes are illustrated only in the two pits at right, which have experienced more primary wall removal by the sectioning process. 4) Portions of three pit membranes, illustrating a range in pore sizes. 5) Portions of four pit membranes; tearing of pit membranes is distinguishable from the natural reticulum. 6) Oblique view of portions of pits from a cut edge of two adjacent tracheids; the pit membranes between the two tracheids are porose. Scale for all figures = 2 μm.




FIGS. 7–12. SEM micrographs of longisections of tracheids of rhizomes of *Platycerium bifurcatum* (Figs. 7–11) and *Pellaea falcata* (Fig. 12). 7) Portions of pit membranes in which prominent reticules are present, although a nonporose membrane is also seen (right). 8) Pit membranes with rather large holes, some of which might represent a degree of artifact formation. 9) Portions of two pit membranes, showing the nature of the reticulum, relatively free from artifacts. 10) Portion of a pit membrane that shows a reticulate nature overlying both pit aperture and pit border. 11) Nonporose pits of a lateral wall, as seen from the inside of a tracheid. 12) Portions of pit membranes that exhibit porose and nonporose areas. Scales = 2 μm.

with the relatively bright underlying pit border, or else the reticule may have collapsed onto the pit border and fused with it as a result of processing. Drying of gel components of the cell wall may account for such pit membrane displacement and adherence. The size of pores in the reticules varies, often appearing to be larger in the center of the membrane, smaller at the margins




FIGS. 13–18. SEM micrographs of longisections of tracheids of rhizomes of *Woodsia obtusa* (Figs. 13–17) and *Cyathea cooperi* (Fig. 18). 13) A pit membrane apparently free from artifact formation. 14) Portions of two pit membranes, showing a range in size of pores. 15) Portion of a pit membrane with relatively large holes in the reticulum. 16) A lateral wall between two adjacent tracheids, cut ends of walls at left. 17) Portions of pit membranes, with a rip in one (center), the others apparently intact. 18) Pit membrane portions showing a range in pore size. Scales = 2 μm.

(Figs. 7–10). The lateral wall pitting of tracheary elements lacks any evidence of porosities (Fig. 11).

*Pellaea falcata* (Fig. 12).—Porose pit membranes were observed on some tracheid walls (Fig. 12). The pores probably occur throughout a given pit membrane, but are less obvious where they overlie a pit border.

*Woodsia obtusa* (Fig. 13–17).—Pit membranes in tracheid end walls contain notably large holes (Figs. 14, 15), although smaller pores are visible also

(Figs. 13, 14, 17). We have no reason to believe that the holes or pores result from artifact formation, because there is very little indication of fracturing in the nonhydrolyzed remnants (= reticulum) of the pit membranes. We were, however, unable to detect pores in marginal portions of pit membranes overlying pit borders (Figs. 13–15, 17). Lateral walls of tracheids were clearly observed to have pit membranes devoid of pores or holes (Fig. 16).

*Cyathea cooperi* (Fig. 18).—Although material of this species proved difficult because of the sinuous course of vascular bundles, porose pit membranes were detected on several tracheids (Fig. 18). The pores occur randomly over the entire surface of a pit membrane.

*Polypodium californicum* (Figs. 19–20).—Despite some fracturing of pit membrane strands, the reticulate nature of pit membranes in this species is evident. There is a tendency toward axial alignment of primary wall strands (Fig. 20). The pit membranes of Fig. 19 have pores generally smaller than those shown in Fig. 20.

*Cyrtomium falcatum* (Fig. 21).—Our material of this species proved difficult to section because the stems have a hard, fibrous texture. Porose pit membranes were evident in pits of a few tracheids.

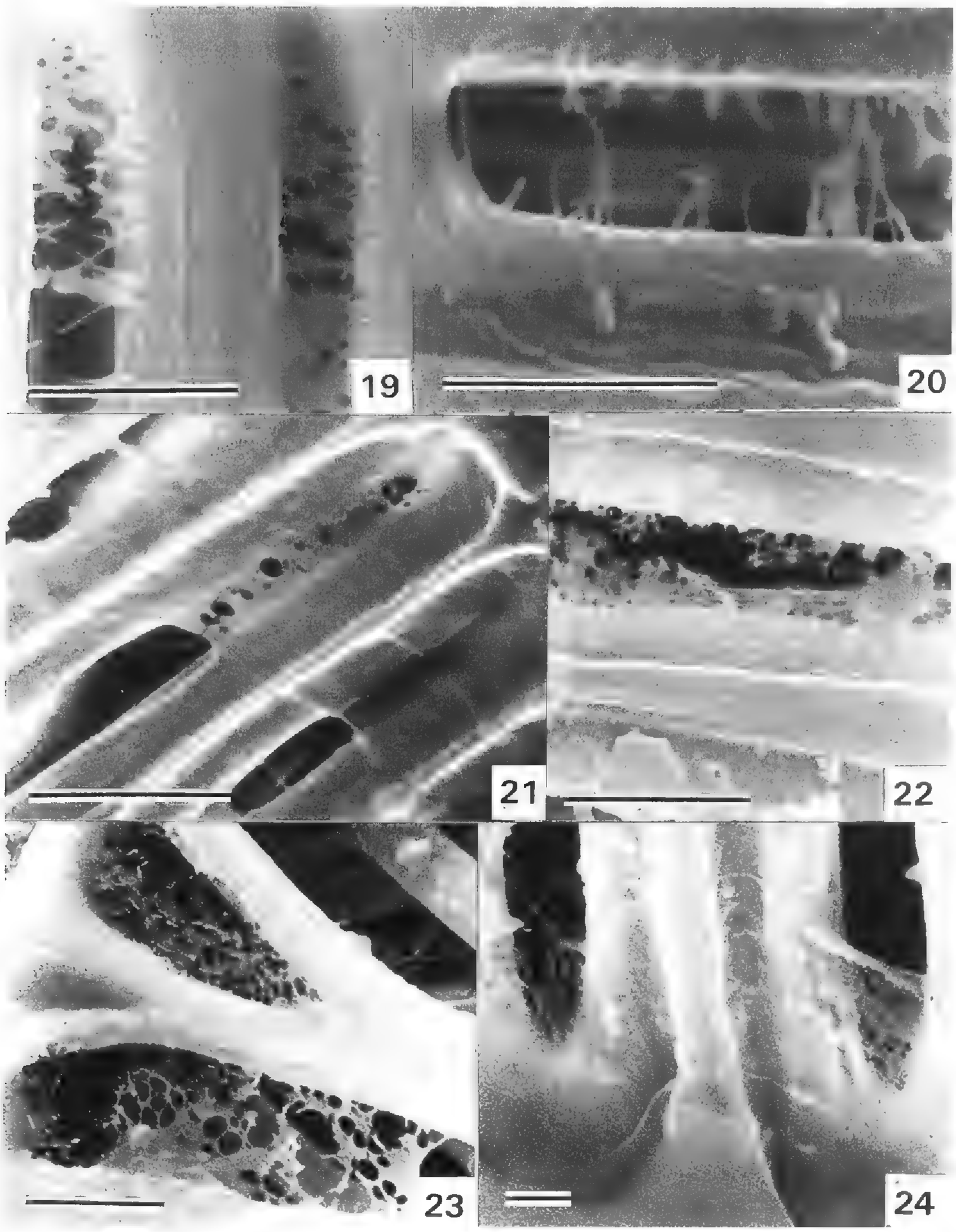
*Davallia fejeensis* (Fig. 22).—Small pores were observed to be characteristic of some tracheid pit membranes in this species, although fracturing of the pit membranes was a common occurrence (Fig. 22).

*Angiopteris evecta* (Fig. 23).—Tracheid pit membranes in roots of this species have a random distribution of pores of various sizes.

*Psilotum nudum* (Fig. 24).—Minute pores, densely distributed, occur in some pit membranes of the tracheids examined.

## Discussion

Our studies using fixation with aqueous ethanol solutions, sectioning by hand with razor blades, and drying in air provided results different from those obtained with macerations. The appearances we report as porosities, reticulate pit membranes, and threadlike pit membrane remnants here are consistent within the specimens studied. Those appearances coexist on specimens with large numbers of pit membranes in which no such structures occur, and the porose to threadlike pit membranes consistently occur in central areas of end walls: the upper and lower tips of end wall surfaces show transitions to nonporose pit membranes. We know of no rigorous comparisons that show that the above methods to be any less reliable than use of methanol or critical point drying in minimizing artifact formation in pit membranes. Cracking in pit membranes, an artifact which probably results from handling as well as to some extent from drying, can easily be differentiated from porose to threadlike pit membrane appearances. We have observed in real time that prolonged exposure to beam current at high magnification can result in fracturing and peeling away of pit membranes, and in production of corrugated pit membrane surfaces. Such appearances are easily recognizable as artifacts and are excluded from our descriptions.




FIGS. 19–24. SEM micrographs of pit membranes from fern tracheids. Figs. 19–22. Longisections from rhizomes. Figs. 19–20. *Polypodium californicum*. 19) Portions of two adjacent pit membranes with different degrees of porousness. 20) Pit membrane remnants tend to be strands oriented in an axial direction. 21) Portions of pits, *Cyrtomium falcatum*; pit membranes present above center, but absent below, apparently because of sectioning. 22) Portion of pit membrane, *Davallia fejeensis*, showing a pit membrane with small pores; the rather extensive tearing is an artifact. 23) *Angiopteris evecta*, pit membranes from longisection of a root, showing reticula broken by some tearing. 24) *Psilotum nudum*, portions of two finely porose pit membranes from a longisection of the subaerial portion of an upright axis. Scales = 2 μm.

Our newer results have produced, in our opinion, reliable images of the nature of pit membranes in tracheary elements in ferns, and therefore we are offering new conclusions here, together with reassessments of our earlier studies. Broader issues, such as the terminology of tracheary elements in the

light of ultrastructural knowledge, and the possible functions of porose and reticulate pit membranes, come into question and are therefore discussed here.

*Distribution of porose pit membranes in fern tracheids.*—The present study could have been extended indefinitely to obtain more images of porose or reticulate pit membranes in fern tracheids, because this feature was observed in all of the ferns studied. The selection was not biased in favor of particular habit types or habitat preferences, so there appears no relationship, in rhizome tracheids at least, between the occurrence of such membranes and plant form or ecology. Epiphytes, tree ferns, rhizomatous ferns, and rosette ferns were all represented. Nonporose pit membranes were observed in lateral wall pits of tracheids or in tracheid-to-parenchyma pits wherever material of xylem was relatively abundant and we could make comparative observations. Our earlier SEM studies of macerated tracheids showed that tracheary end walls free of pit membranes occurred in roots of *Astrolepis*, *Marsilea*, *Pteridium*, and *Woodsia*. Discounting reports of vessels in ferns other than these, our work showed finely porose pit membranes in a surprising number of ferns (Carlquist and Schneider, 2001). Our present study confirms that porose and reticulate pit membranes do occur widely in fern tracheids. Our renditions of these delicate structures seem much more accurate in the present study. Oxidative action of the macerating fluid in our earlier studies, we believe, did not create the porous appearances, but its action in removing portions of pit membranes is now evident. The picture that emerges is that reticulate to porose pit membranes are characteristic on end walls of fern tracheids. Variations in these networks occur, and need further exploration.

*Defining the vessel element.*—In dicotyledons, vessels are defined by having four features (Carlquist and Schneider, 2002a): (1) there are one or more perforations (free of pit membranes) on the end wall; (2) the end wall architecture (perforations) is different from that of the lateral wall (pits); (3) vessel elements are shorter than the imperforate tracheary elements they accompany; (4) vessel elements are wider than the imperforate tracheary elements they accompany. The last two features represent a division of labor between two conductive cells possible in vascular plants with vascular cambia, and aside from dicotyledons, only *Ephedra* and *Gnetum* possess this feature. Thus, only the first two criteria are applicable to ferns and monocotyledons. In ferns, only the genera *Astrolepis*, *Marsilea*, *Pteridium*, and *Woodsia* (see Introduction) satisfy the second criterion by having secondary wall architecture of end walls different from that on lateral walls. Such distinctive end walls possibly may occur in a few other genera.

The first criterion, presence or absence of pit membranes on the end walls of tracheary elements, has been judged until the past two decades on the basis of light microscopy. Light microscopy thus is the source of definitions currently used in textbooks. When SEM study is used, one does find genuine absence of pit membranes in end walls (thus perforation plates) of many monocotyledons and dicotyledons, but in some putatively primitive families of both, pit membrane remnants do occur (e.g., Carlquist, 1992; Carlquist and Schneider, 2002b, Schneider and Carlquist, 2003). In fact, within the genus *Illicium*,

various degrees of pit membrane occurrence occur. In *I. anisatum* L. and *I. floridanum* J. Ellis, one could designate vessels present or absent in terms of pit membrane presence in end walls: pit membranes may be nonporose, porose, strandlike, and reticulate. In *Illicium*, the three criteria of vesselhood other than pit membrane absence in end walls are satisfied.

