Cryptogamie, Bryologie, 2006, 27 (1): 85-96

Notes on neotropical *Cyathodium*

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(Received 3 October 2005, accepted 12 December 2005)

Resumen – Se reconocen cinco especies de *Cyathodium* para el Neotrópico. Las especies crecen en hábitats sombreados que varían desde laderas inestables de los ríos hasta potes y desagües de cemento de las carreteras. Las especies de *Cyathodium* son especies de selección r. Dos especies son dioicas y tres monoicas. La reproducción vegetativa por tubérculos está restringida a las especies dioicas las que también producen abundantes esporofitos. Todas las especies monoicas producen abundantes esporofitos. Los patrones de germinación de esporas de *C. cavernarum y C. foetidissimum* que crecen en medios de cultivo, incluyen una fase inicial filamentosa seguida por el desarrollo de los talos en el ápice del filamento protonemático. *Cyathodium spruceanum* produce un protonema multiseriado a manera de brote del cual desarrolla el talo. Se seleccionaron doce caracteres gametofíticos y dos esporofíticos para discriminar entre las species, tanto en estado vegetativo como fértil. Para cuatro especies se analizaron las variaciones en la secuencias de nucleótidos del ADN ribosomal del núcleo, en la región ITS1-5.85S rRNA-ITS2. Cada especie es genética y morfológicamente distintiva. Las diferencias genéticas más grandes se encontraron entre *C. foetidissimum* y *C. spruceanum*. Las secuencias para *C. bischlerianum* no resultaron y por lo tanto no se incluyeron en el análisis. Los especímenes de *C. cavernarum* y *C. spruceanum* de áreas geográficas cercanas están genéticamente más relacionados entre sí que con aquéllos de sitios más distantes.

Abstract – Five species of *Cyathodium* occur in the Neotropics. The species grow in shaded habitats that range from unstable river banks to cement pots and road ditches. *Cyathodium* species are r-selected species. Two species are dioicous and three are monoicous. Vegetative reproduction by tubers is restricted to dioicous species that also produce abundant sporophytes. All monoicous species produce abundant sporophytes. Sporeling patterns of *C. cavernarum and C. foetidissimum* grown under culture conditions include an initial filamentous phase followed by the apical development of thalli. *Cyathodium spruceanum* produces a multiseriate bud-like protonema from which the thallus develops. Twelve gametophytic and two sporophytic characters were selected to discriminate between species in vegetative and fertile states. Nucleotide sequence variation in the nuclear ribosomal DNA region, ITS1-5.8S rRNA-ITS2 was analyzed for four species. Each species is genetically and morphologically distinctive. The largest genetic differences were found between *C. foetidissimum* and *C. spruceanum.* Sequences for *C. bischlerianum* failed and were not included in the analysis. Samples of *C. cavernarum* and *C. spruceanum* from nearby geographical areas were shown to be genetically more closely related than to those of geographical distant areas.

Ephemeral life-cycle / r-selected species / monoicous and dioicous species / sporeling patterns / molecular phylogeny

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INTRODUCTION

Cyathodium Kunze is a pantropical Marchantialean liverwort with 12 species worldwide, eight in the Paleotropics and five in the Neotropics (Srivastava & Dixit, 1996; Salazar Allen, 2005). Of the Paleotropical species two occur in tropical America: *C. cavernarum* Kunze and *C. foetidissimum* Schiffn. Three species are endemics to the Americas, *C. bischlerianum* Salazar Allen, *C. spruceanum* Prosk. and *C. steerei* Hässel (Salazar Allen, 2005). Two of the Neotropical species, *C. foetidissimum* and *C. steerei* have a restricted distribution. *Cyathodium foetidissimum* is known from collections from Costa Rica (Parque Nacional Tapantí) and a dubious collection from Ecuador (Salazar Allen *et al.*, 2004) and *C. steerei* from the type locality in Tucumán, Argentina (Hässel de Menéndez, 1961, 1962). Recently a population of *C. foetidissimum* has been found in Southern Italy (Duckett & Ligrone, 2005). The authors suggested that this population is most likely a pre-glacial relic rather that a recent acquisition.