In ferns other than *Astrolepis*, *Marsilea*, *Pteridium*, and *Woodsia*, degree of pit membrane presence is the only criterion by which vessel presence might be claimed. If one says that possession of reticulate pit membranes, as in *Blechnum* and *Platycerium*, could constitute a criterion, one could conceivably develop a criterion that hydrolysis of more than 50% of the pit membrane would result in formation of vessels, and thus *Blechnum* and *Platycerium* might be said to have vessels. Clearly, ultrastructure erases any usable boundary between tracheids and vessel elements in a number of cases. The most sensible solution seems to be to call attention to intermediate conditions when they occur. The inherent interest is not in the definition, but in the evolutionary and physiological significance of this phenomenon.

*Possible functional significance of tracheid ultrastructure.*—Given the widespread occurrence of weblike or porose pit membranes or pit membrane remnants in ends walls of fern tracheids, the probability exists that this structural mode represents an adaptive system. Reticulate or porose pit membranes would represent an intermediate stage on the way to attainment of vessels. Intermediate stages, however, are often labile, whereas the tracheid end-wall pit membranes of ferns are not. One can compare the condition in ferns to the similar differentiation in end walls of *Tetracentron* tracheids (see Carlquist, 1992). Porosities in the margos of coniferous tracheid pit membranes also bear comparison. The margo pores illustrated for Podocarpaceae by Meylan and Butterfield (1978) are in the same size range as the pores or reticular spaces we figure for ferns. In coniferous tracheids, the margo pores may increase conductive rate (Zimmermann, 1983). The larger pores of a coniferous margo can allow air passage under some conditions, a process shut down by aspiration of the pit as the torus is forced against the pit border. The nonhydrolyzed cellulosic strands of the conifer pit margo permit the torus to function in pit aspiration; the openings among the strands are relatively large. The margo pores in conifers that are likely to transmit air are often about 1 μm in diameter (Zimmermann, 1983), whereas the pores in fern tracheid pit membranes are much smaller, as our illustrations show. Fern tracheids do not have tori, and thus pores as large as those of a conifer pit margo would risk embolism passage. The adaptive significance of the porose or reticular pit membranes in end walls of fern tracheids thus appears to be enhancement of conductive rates without increase in the risk of embolism. Understanding of the physiological action of fern tracheids has lagged behind understanding of the relationship between ultrastructure and physiology in the case of conifer woods, very likely because of the economic value of conifer woods. Porose pit membranes certainly do occur between adjacent tracheids of ferns, as shown in Fig. 6. The possibility remains that where nonporose membranes exist on the

same facet as porose membranes (e.g., Figs. 3, 7), pores may only partially perforate the pair of adherent primary walls between tracheids.

In those ferns that do have vessels in the roots, one can cite ecological correlations. A number of monocotyledons have vessels in the root only. These monocotyledons exist in wet areas that may dry seasonally. Under such conditions, rapidity of conduction in roots is of selective value, whereas the lack of vessels in stems of those monocotyledons may inhibit spread of embolisms into stems, which are the perennating organs of monocotyledons (Carlquist, 1975). This statement would apply to *Marsilea*, which often grows in ponds of limited duration. *Pteridium* and *Woodsia* grow in habitats where temperatures and water availability show strong fluctuation.

*Artifacts; reinterpretation of earlier data.*—Our earlier studies on fern xylem were based on macerations prepared with Jeffrey's fluid. Macerations of dicotyledonous woods and even of xylem of monocotyledonous roots and stems (Carlquist and Schneider, 1998b; Schneider and Carlquist, 1998b) tend to leave pit membranes in lateral walls of vessel elements intact. We therefore assumed that these methods would result in similar results with fern xylem. That assumption proved faulty because fern roots and stems are refractory when treated with Jeffrey's fluid. Successful macerations require prolonged treatment, apparently because the xylem is associated with thick fibrous sheaths. Although secondary wall architecture was not damaged by prolonged maceration, we believe that the integrity of primary walls was lost to various degrees by prolonged oxidative treatment. We therefore turned to nonoxidative methods, such as those used by Sano (2005) in ultrastructural studies of cell walls. The preparation methods of Meylan and Butterfield (1978) were similar, where study with SEM was concerned.

We believe that revisions in our earlier reports are therefore required. Certainly presence of porous pit membranes, reported in our earlier studies, has been confirmed in the present study, although with better preservation and imaging. However, the reports of multiple perforation plates, lateral perforation plates, and intermittent perforations in fern tracheids should be regarded as erroneous. Reports of perforation plates lacking any pit membrane remnants in such genera as *Microgramma* and *Vandenboschia* (Carlquist and Schneider, 2001) are probably the result of excessive maceration. All reports of vessels in genera other than *Astrolepis*, *Marsilea*, *Pteridium* and *Woodsia* should be regarded as incorrect or possibly incorrect. Likewise, reports of pit dimorphism (alternately wide and narrow pits or perforations) should be regarded as results of artifact formation.

Air drying of sections, employed by Meylan and Butterfield (1978) and by Sano (2005) as well as in our present study, does not induce any serious artifact formation and preserves reticulate or porose pit membranes, the "microfibrillar webs" of Meylan and Butterfield (1978). We compared the results of varied settings for accelerating voltage and beam current in our present study and did not find that differences in these settings produced artifacts. Artifacts, such as tearing or cracking of pit membranes, are readily recognized as stress-induced phenomena. Pit membranes that are "striated"

(minutely corrugated) occurred both in our earlier studies and in the present study (Fig. 3, upper left) and very likely result from kinds of stress, such as heating by the SEM electron beam. Such stress artifacts are easily recognizable as different from the reticulum of pit membranes that results from natural hydrolysis of the cell wall. In our present study, we are aware that sections that show both reticulate and nonreticulate pit membranes may reveal differences in layers of the pit membrane (e.g., Fig. 12), although pores can be observed to perforate the entire thickness of the primary wall (as in Fig. 6). Thus, sections must be studied with care and with regard to context. On one side of a pit pair, a pit membrane may be reticulate, whereas the pit membrane of the adjacent cell may be nonporose. Portions of both pit membranes can be exposed by our sectioning technique. We hope that our work will be helpful to those dealing with xylem ultrastructure by pointing the way toward reliable methodology.

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# *Doryopteris majestosa* (Pteridaceae), a New Species from South America

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Abstract.—A new species of *Doryopteris* is here described, illustrated and its diagnostic characters discussed. Material of the new taxon, *Doryopteris majestosa*, has been misinterpreted as *Doryopteris nobilis* as it has features similar to it, such as the lamina architecture and large fronds, but differs mainly in having proliferous buds at the base of the lamina amongst other characters.

Key Words.—Cheilanthoideae, Doryopteris, Neotropical, Pteridaceae, South America

During a revision of the genus *Doryopteris* J. Sm. (Yesilyurt, in preparation), detailed nomenclatural studies were also carried out, taking into account ca. 218 names published within *Doryopteris*. Among these, *Doryopteris nobilis* (T. Moore) C. Chr. is well known, probably because it is one of the largest species of the genus, and several synonyms have been assigned to this taxon (Tryon, 1942). However, on checking its synonyms and types, it was noticed that all the names referred to plants without proliferous buds at the base of the lamina. The similarities of the lamina architecture (Fig. 1, A–D) and the size probably led to misidentification. Moreover, Tryon (1942) stated that *Doryopteris nobilis* '*.. is characterized by ... the usual presence of buds at the base of the blade.*,' thereby applying the name *D. nobilis* to what are considered here as two distinct taxa. All specimens with buds I therefore describe here as a new species, confirmed not only on morphological but also on the cytological evidence (Yesilyurt and Gibby, unpubl. data). Most of the terms used in this work follow Lellinger (2002).

***Doryopteris majestosa*** J. C. Yesilyurt, ***sp. nov.*** TYPE.—BRAZIL. **Rio de Janeiro, Itatiaia**: Estrada para os chales Terra Nova. Crescendo sobre rocha, próximo de córrego, local sombreado e úmido. *J. C. Yesilyurt 564, J. Prado & P. H. Labiak*, 18/01/1999 (holotype SPF; isotype BM). **Figs. 1–4**.

*Doryopteris majestosa* sp. nov. *D. nobilis* (T. Moore) C. Chr. affinis, sed gemmis ad basin laminae locatis (nec gemmis e lamina carentibus), petiolo tereti, lamina adaxialiter pubescenti, indumento ad basin laminae atque in venis maioribus densiore (nec pilis omnino sparsae) differt.

Plants terrestrial, occasionally rupicolous. *Rhizome* decumbent; scales lanceolate to ovate-lanceolate, with sporadically dentate margin, light-brown. *Sterile and fertile fronds* dimorphic, sub-coriaceous, with proliferous buds at the base of the lamina, adaxial surface with glandular hairs, usually

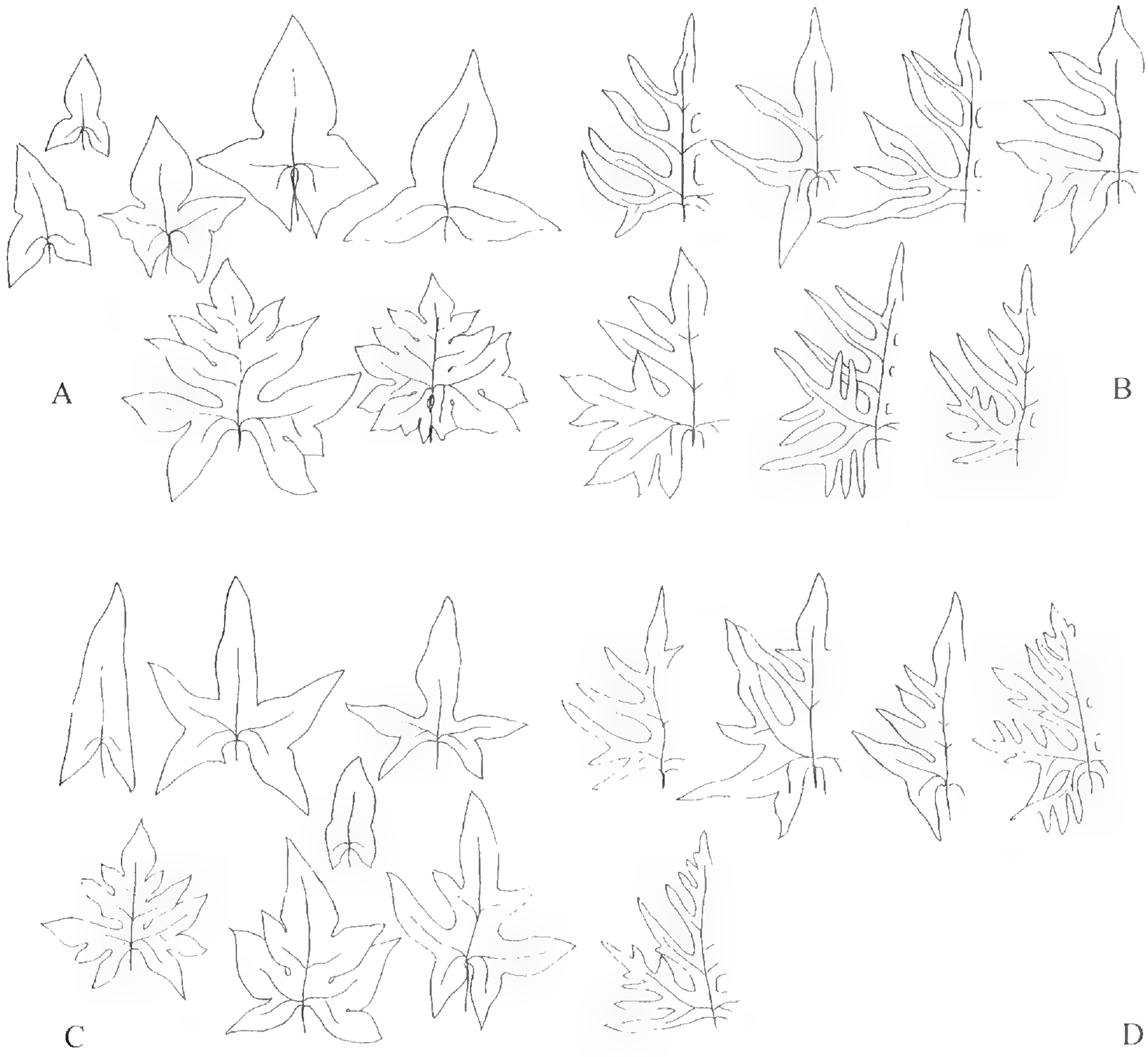


FIG. 1. Frond variation in *Doryopteris*. *D. majestosa* (A, B): A, sterile fronds; B, fertile fronds; *D. nobilis* (C, D): C, sterile fronds; D, fertile fronds.

concentrated at base of lamina and extending along the axes; abaxial surface glabrescent, indument of glandular hairs, microscales (simple and furcate) on the axes; veins anastomosing. *Petiole* terete to slightly sub-terete, brown to rarely dark brown, glabrescent, with hairs usually on the upper side of the petiole and more concentrated towards the base of lamina; scales same as those of rhizome, concentrated at the base of the petiole, those above more scattered, lanceolate to linear-lanceolate, usually caudate. *Sterile fronds* to 46 cm long; lamina to 29 cm long, sagittate, ovate-lanceolate to 3-5-lobate, occasionally pentagonal, pedate, usually broadly pinnatilobed, apex acute to acuminate; margin serrate with ascending teeth; hydathodes present. *Fertile fronds* to 80 cm long; lamina to 30 cm long, pentagonal, pedate to usually deeply 5-lobed, with 3–5 pairs of pinnae/segments, slightly ascending; apices acute to acuminate; sterile tips conspicuous, up to 1 cm long, with crenate to serrate margin; basal pinnae/segments pinnatifid to deeply pinnatilobed, asymmetric,

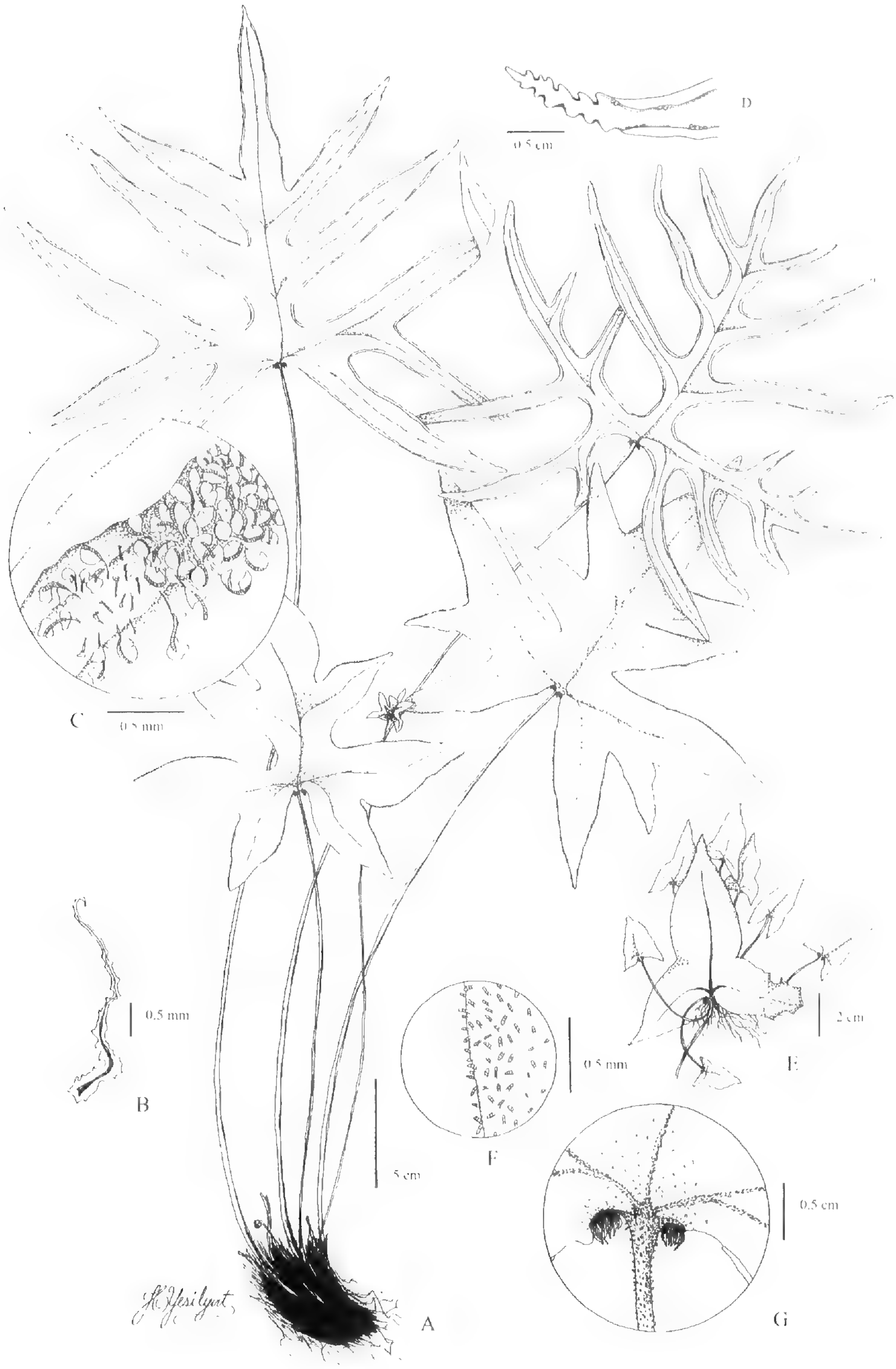


FIG. 2. *Doryopteis majestosa* J. C. Yesilyurt (*Yesilyurt 564 et al.*, SPF, holotype). A, habit; B, petiole indument: scales; C, sorus detail showing sporangia and indusium; D, segment apex; E, proliferous buds with new plants; F, petiole indument: hairs; G, proliferous buds at the base of the lamina (adaxial surface). Drawings by the author.

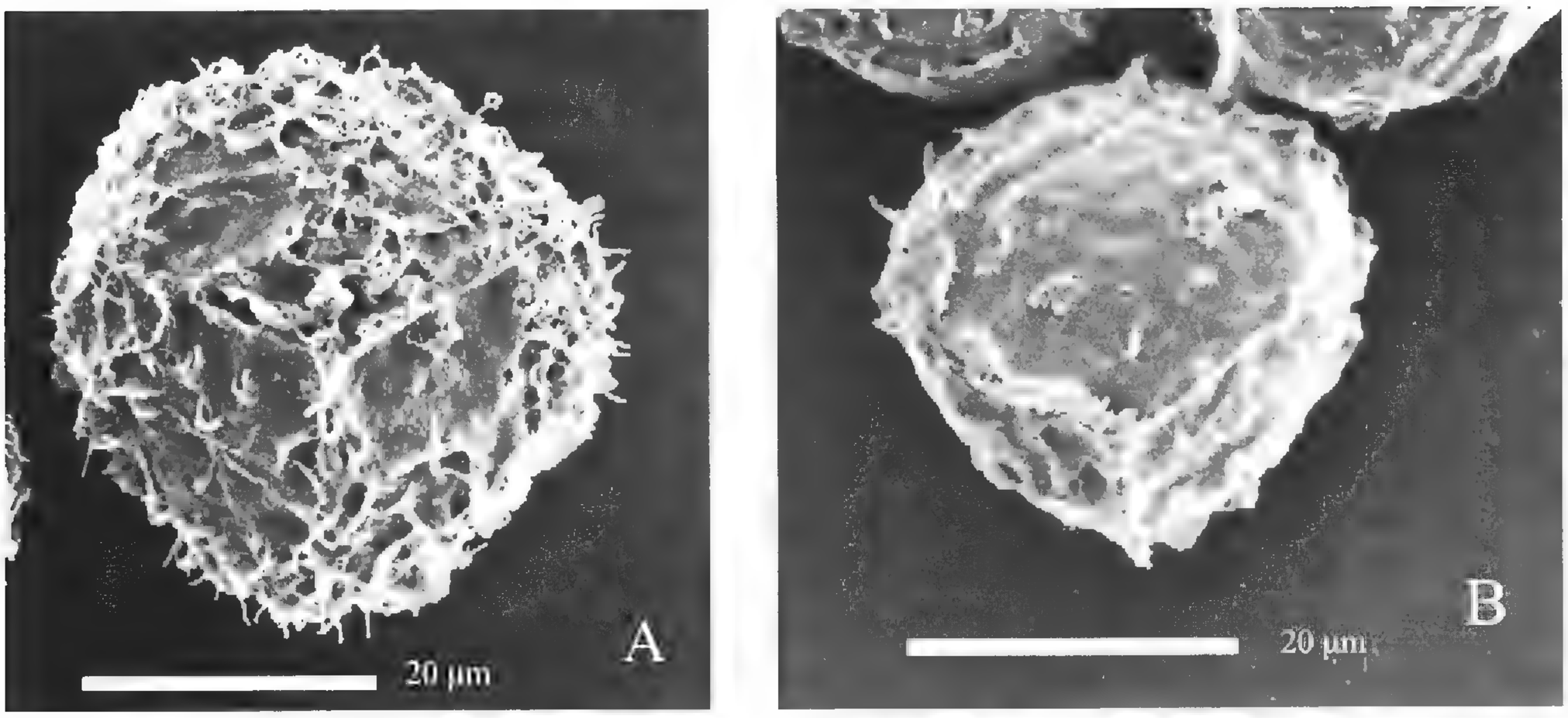


FIG. 3. SEM photomicrographs of *Doryopteris* spores. *D. majestosa* (A. *Yesilyurt 564 et al.*); *D. nobilis* (B. *Yesilyurt, J. C. 542 & Prado, J.*).

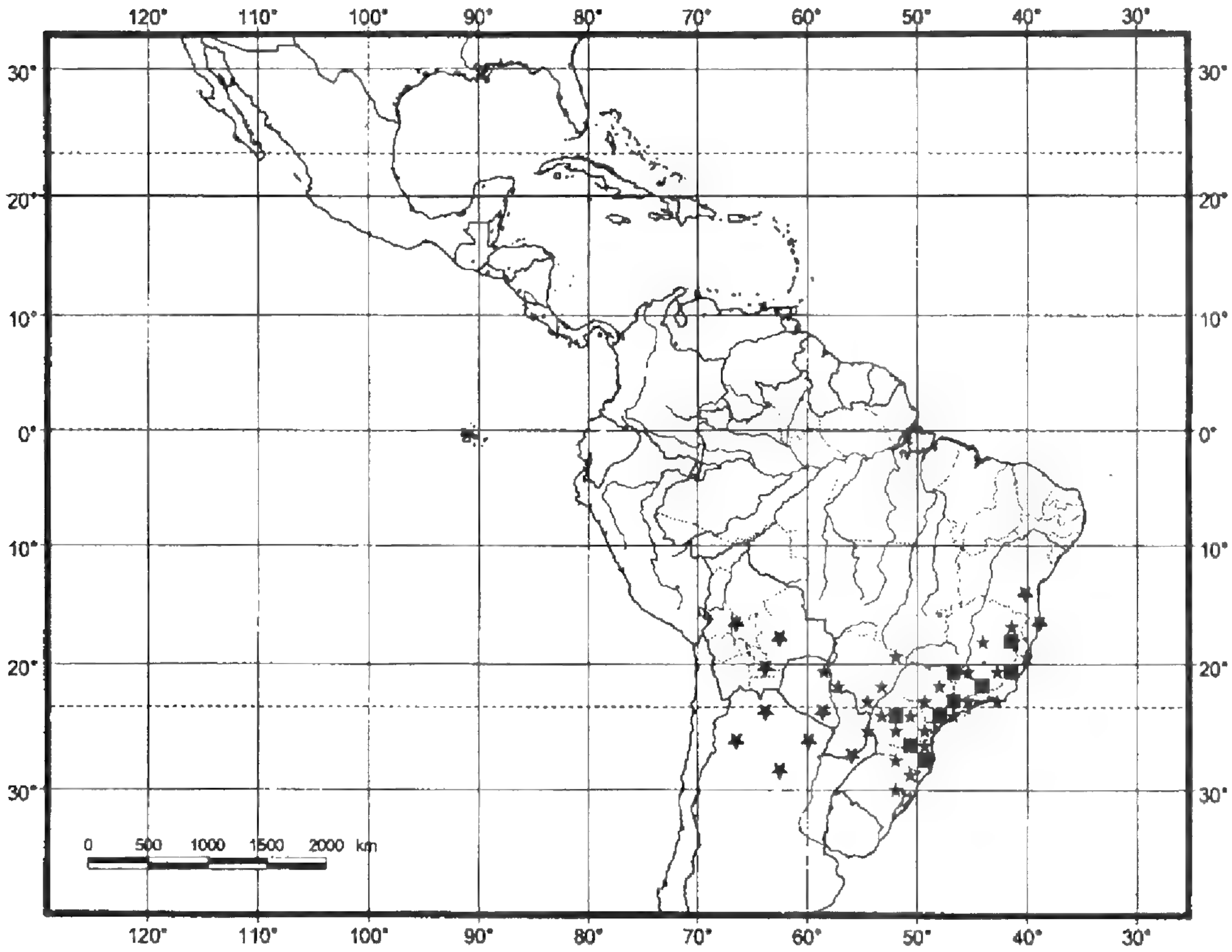


FIG. 4. Distribution of *Doryopteris*. *D. majestosa*: stars; *D. nobilis*: squares.

the basiscopic side being more developed and more dissected than the acroscopic side, broadly surcurrent; distal pinnae/segments predominantly lanceolate, rarely lobed, broadly sursumcurrent; inter-pinnae sinus inconspicuous to broad, rounded; apex of the lamina long-acuminate, tapering. *Sori* along a marginal vascular commissure, continuous around all sinuses; *receptacle and indusium* continuous, indusium with margin entire; *sporangium* up to 0.4 mm long, with stalk almost same length as capsule; capsule basal cell inconspicuous (to absent); annulus with ca 15–17 indurated cells. *Spores* light castaneous (whitish), cristate, 30–35 µm.

The specific epithet given to this new *Doryopteris* species refers to its majestic appearance, due to the outstanding size and shape of the fronds. The specimens mentioned here as *Doryopteris majestosa* were previously identified as *D. nobilis*. Both species have similar lamina architecture (Fig. 1, A–D). *Doryopteris majestosa* however is a much larger plant and has a wider range of distribution than *D. nobilis* (Fig. 4). It can be distinguished from *D. nobilis* most easily by the presence of the proliferous buds at the base of the lamina (Fig. 2, E, G). These buds are also present in juvenile fronds. New plants of both species were grown from spores collected either from herbarium specimens or from the original plants and were cultivated under the same conditions for about two years at Chelsea Physic Gardens, London, UK. Buds at the base of the lamina were always present in *Doryopteris majestosa*. Furthermore, *Doryopteris majestosa* has a brown to dark red-brown, terete to sub-terete petiole with denser indument (ferrugineous hairs) especially towards the base of the lamina (Fig. 2, F–G); light castaneous (whitish), cristate spores (Fig. 3A) and pusbescent on both surfaces of the lamina (Table 1), especially on the main axis. Cytological data (Yesilyurt and Gibby, unpubl. data) also supports the distinction of both species; *Doryopteris majestosa* is triploid and *D. nobilis* diploid.

*Doryopteris majestosa* is relatively widely distributed in east central South America (Fig. 4). It occurs in northeastern (Bahia), southeastern (Minas Gerais) and southwards to southern Brazil (Rio Grande do Sul). It also occurs in central west Brazil (Mato Grosso do Sul), Paraguay, Argentina and southeastern Bolivia.

*Doryopteris majestosa* occurs in different ecological habitats from the Atlantic, Parana pine (south Brazil), Galery and secondary forests, alongside streams, slopes of mountains or usually along forest borders. The species has usually been found growing in rather large populations when compared to other species of the genus *Doryopteris*. Therefore, I do not believe that the species suffers from any strong threat.

Specimens examined.—BRAZIL. **Bahia**: *T. S. Santos et al. 4121* (US); *M. Blanchet* s.n. (G). **Espirito Santo**: *A. C. Brade et al. 18503* (RB). **Rio de Janeiro**: *Luetzenberg 12917* (M, S); *R. M. Tryon & A. Tryon 6620* (BM, GH); *C. Rizzini 452* (RB); *A. C. Brade 12709* (RB); *A. C. Brade 9495* (BM). **Minas Gerais**: *Mosén 2082* (B, K, M, S); *T. Santos 4121* (US); *G. Lindberg 601* (B); *Regnell 329* (BR, U); *E. P. Heringer 5709* (M); *L. S. Leoni 745* (UC). **São Paulo**: *M. Kuhlmann 804*

TABLE 1. Main morphological features to distinguish *Doryopteris majestosa* from *D. nobilis*.

| CHARACTERS | D. majestosa | D. nobilis |
|---|---|---|
| **Proliferous buds at the base of the lamina** | present (Fig. 1E) | absent |
| **Size of the fertile frond (length in cm)** | frond up to 80, lamina up to 30 | frond 55–60; lamina up to 45 |
| **Size of the sterile frond (length in cm)** | frond up to 46, lamina up to 29 | frond 35–40 long; lamina 30 |
| **Petiole shape (especially towards base of the lamina)** | terete (rarely sub-terete), | predominantly sulcate, |
| **Petiole colour** | brown to dark red-brown, | red to light red-brown, |
| **Petiole indument** | pubescent (ferrugineous hairs, Fig. 1F), with denser indument towards the base of the lamina (Fig. 1G) and scales (Fig. 1B) | with hairs usually on the upper side of the petiole and more concentrated towards the base of the lamina |
| **Indument on the adaxial surface of the lamina** | pubescent, denser at the base of the lamina, extending on the main axes (Fig. 1G) | puberulous to glabrous, hairs scattered along the main axes |
| **Indument on the abaxial surface of the lamina** | glabrescent, with glandular hairs and scattered microscales along the axes | glabrescent and with microscales usually concentrated at the base of the lamina |
| **Margin of sterile tips of the fertile lamina and/or margin of the sterile lamina** | predominantly serrate (Fig. 1D) | predominantly dentate |
| **Size of the sterile tips of the fertile lamina** | usually long (up to 1 cm) (Fig. 1D) | short to medium (up to 0.7 mm) |
| **Spores in light microscope (color en masse) and SEM** | light castaneous (whitish), cristate (cristae with threads) (Fig. 3A) | light castaneous (golden), sparsely cristate (cristae lamellate) (Fig. 3B) |
| **Spore size (in μm)** | 30–35 | 27.5–30 |
| **Sporangium, size (in mm)** | up to 0.4, with stalk almost same length as capsule | up to 0.3, with stalk ca. 2/3 of capsule length. |
| **Distribution** (Fig. 4) | in Brazil, from Bahia down to Rio Grande do Sul states, towards Paraguay, Argentina and Bolivia | only in Brazil, usually along coastal rainforest, from Bahia to Santa Catarina states. |

(SP); *M. Kuhlmann 1053* (SP); *C. Duarte* s.n. (SP); *F. C. Hoehne* s.n. (SP) *H. Luederwaldt 21312* (SP); *H. Luederwaldt* s.n. (SPF); *O. Yano 3663* (SP); *F. Tamandare & A. C. Brade 6516* (SP); *A. C. Brade 8599* (UC); *Duarte 4979* (S); *M. R. Silva, 400* (MO, PACA, SPF); *M. R. Silva, 1571* (SPF); *Guedes 72* (NY); *M.*

*Albricht 130* (NY); *A. B. Joly 841* (RB, SPF). **Mato Grosso do Sul**: *A. Sehnem 8063* (PACA). **Paraná**: *G. Hatschbach 21520* (MBM, MO, PACA); *G. Hatschbach 24144* (C, PACA, S, UC, US); *G. Hatschbach & O. Guimaraes 19338* (C); *G. Hatschbach 21520* (PACA, MO); *P. Dusen 11621* (S); *G. Hatschbach & E. Perreira 10406* (PACA); *J. R. Pirani et al. 406* (SP); *O. S. Ribas & J. M. Silva 109* (B); *J. C. Lindenam & J. H. Hass 3357* (B, U); *J. C. Lindenam & J. H. Hass 4969* (GH, U); *J. C. Lindenam & J. H. Hass 1219* (BM, RB, U); *E. Perreira 5322* (B); *Reis 129* (GH); *A. C. Cervi 2536* (NY); *A. P. Duarte & E. Pereira 1678* (NY, RB); *U. A. Dietrich & C. Kazera 168* (NY); *E. Pereira 7790* (M); *A. Sehnem 971* (NY); *G. Tessman 26* (RB); *G. Tessman 6026* (BR); *J. R. Pirani et al. 406* (SPF); *J. Cordeiro & J. M. Silva 436* (S, UC); *C. B. Poliquesi & J. Cordeiro 304* (UC). **Santa Catarina**: *M. Klein 7982* (PACA); *Reitz & Klein 6673* (PACA, US); *R. Reitz c427* (RB); *R. Reitz 4723* (BM); *J. R. Pirani et al. 450* (SP, SPF); *Spanagel* s.n. (NY, SP, UC); *B. Rambo 49940* (S); *A. Schmalz 4* (MO, NY); *H. Gauthier* s.n. (RB); *L. B. Smith et al. 9614* (US). **Rio Grande do Sul**: *A. Sehnem 3330* (B, C, GH, PACA, US); *A. Sehnem 3576* (C); *A. Sehnem 3574* (GH); *Brauner 148* (PACA); *A. Kunnert n.0* (B), *J. E. Leite 2186* (US); *J. E. Leite 2579* (SP); *J. E. Leite 706* (NY); *L. Stier 115* (S); *E. Hassler 5701b* (G, K); *E. Hassler 5388* (G); *C. A. M. Lindman 1019* (B, S); *C. Juergens 126* (GH, L, U, UC); *C. Juergens* s.n. (B, L, M, UC); *R. Reitz 81* (GH); *Mackhieske 9* (NY, UC); *B. Rambo 41710* (RB); *B. Rambo 42096* (RB).

BOLIVA. *T. Herzog 2* (US); *E. L. Ekman 26* (S). **Tablas-Thales**: *T. Herzog 2143* (L, M, UC). **La Paz**: *T. Plowman & E. W. Davis 5166* (GH); *M. Kessler et al. 10295* (UC). **Tarija**: *J. L. Solomon 10114* (NY, UC). **Chusquisaca**: *L. Amayo et al. 1000* (UC).

PARAGUAY. *E. Rojas 1813a* (GH); *P. Joergensen 4061* (GH). **Guarapi**: *B. Balansa 2842* (B, BM, C, G, GH; K, L, U); *E. Zardini 7885* (G); *E. Zardini 7772* (MO); *M. Ortiz 492* (G). **Caazapa**: *M. Ortiz 945* (MO); *R. Degen 1295* (MO). **Canindeyú**: *M. Penna-Chocarro 268* (BM). **La Soledade**: *T. Pedersen 5973* (C, K, L). **Serra Maracayī**: *E. Hassler 4372* (B, BM, G, GH, NY, UC); *E. Hassler 5701a* (B, BM, GH, NY). **Alto Paraguay**: *A. L. Woolston* 707 (BM, NY, SP, S, U, UC). **Alto Paraná**: *K. Fiebrig 5796* (B, GH, US). **Itapuá**: *J. F. Casas & J. Molero 3738B* (MO, NY); *P. Jorgensen 4062* (MO, NY, UC); *I. Bassualdo 002123* (MO); *M. S. Foster 7640* (UC). **Gran Chaco**: *C A. M. Lindman 2077* (GH, NY); *T. M. Pedersem 5973* (GH); *F. Billiet & B. Jardin 3390* (BR).

ARGENTINA. *G. Niedulein* s.n. (B); *E. Hassler 782* (G). **Tucuman**: *J. E. Montes 2446* (MO); *L. Castilhum 23493* (GH); *S. Vemturi 9645* (GH, US); *F. Vervoorst & A. R. Cuezzo 7806c* (GH); *Lillo 2918* (GH); *S. Vemturi 1231* (U). **Missiones**: *J. E. Mortes 1673* (BM, C); *J. E. Mortes 10756* (MO); *J. E. Mortes 14743* (NY); *R. Huidabro 5540* (BM, MO); *F. O. Zulloaga et al. 5472* (MO); *A. Buskart 1355* (GH); *A. Scala 287* (GH); *T. Meyer 5498* (GH); *J. H. Henziker 908* (BM, RB); *J. Diem 1448* (UC); *Rodriguez 130* (G, UC). **La Laguna**: *E. Zardini & R. Velazques 15752* (MO); *A. G. Schulz 722* (GH); *L. Castilhum 41031* (U). **Salta**: *Willink 236* (U); *J. Novara 9999* (M). **Jujuy**: *Dinelli 41026* (U); *E.R. Sota 4481* (GH, US).

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# *Isoetes araucaniana*, a New Species from Southern South America

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Abstract.—*Isoetes araucaniana* is newly described from central Chile. This aquatic species is endemic to Araucanía and is the only southern Andean species with strongly and densely reticulate megaspores.

Key Words.—*Isoetes*, new species, South America

As part of studies of *Isoetes* in southern South America, a new species was discovered among undetermined specimens from Chile. This new species is distinct among species of the southern Andes in having reticulate megaspores, an incomplete velum, and essentially laevigate microspores.

***Isoetes araucaniana*** Macluf & Hickey, ***sp. nov.*** TYPE.—CHILE. Provincia de Malleco, Parque Nacional Nahuelbuta, sector Piedra del Aquilla, 37 42′S, 72 59′W, 800 m, at the edge of a small stream, in the water, 8 Feb 1991, *M. DeVore 1590 & M. Baeza* (holotype: MU; isotypes: CONC, F, OSH). **Figs. 1–10**.

*Cormus* globosus, 2–3-lobatus, 10–18 mm latus, 11–14 mm elatus; *radices* dichotomae. *Folia* 10–38, erecta, 11–30 cm longa, 4.0–8.0 mm basi lata, 1.0–3.0 mm medio lata; *alae* hyalinae, chartaceae, 2.0 mm latae, 5–10 cm longae (15–40% per foliae longitudinem ascendentes), apicibus attenuates; *subula* teres, atroviridis, attenuata; *stomata* praesentia; *squamae* et *phyllopodia* absentia. *Sporangium* ellipticum, concolor, aetate hyalinum, 3.0–9.0 mm longum, 2.0–5.0 mm latum, basale. *Velum* incompletum. *Ligula* lanceolata, auriculata, 2.0–6.0 mm longa, 1.5–3.5 mm lata. *Labium* ligulatum, 1.0–1.5 mm longum, 0.2–0.25 mm latum. *Meagsporae* albae, 630–(750)–870 µm diametro, reticulatae, zona absentia. *Microsporae* murinae, 38.0–41.0 µm longae, 25.0–28.0 µm latae, laevigatae.

*Corm* globose, 2–3-lobed, 10–18 mm wide, 11–14 mm high; *roots* dichotomously branched. *Leaves* 10–38, erect, 11–30 cm long, 4.0–8.0 mm wide at the base, 1.0–3.0 mm wide at mid length; *alae* hyaline, chartaceous, 2.0 mm wide

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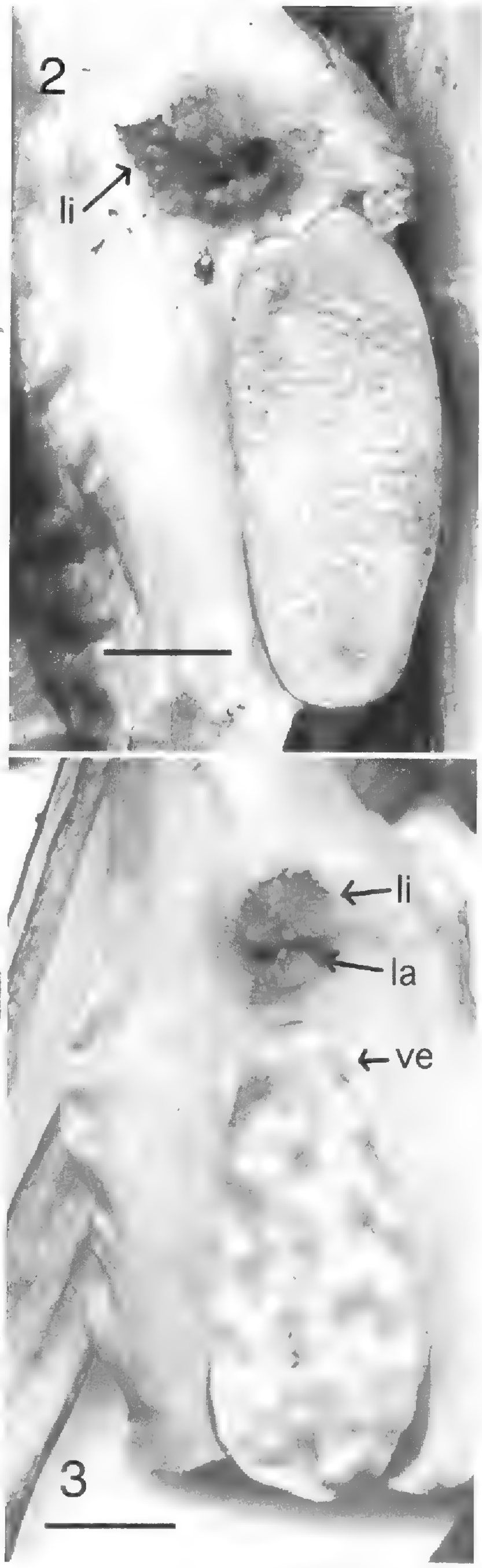
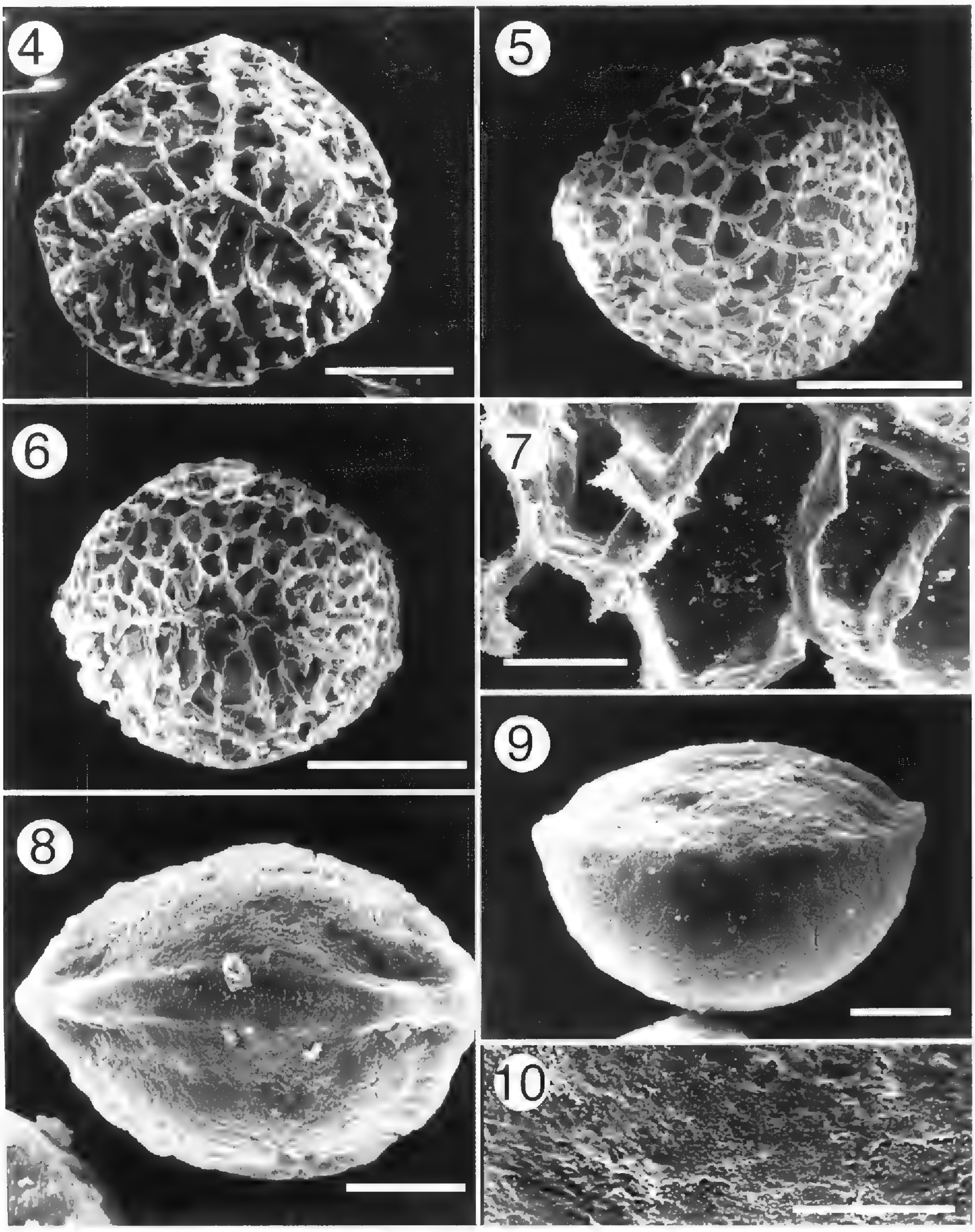



FIGS. 1–3. Holotype of *Isoetes araucaniana* (*M. DeVore 1590 & M. Baeza*, MU). 1. Habit showing corm and leaf bases; scale bar = 5 cm. 2. Adaxial view of microsporophyll base showing sporangium, incomplete velum, ligule fragment and ligulate labium; scale bar = 2 mm. 3. Adaxial view of megasporophyll and megasporangium with incomplete velum, ligule fragment and labium; scale bar = 2 mm. li = ligule; la = labium; ve = velum.




FIGS. 4–10. SEM images of *I. araucania* megaspores and microspores (*K.H. & W. Rechinger 63165*, M). 4. Proximal view of megaspore; scale bar = 250 μm. 5. Distal view of megaspore; scale bar = 500 μm. 6. Equatorial view of megaspore; scale bar = 500 μm. 7. Detail of the proximal sculpture; scale bar = 50 μm. 8. Proximal view of microspore; scale bar = 10 μm. 9. Equatorial view of microspore; scale bar = 10 μm. 10. Detail of the distal sculpture; scale bar = 5 μm.

at the sporangium, 5–10 cm long (extending up for 15–40% of the leaf length), apices attenuate; *subula* terete, dark green, attenuate; *stomata* present; *scales* and *phyllopodia* absent. *Sporangium*, elliptic, concolorous, hyaline with age, 3.0–9.0 mm long, 2.0–5.0 mm wide, basal. *Velum* incomplete, occasionally

TABLE 1. Morphological comparison between *Isoetes araucaniana* and *Isoetes chubutiana*.

| | *I. araucaniana* | *I. chubutiana* |
|---|---|---|
| Leaf color | Light to medium green | Dark green |
| Labium | Ligulate, occasionally bifid, 1–1.5 mm long, 0.2–0.25 mm wide | Inconspicuous to absent |
| Ligule | Lanceolate with pronounced basal lobes, to 2.0–6.0 mm high, 1.5–3.5 mm wide | Deltate to widely ovate, chordate to hastate, dark 1.5–3.0 mm high, 1.7–2.3 mm wide |
| Microspores | Laevigate | Sparsely to densely echinate |
| Megaspores size Mean: range | 750: 630–870 µm | 595: 460–750 µm |
| Megaspore ornamentation | Densely and distinctly reticulate; the muri generally as tall or taller than the diameter of the included areole | Ruglulate to low and sparsely reticulate; the muri low, much lower than the diameter of the included areole |
| Megaspore girdle | Absent | Weakly differentiated to distinctly smooth |

covering 25% of the upper portion of the sporangium. *Ligule* lanceolate with pronounced basal lobes, to 2.0–6.0 mm high, 1.5–3.5 mm wide. *Labium* ligulate, occasionally bifid, 1–1.5 mm long, 0.2–0.25 mm wide, delicate and often ephemeral. *Megaspores* 630–(750)–870 µm in equatorial diameter, reticulate, the muri strongly developed and distinct; girdle undifferentiated. *Microspores* 38–41 µm long, 25–28 µm wide, laevigate, minutely rugulate to granulate at higher magnification.

*Isoetes araucaniana* is endemic to Chile and is known only from collections made in Nahuelbuta National Park in Araucanía. It grows in streams at elevations of 800 to 1500 m, in *Araucaria araucana-Nothofagus dombeyi* mixed forest.

The only other described species of *Isoetes* growing even marginally close to *I. araucaniana* is *I. chubutiana* Hickey, Macluf & Taylor. *Isoetes araucaniana* is distinctive (Table 1) in possessing smooth microspores, a narrowly ligulate (sometimes bifid) labium, and larger megaspores with a tightly reticulate ornamentation of tall, distinctive muri (Hickey *et al.*, 2003). Undamaged muri in megaspores of *I. araucaniana* are tall and thin, typically as high as the diameter of the included areoles (Figs. 4–6). In contrast, the muri of *I. chubutiana* are much shorter than the diameter of the included muri. Finally, the megaspore reticulations of *I. araucaniana* continue uninterrupted across the girdle region; in *I. cubutiana* the girdle typically has little or no ornamentation.

PARATYPES.—CHILE. **Araucanía**: Nahuelbuta, 1000 m, 30 Jan 1901, *T. G. s.n.* (CONC); Prov. Malleco, Dpto. Angol, Parque Nacional de Nahuelbuta, Centro del Parque, 1250 msm, 37°46′S, 73°02′W, 7 Jan 1968, *Ricardi, Marticorena y Matthei 1832, 1833* (CONC); Angol, Parque Nac. Nahuelbuta-Vag, 1460 msm, 7 Jan 1968, *G. Montero O. 8116* (CONC); Küstenkordillere, am Nahuelbuta, Sumpfstellen und Araukarien-Nothofagus-Wald, 1250 m, 28 Dec 1968, *H.*

*Merxmüller 25085* (M); Prov. Malleco, Parque Nacional de Nahuelbuta, 1460 msm, 15 April 1972, *R. Rodríguez* (CONC); VIII Regíon, Prov. Arauco, Cordillera de Nahhuelbuta, loma del Consorcio, en mallín, semi enterrado, en pequeñas pozas de agua estancada, 925 msm, 37 34′S, 7° 12′W, 13 Dec 1983, *O. Matthei y M. Quezada 35* (CONC); Region of Araucanía, Prov. Malleco. Alt.1300 m, 28 Oct, 1987. *K. H. & W. Rechinger 63165* (M); Nahuelbuta Nacional Park, near Agua Caliente, ca. 42 km from Angol, *Araucaria araucana – Nothofagus dombeyi* mixed forest, 22 Jan 1975, *M. Nishida, M. Ono, T. Hashimoto & N. Ohga 750002 [751302], 750003 [751303]* (SGO).

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# *Blechnum* ×*rodriguezii* Hyb. Nov., a Deer Fern Hybrid from Southern Chile

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Abstract.—A new deer fern hybrid, *B. corralense* × *B. mochaenum* subsp. *mochaenum*, is described on the basis of macro- and micromorphological characters. Previous reports of this hybrid combination are excluded. In addition, new data on the distribution of *B. corralense* are reported, including its presence in Argentina.

Key Words.—*Blechnum*, Chile, fern, hybrid, morphology

*Blechnum* L. (deer ferns) is one of Chile's most speciose fern genera. Thirteen species were recognized in this territory by Rodríguez (1995). Maximum species richness is reached in southern mainland Chile (Maule to Magallanes regions) and in the Juan Fernández Archipelago. Morphology is likewise very diverse, ranging from the smallest species in the genus, *B. corralense* Espinosa, to subarborescent forms, such as *B. cycadifolium* (Colla) Sturm, and including species with monomorphic, dimorphic and even trimorphic fronds. The different species of *Blechnum* have been tentatively classified into subgeneric groups (Tryon and Tryon, 1982), but their evolutionary relations remain poorly understood. It is not known whether hybridization and reticulate evolution, so widespread in homosporous ferns (see e.g. Grant, 1981; Barrington *et al.*, 1989; Haufler, 2002), play an important role in Chilean deer ferns.

*Blechnum corralense* × *B. mochaenum* subsp. *mochaenum* is the only hybrid combination reported to date among the Chilean deer ferns (Villagrán *et al.*, 1986; Rodríguez, 1995); though as detailed below we consider that this report was probably erroneous. *Blechnum mochaenum* G. Kunkel comprises three subspecies according to Rolleri and Prada (2006): subsp. *mochaenum* (southern Chile and southern Argentina), subsp. *achalense* (Hieron.) Prada & Rolleri (central and northwestern Argentina), and subsp. *squamipes* (Hieron.) Prada & Rolleri (central and northwestern Argentina and southern Brazil). *Blechnum corralense* was until now known only from the Chilean region of Los Lagos (Rodríguez, 1995), where it frequently co-occurs with *B. mochaenum* subsp. *mochaenum*. According to the most recent revisions of the Chilean

TABLE 1. Morphological characters of *Blechnum corralense*, *B. mochaenum* subsp. *mochaenum* and the new hybrid. The intervals cover mean values for 5 individuals in each taxon.

| Character | *B. corralense* | *B. ×rodriguezii* | *B. mochaenum* |
|---|---|---|---|
| Lamina texture | herbaceous | intermediate | coriaceous |
| Aphlebia | present | present | present |
| Sterile frond length (cm) | 4–11 | 5–10 | 12–30 |
| Fertile frond length (cm) | 11–20 | 8–20 | 13–36 |
| Number of pinna pairs per sterile frond | 7–16 | 12–17 | 14–27 |
| Number of main veins per sterile pinna | 5–11 | 8–10 | 13–29 |
| Length of the petiole paleae (mm) | 2–5 | 2–3 | 6–11 |
| Length/width ratio of the petiole paleae | 2–4 | 2–3 | 5–9 |
| Guard cell length (μm) | 71–80 | 60–76 | 58–69 |
| Exospore length (μm) | 37–42 | misshapen | 32–35 |

deer ferns (Rodríguez, 1995; Rolleri and Prada, 2006), the main characters for distinguishing these two taxa are lamina texture and number of veins of sterile pinna. We have identified some other diagnostic characters, as shown in Table 1. On the basis of a study of material in the Chilean herbaria CONC and SGO, and our own collections, we have concluded that *B. corralense* is also present in the regions of La Araucanía (*Amigo, Pajarón, Pangua & Quintanilla LGQ541*, SANT) and Aysén (*Landrum 8179*, CONC, and *Amigo CL2324*, SANT). In addition, two specimens collected by José Diem in the Nahuel Huapi National Park, Neuquén Province, Argentina (*Diem 50a*, *50b*; CONC) turned out to be *B. corralense*.

We have also revised the putative hybrids between *B. corralense* and *B. mochaenum* subsp. *mochaenum* collected by Carolina Villagrán and co-workers in the Los Lagos region (Villagrán *et al.*, 1986), totaling six specimens (*Meza & Aguila 6510*, *Villagrán 6329*, *Villagrán & Aguila 5937*, *Villagrán & Leiva 7325*, *7452*, and *Villagrán, Aguila & Leiva 7021*; CONC). Two of these specimens (*7325* and *7021*) lack fertile fronds, so it was not possible to determine whether they are hybrids. The other four specimens have well-formed spores indicating that they are not hybrids (though see Mayer and Mesler, 1993), and almost all characters suggest that these specimens are *B. mochaenum* subsp. *mochaenum*. However, two of these specimens (*6510* and *7452*) have exceptionally large spores (mean exospore lengths $> 40$ μm), and thus merit further study.

We have found the hybrid *B. corralense* × *B. mochaenum* at various locations in the Los Lagos region. In addition to abortive spores, this hybrid is distinguished by various characters that support its origin from a cross between *B. corralense* and *B. mochaenum* subsp. *mochaenum* (Fig. 1, Table 1). Characters shared by the hybrid and its hypothesized parents include dimorphic fronds, the fertile fronds being longer than the sterile fronds, and fertile and sterile pinnae slightly angled towards the frond apex, with numerous bi- and tricellular hairs 250–450 μm long on their abaxial face. In addition, the sterile fronds of the three taxa have aphlebia, i.e., markedly reduced pinnae at the base of the lamina. The texture of the lamina is

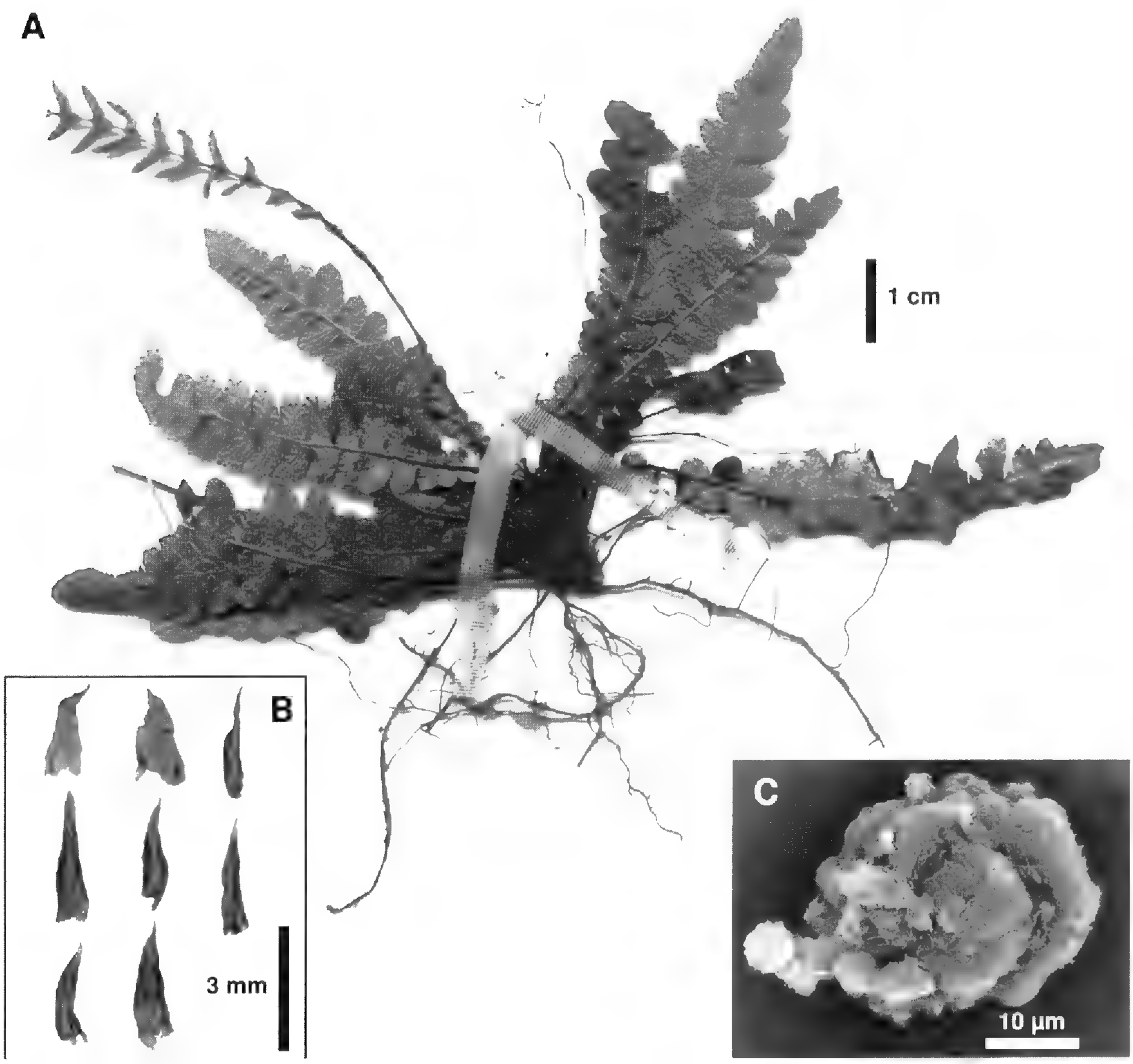


FIG. 1. Rodríguez's hybrid deer fern, *Blechnum* ×*rodriguezii*. A) type specimen (*Amigo & Rudloff CL1956*, SANT); B) petiole paleae (*Amigo CL1978*, SANT); C) scanning electron micrograph of spore (*Amigo & Quintanilla LGQ582*, SANT).

intermediate between that of *B. corralense* (herbaceous) and *B. mochaenum* subsp. *mochaenum* (coriaceous). Stoma length is likewise intermediate. However, some characters coincide with those in *B. corralense*: lengths of sterile and fertile fronds, number of pinnae per sterile frond, number of veins per sterile pinna, and size and shape of paleae. At several sites in the immediate vicinity of the hybrids, we have found *B. chilense* (Kaulf.) Mett. and *B. penna-marina* (Poir.) Kuhn, in addition to both hypothesized parents. *Blechnum chilense* is a giant deer fern with sterile fronds up to 150 cm long, morphologically very different from the hybrids. *Blechnum penna-marina* is more similar to the hybrids, but its sterile and fertile pinnae are oblong, perpendicular to the rachis, and subglabrous. This species, moreover, lacks aphlebia.

***Blechnum* ×*rodriguezii*** Aguiar, Quintanilla & Amigo, ***hyb. nov.*** TYPE.—CHILE. Los Lagos Region, **Valdivia Province**: Valdivia National Reserve, El Peuco, in close association with both putative parents, 585 m, 26 Jan 2000, *Amigo & Rudloff CL1956* (holotype: SANT; isotype: CONC).

Planta hybrida, media inter parentes putatos, sed *Blechno corralensi* aliquid similior. Ab hoc distinguitur: lamina magis coriacea et stomata minora. Sporae abortivae.

Dedicated to Roberto Rodríguez, Concepción, who has added very considerably our knowledge of the Chilean ferns, especially of the genus *Blechnum*.

PARATYPES.—CHILE. Los Lagos Region, **Valdivia Province:** Oncol hill, Sendero Bonifacio, 530 m, 11 Feb 2000, *Amigo CL1978* (SANT); Cordillera Pelada, Chivería river, 555 m, 13 Feb 2001, *Amigo & Quintanilla LGQ582* (SANT); **Palena Province:** ascent to Hornopirén National Park, 150 m, 23 Jan 2001, *Amigo, Pajarón, Pangua & Quintanilla LGQ545* (SANT); ascent to Termas El Amarillo, 330 m, 26 Jan 2001, *Amigo, Pajarón, Pangua & Quintanilla LGQ548* (SANT).

In these locations, the vegetation consists of Valdivian rainforest dominated by evergreen trees (*Nothofagus nitida, Laureliopsis philippiana, Podocarpus nubigena, Amomyrtus luma, Saxegothaea conspicua, Weinmannia trichosperma, Drimys winteri*, etc.; Amigo *et al.*, 2004). *Blechnum* ×*rodriguezii* appears most commonly on dripping soil banks and rock surfaces, on which it may be present at high density, possibly as a result of recurrent hybridization events and extensive clonal growth via stoloniferous axes (also present in *B. corralense* and *B. mochaenum* subsp. *mochaenum*). Given that both parents are endemic to southern Chile and Argentina, the potential area of distribution of this hybrid is limited.

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# Shorter Notes

**The Gametophyte of *Lycopodiella prostrata*.**—As part of an extended study on mycorrhizal and photosynthetic gametophytes of the Lycopodiaceae, spores of *Lycopodiella prostrata* (Harper) Cranfill, a species with an undescribed gametophyte, were cultured. The spores were obtained from plants collected in Cook County, Georgia and a voucher was deposited at VSU (*Carter #14616*). The conditions, techniques, and nutrient medium used were those of Whittier and Renzaglia (Amer. Fern J. 95:153–159. 2005). The system of classification followed in this report is that of Øllgaard (Opera Bot. 92:153–178. 1987).

There are five gametophyte types in *Lycopodium (s.l.)*. Four of the five are mycorrhizal with the following shapes – carrot-shaped, disk-shaped, uniaxial strap-shape, and branched cylindrical. The last type, which has been reported for *Lycopodiella*, is photosynthetic with a solid, more or less cylindrical base topped with photosynthetic lobes. This study was carried out to determine if the gametophyte of *L. prostrata* is this type.

Spore germination was slow. The earliest germination occurred two months after sowing spores in illuminated cultures, and at one year, 61 spores out of 10,000 (0.6%) had germinated. Spores cultured in the dark for one year did not germinate; however, spores from these dark cultures remained viable and 142 of them out of 10,000 (1.4%) germinated after moving them into the light for seven months.

Although spores of the mycorrhizal species of *Huperzia* and *Lycopodium* germinate slowly and at low percentages (Whittier, Amer. Fern J. 88:106–113. 1998), it is generally believed that *Lycopodiella* spores germinate rapidly and at high percentages (Whittier, Amer. Fern J. 88:106–113. 1998). This is not completely true because spores from some *Lycopodiella* species germinate slowly (Whittier, Amer. Fern J. 88:106–113. 1998).

Cell divisions in various planes formed a small mass of gametophyte tissue that remained partially contained by the spore coat. At about six weeks of growth, the young gametophyte escaped from the spore coat. At this time a small, dark green, ellipsoidal mass of cells formed – the young primary tubercle (Fig. 1A). Once the main body of the tubercle had a width of 150 µm or more, the first photosynthetic lobe developed at its apical end (Figs. 1B, 1C). Further enlargement of the tubercle resulted in a larger apical region where additional photosynthetic lobes formed. The lobes were erect, narrow, and strap-shaped with tapering distal ends.

The early mature gametophytes had a short, solid, more or less cylindrical base topped with numerous photosynthetic lobes. As the gametophytes aged, more lobes formed, and the previously formed lobes were displaced to the sides of the larger base. Gametophytes at this stage are illustrated in Figs. 1D and 1E.

The gametangia usually formed at the junction of the photosynthetic lobe and the gametophyte base. Both archegonia and antheridia developed on the

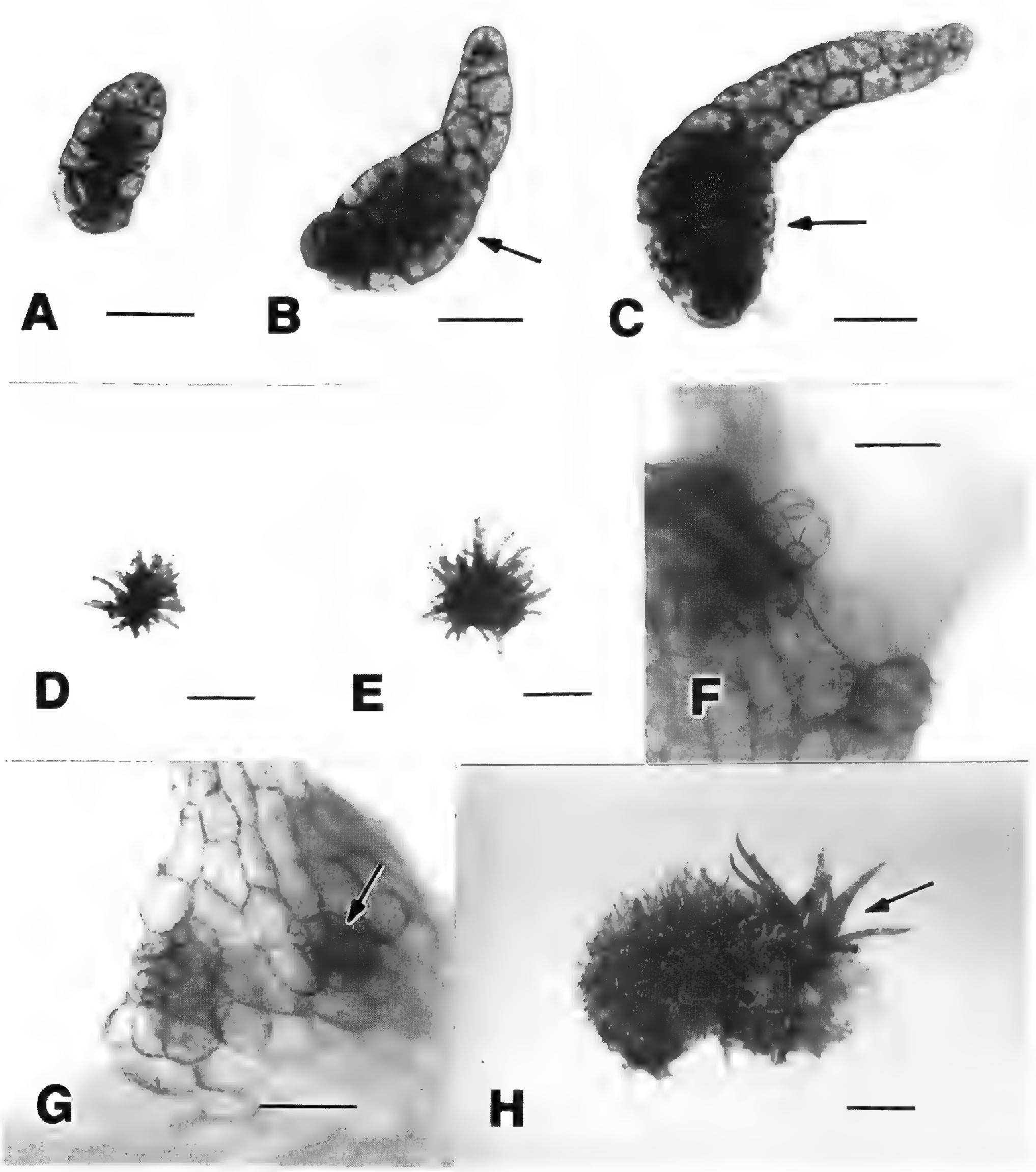


FIG. 1. Gametophytes of *Lycopodiella prostrata*. A. Primary tubercle. B–C. Primary tubercles (arrows) with young photosynthetic lobes (ca. 2 mo old). D. Oblique view of early mature gametophyte (ca. 5 mo old). E. Apical view of gametophyte (ca. 7 mo old). F. Two archegonia with short necks. G. Two antheridia – one with view of opercular cell (arrow). H. Large gametophyte (ca. 18 mo old) with young sporophyte (arrow). Bars = 100 μm for Figs. A–C & F–G, 1 mm for Figs. D–E, and 2 mm for Fig. H.

young mature gametophytes. The archegonia had short necks made up of two tiers of neck cells exposed above the gametophyte surface (Fig. 1F). The length of the archegonial neck was about 70 μm long. The length from the tip of the neck to base of egg was about 110 μm as determined with optical sections. Each antheridium had one opercular cell in the antheridial jacket at the gametophyte surface (Fig. 1G). Optical sections showed the gamete masses of the antheridia to be essentially spherical with diameters of about 70 μm.

The small, young gametophytes with both antheridia and archegonia continued to grow on the nutrient medium without undergoing sexual reproduction. With age these medium-sized gametophytes took on a pincushion shape (Fig. 1D, 1E). After a year or more in culture, large pincushion-shaped gametophytes formed. The solid basal portions of these gametophytes were obscured by the numerous photosynthetic lobes (Fig. 1H).

Mature gametophytes were capable of fertilization if water was added to the cultures. Fifty older gametophytes growing in separate cultures produced 24 sporophytes after flooding with water. The first microphylls, which were larger than the photosynthetic lobes, were evident two weeks after flooding. Within three months the young sporophytes became well established with numerous microphylls growing above the photosynthetic lobes (Fig. 1H).

The development of the primary tubercle is typical for *Lycopodiella* gametophytes and the ellipsoidal or oblong shape is known from other species (Whittier & Renzaglia, Amer. Fern J. 95:153–159. 2005). A growth from the top of the tubercle, the intermediate shaft, which was reported for *Lycopodiella* gametophytes growing on soil (Holloway, Trans. New Zealand Inst. 48:253–303. 1916; Bruce, Amer. J. Bot. 66:1156–1163. 1979), does not develop in *L. prostrata* under these conditions. It appears that the growth of *Lycopodiella* gametophytes in well-illuminated cultures prevents the development of the intermediate shaft (Whittier & Renzaglia, Amer. Fern J. 95:153–159. 2005).

Photosynthetic lobes develop from the top of the tubercle in *L. prostrata* as was observed with the gametophyte of *Lycopodiella lateralis* (R.Br.) B. Øllg. (Whittier & Renzaglia, Amer. Fern J. 95:153–159. 2005). The formation of the pincushion-shaped gametophyte with many green lobes arising from a solid base is typical for *Lycopodiella* (Wagner & Beitel, Flora North America 2:18–37. 1993). The young pincushion-shaped gametophytes with photosynthetic lobes arising from the apex and sides of the solid base appear to have a radial symmetry (Figs. 1D, 1E). The symmetry of the larger pincushion-shaped gametophytes (Fig. 1H) appears dorsiventral as was reported for *Lycopodiella carolinianum* by Bruce (Amer. J. Bot. 66:1156–1163. 1979). The long strap-shaped lobes have been described for *Lycopodiella* gametophytes previously (Whittier & Renzaglia, Amer. Fern J. 95:153–159. 2005).

Both gametangia form on these gametophytes at the base of the photosynthetic lobes. Descriptions of *Lycopodiella* archegonia indicate that they have short necks (Bruce, Amer. J. Bot. 63:919–924. 1976; Wagner & Beitel, Ann. Mo. Bot. Gard. 79:676–686. 1992). The antheridia are smaller than those reported for the terrestrial species of *Huperzia* (Whittier, Pintaud, & Braggins, Amer. Fern J. 95:22–29. 2005) and much smaller than those of *Lycopodium* (Bruce,

Amer. J. Bot. 66:1138–1150. 1976; Whittier, Canad. J. Bot. 55:563–567. 1977). The gametangia of *Lycopodiella appressa* (F.Lloyd & L.Under.) Cranfill and *Lycopodiella cernua* (L.) Pichi-Serm. have essentially the same sizes as those of *L. prostrata*. The gametangia of *L. prostrata* are typical for *Lycopodiella*.

The development of the other types of gametophytes of the Lycopodiaceae is quite different from that found in *Lycopodiella*. The mature gametophyte of *Phylloglossum* is photosynthetic but it starts out as a subterranean, mycorrhizal gametophyte that is negatively gravitropic. After its exposure to light at the soil surface it becomes a green, bilaterally symmetrical, tuberous gametophyte lacking photosynthetic lobes (Whittier & Braggins, Amer. J. Bot. 87:920–924. 2000).

The remaining gametophytes of the Lycopodiaceae are subterranean, mycorrhizal, and nonphotosynthetic. Their development is initiated underground by the dark germination of their spores and requires a mycorrhizal association for continued growth. Early growth forms a solid, teardrop-shaped gametophyte that gives rise to the four other gametophyte shapes found in the Lycopodiaceae. Larger teardrop-shaped gametophytes develop ring meristems that form the radially symmetrical disk- and carrot-shaped gametophytes of *Lycopodium* (Whittier, Canad. J. Bot. 55:563–567. 1977; Whittier, Bot. Gaz. 142:519–524. 1981).

The uniaxial, dorsiventral, strap-shaped gametophyte of the terrestrial *Huperzia* species lacks a ring meristem. The meristem arises from a portion of the apical region of a larger teardrop-shaped gametophyte (Bruchmann, Flora 101:220–267. 1910). This meristem occurs in a subterminal groove overarched by young dorsal tissue on these strap-shaped gametophytes. With the epiphytic *Huperzia* species, the teardrop-shaped gametophyte enlarges and grows into the branched, cylindrical, mycorrhizal gametophyte (Whittier unpublished).

The gametophyte of *L. prostrata* has the typical structure and development of *Lycopodiella* gametophytes; thus it is different from the other gametophyte types of the Lycopodiaceae.—Dean P. Whittier, Department of Biological Sciences, Box 1634, Vanderbilt University, Nashville, TN 37235-1634, and Richard Carter, Department of Biology, Valdosta State University, Valdosta, GA 31698-0015.

**Three New Flavonoid Glycosides, Kaempferol 3-*O*-(caffeoylrhamnoside), Apigenin 4′-*O*-(caffeoylglucoside) and 4′-*O*-(feruloylglucoside) from *Dryopteris villarii*.**—Ten flavonol *O*-glycosides (based on kaempferol and quercetin), two flavanone *O*-glycosides (based on naringenin and eriodictyol) and three *C*-glycosylflavones ( vitexin, vitexin 7-*O*-glucoside and orientin) have previously been identified by Hiraoka (Biochem. Syst. Ecol. 6: 171-175. 1978) in eighteen *Dryopteris* species whereas 3-desoxyanthocyanins have been found in red sori of *Dryopteris erythrosora* (Eat.) Kuntze by Harborne (Phytochemistry 5: 589–600. 1966). In addition kaempferol 7-*O*-(6″-succinyl-

glucoside) was found in four *Dryopteris* species and an unusual flavan was isolated from *Dryopteris filix-mas* (L.) Schott as shown in a review by Markham (pp. 427–468, *in* J.B. Harborne ed., *The Flavonoids, Advances in Research since 1980*. Chapman and Hall, London and New York. 1988). Eighteen flavonoids (14 flavonol glycosides, one flavone glycoside and three aglycones) have been found recently in *Dryopteris villarii* by Imperato (Amer. Fern J. 96: 93–96. 2006; Amer. Fern J. 97(2): 124–126. 2007; Nat. Prod. Commun. 2: 909–912. 2007).

This paper deals with identification of three flavonoids (I–III) from aerial parts of *Dryopteris villarii* (Bellardi) Schinz & Thell collected in the Botanic Garden of the University of Naples (Italy). The fern was identified by Dr. R. Nazzaro (Università "Federico II", Naples); a voucher specimen (*NAPEA 3496*) has been deposited in Herbarium of Dipartimento di Biologia, Università "Federico II", Naples, Italy (NAP).

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

Color reactions (brown to yellow in UV+$NH_3$), $R_f$ values on Whatman N.1 paper (0.75 in BAW; 0.23 in 15% AcOH; 0.08 in water) and UV spectral analysis in the presence of usual flavonoid shift reagents ($\lambda_{max}$ (nm) (MeOH) 266, 324; +$AlCl_3$ 274, 303, 347, 398; +$AlCl_3$/HCl 274, 300, 343, 396; +NaOAc 272, 300, 370; +NaOMe 272, 325, 395) suggested that flavonoid (I) may be a flavonoid glycoside with free hydroxyl groups at positions 5, 7 and 4′ (shifts with $AlCl_3$/HCl, NaOAc and NaOMe respectively); in addition flavonoid (I) may be acylated with a hydroxycinnamic acid since the UV spectrum of hydroxycinnamic acid is superimposed on the flavonoid spectrum as shown in a review by Harborne and Williams (pp. 376–441 *in* J.B. Harborne, T.J. Mabry, H.Mabry, eds. *The Flavonoids*, Chapman and Hall, London. 1975). Both total acid hydrolysis (2 N HCl; 2 hr at 100°C) and controlled acid hydrolysis (10% AcOH, 3.5 hr under reflux) gave kaempferol and L-rhamnose whereas alkaline hydrolysis (2N NaOH, 2 hr at room temperature in a sealed tube) gave 3, 4-dihydroxycinnamic acid (caffeic acid) and kaempferol 3-*O*-rhamnoside. CID (collision induced dissociation) mass spectrum (negative mode) gave a quasi-molecular ion $[M-H]^-$ at m/z 593 and fragment ions at m/z 431 (kaempferol 3-*O*-rhamnoside) and m/z 285 (kaempferol). These results show that flavonoid (I) is kaempferol 3-*O*-(caffeoylrhamnoside), a new natural product (Fig. 1).

Color reactions (brown to yellow in UV+$NH_3$), $R_f$ values on Whatman N.1 paper (0.78 in BAW, 0.21 in 15% AcOH, 0.04 in water) and UV spectral analysis in the presence of usual shift reagents ($\lambda_{max}$ (nm) (MeOH) 262, 322; +$AlCl_3$ 272, 305 (sh), 340, 383; +$AlCl_3$/HCl 270, 294 (sh), 326, 386 (sh); +NaOAc 268, 340; +NaOMe 271, 347) showed that flavonoid (II) may be a flavonoid glycoside with free hydroxyl groups at positions 5 and 7 (shifts with $AlCl_3$/HCl and NaOAc respectively). In addition flavonoid (II) may be acylated with a hydroxycinnamic acid since the UV spectrum of hydro-

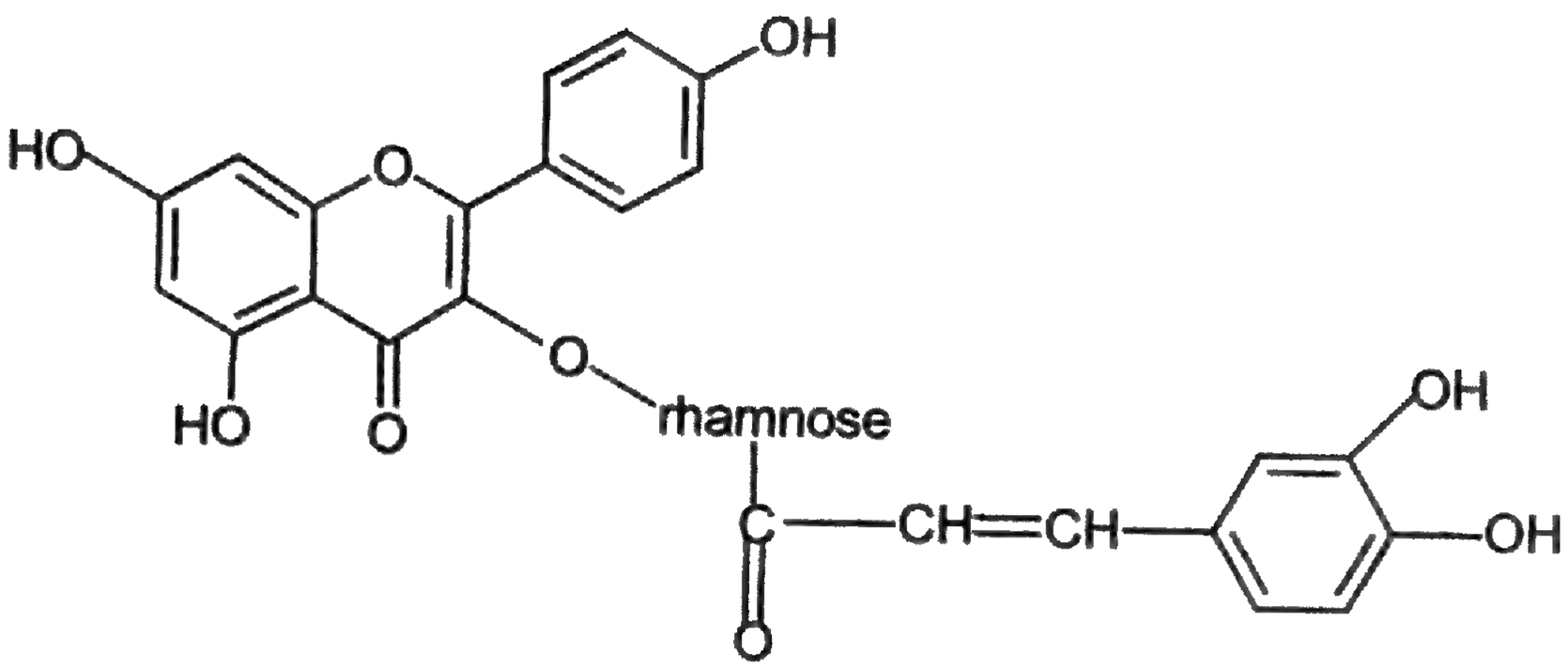


FIG. 1. Kaempferol 3-*O*-(caffeoylrhamnoside).

xycinnamic acid is superimposed on the flavonoid spectrum (Harborne and Williams, 1975). Both total acid hydrolysis (2N HCl; 1 hr at 100°C) and controlled acid hydrolysis (10% AcOH; 3.5 hr under reflux) gave apigenin and D-glucose whereas alkaline hydrolysis (2 N NaOH; 2 hr at room temperature in a sealed tube) gave apigenin 4′-*O*-glucoside and 3, 4-dihydroxycinnamic acid (caffeic acid). CID (collision induced dissociation) mass spectrum (negative mode) showed a quasimolecular ion at m/z 593 $[M-H]^-$ and fragment ions at m/z 431 (apigenin 4′-*O*-glucoside), m/z269 (apigenin) and m/z 253 (glucosylated B-ring). These results show that flavonoid (II) is apigenin 4′-*O*-(caffeoylglucoside), a new natural product (Fig. 2).

Color reactions (brown to yellow in UV+$NH_3$), $R_f$ values on Whatman n.1 paper (0.72 in BAW; 0.26 in 15% HOAc; 0.04 in water) and UV spectral analysis with the usual shift reagents ($\lambda_{max}$ (nm) (MeOH) 263, 319; +$AlCl_3$ 272, 305, 340, 382; +$AlCl_3$/HCl 270, 294, 326, 380 (sh); +NaOAc 268, 340; +NaOMe 271, 351) suggested that flavonoid (III) may be a flavonoid glycoside with free hydroxyl groups at positions 5 and 7 (shifts with $AlCl_3$/HCl and NaOAc respectively); in addition flavonoid (III) may be acylated with hydroxycinnamic acid since the UV spectrum is superimposed on the flavonoid spectrum (Harborne and Williams, 1975). Both total acid hydrolysis (2N HCl; 1 hr at 100°C) and controlled acid hydrolysis (10% AcOH; 3.5 hr under reflux) gave apigenin and D-glucose whereas alkaline hydrolysis (2N NaOH; 2 hr at room temperature in a sealed tube) gave 3-methoxy-4-hydroxy-cinnamic acid (ferulic acid) and apigenin 4′-*O*-glucoside. The electrospray mass spectrum exhibits a pseudomolecular ion at m/z 632 $[(M+H)+Na]^+$ and fragment ions at m/z 455 (apigenin glucoside+Na) and 271 (apigenin). These results show that flavonoid (III) is apigenin 4′-*O*-(feruloylglucoside), a new natural product (Fig 2).

As shown in a review by Williams (pp. 749–856 *in* O.M. Andersen and K.R. Markham eds., *Flavonoids, Chemistry, Biochemistry and Applications*, CRC Press, London, New York. 2006) flavonoids having an acyl group linked to a carbohydrate attached at position 4′ of B-ring (as flavonoids (II) and (III)) are rare natural products. Such compounds have previously been reported by

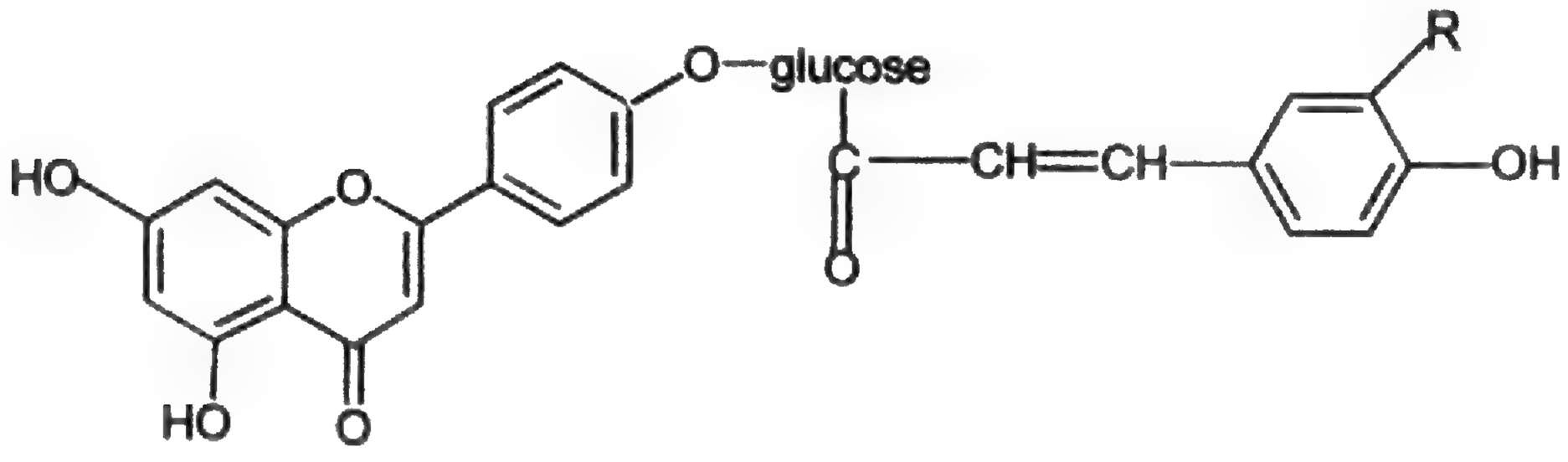


FIG. 2. Apigenin 4′-*O*-(caffeoylrhamnoside). R=OH; Apigenin 4′-*O*-(feruloylglucoside). R=$OCH_3$.

Horie *et al.* (Phytochemistry 25: 2621–2624. 1986) who isolated 6, 8-dihydroxyluteolin 6, 8, 3′-trimethyl ether 4′-*O*-(6″-(3-hydroxy-3-methylglutaryl) glucoside) (sudachiin B) from *Citrus sudachi* green peel (Rutaceae) and by Stockmalia *et al.* (Phytochemistry 57: 1223–1226. 2001) who found apigenin 4′-*O*-(2″ feruloylglucuronosyl (1→2) glucuronide) from aerial parts of *Medicago sativa* L. var. *artal* (Leguminosae). The first occurrence of these compounds in ferns has been described by Imperato (Nat. Prod. Commun. 2: 909–912. 2007) who identified apigenin 4′-*O*-(*p*-coumaroylglucoside) in aerial parts of *Dryopteris villarii.* The presence of acylated flavonoid glycosides previously reported in *Dryopteris villarii* by Imperato (Amer. Fern J. 96, 93–96. 2006; Nat. Prod. Commun. 2: 909–912. 2007; Amer. Fern J. 97(2): 124–126. 2007) and identification of three further acylated flavonoid glycosides (I–III) in *Dryopteris villarii* show that this fern has a number of acylated flavonoid glycosides which are generally absent from *Dryopteris* species with the exception of kaempferol 7-*O*-(6″ succinylglucoside) in four *Dryopteris* species and a flavan acetate in *Dryopteris filix-mas* (L.) as shown in a review by Markham (1988).

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# AMERICAN FERN JOURNAL

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*2007*

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

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