ECOLOGICAL NOTES

Plants of *Cyathodium* grow in a variety of shaded habitats, along river banks on soil or rocks, on water falls, caves, cement floors, stairs and flower pots, and may also be corticolous (on bark of several species including *Ficus, Anacardium excelsum* (Bertero & Balb.) Skeels, *Attalea butyraceae* (L.f.) Wess. Boer and *Socratea* sp.). Large communities may be observed (Costa Rica and Panama) on unstable river banks, where they can be subject to submergence or be detached from substrate by river floods or drying of the soil and along cement road ditches (Costa Rica) in humid areas. Plants have been found on soils with pH 5.45 to 7.84, mostly on loamy clay to sandy loam. *Cyathodium cavernarum* and *C. bischlerianum* are the only corticolous species. *Anacardium excelsum* cortex has a pH of 6.50 to 6.98. All *Cyathodium* species have been found associated with filamentous cyanobacteria. Nevertheless, none of the bacteria have been found inside the thallus. Fungal endophytes have been isolated from wild populations of *C. spruceanum* and *C. cavernarum* from Panama (Salazar, unpublished data). Association of fungi with *Cyathodium* has also been observed in populations of *C. foetidissimum* growing in the Malay Peninsula (Perak) (Lang, 1905) and in Italy (Duckett & Ligrone, 2005). Other liverworts found in association with *Cyathodium* are *Lejeunea cladogyna* Evans, *Caudalejeunea lehmanniana* (Gottsche, Lindenb. & Nees) A. Evans, *Dumortiera hirsuta* (Sw.) Nees and *D. hirsuta* subsp. *nepalensis* (Taylor) R. M. Schust., as well as various species of *Marchantia, Riccia* and *Radula* and the hornworts *Notothylas* and *Phaeoceros.* Among the mosses are various species of *Fissidens* (e.g., *F. flaccidus* Mitt.), *Taxiphyllum taxirameum* (Mitt.) Fleisch., *Cyrtho-hypnum scabrosulum* (Mitt.) W. R. Buck & H. A. Crum, *Chryso-hypnum diminutivum* (Hampe) W.R. Buck, *Racopilum tomentosum* (Hedw.) Brid., *Brymela* sp., *Philonotis* and some Pottiaceae (Salazar Allen, 2005). Diatoms have also been observed in plants growing along river banks including species of *Surirella, Nitzschia, Amphora, Rhopalodia, Eunota* and possibly *Diploneis*. A specimen of *Enochrus* sp. (Coleoptera: Hydrophilidae) was also observed among thalli collected from a rock

in a creek. Species of this family are aquatic and a few live in feces of animals or in humid soil. Most of these Coleoptera are scavengers though some species are predators of other aquatic animals (R. Cambra, pers. comm.). Some predation has been observed in thalli of *C. spruceanum*, particularly for male receptacles. It is unknown what organism is involved in this phenomenon.

Species of *Cyathodium* have an ephemeral life cycle, disappearing during the dry season except in places where water supply is constant. Cultures on soil have been maintained in a glass container for two years, but produced no sex organs (Salazar Allen, unpublished data). Thus, mortality seems to be determined by environmental rather than by biotic factors as suggested by Joenje & During (1977) and During (1979) for ephemeral bryophytes and by Pianka (1970) for r-selected organisms. An ephemeral ("fugitive species"— During, 1979) life cycle is also known for various other bryophytes, among these, some liverwort species of *Riccia* and *Ricciocarpus* and the moss *Funaria hygrometrica* Hedw.

According to the concepts of r- and K- selection (Pianka, 1970; Gadgil & Solbrig, 1972; During, 1979), *Cyathodium* species are r-selected. In such species (ephemerals, fugitives and pioneers), selection acts to increase r, the intrinsic reproductive rate (Slack, 1982). This could result in abundant production of sporophytes and/or in various types of asexual diaspores. *Cyathodium spruceanum* usually grows in homogeneous patches on exposed places and populations are rather large in years that have high rainfall. On river banks, *C. spruceanum* occupies the upper slopes while *C. cavernarum* and *C. bischlerianum* grow in more shaded, damper habitats closer to the river or in areas of seepage. *Cyathodium cavernarum, C. bischlerianum, C. foetidissimum* and *C. steerei* appear to be more mesic than *C. spruceanum*.

According to Pianka (1970), in r-selected organisms population size is variable in time, and recolonization occurs each year with variable or lax intra and interspecific competition, as observed in *Cyathodium*.

The dioicous *Cyathodium spruceanum* produces ventral tubers in the middle of the rainy season that generally remain attached to the parent thalli until these decay. Cultures of *C. spruceanum* produce abundant tubers when culture media is depleted of nutrients (Salazar Allen, unpublished data). Tubers consist of a central mass of parenchymatous tissue, an apical area overarched by thallus flaps, and many rhizoids on the distal part (Fig. 1, A-B). Under laboratory conditions (agar with Knop nutrient media and in soil), detached tubers develop into new gametophytes - but in nature they have not been seen germinating. It seems likely that, covered by mud, the tubers may remain latent until the next season, or that they act as a pool of diaspores that may reproduce new thalli if the population is decimated or if the growing season is extended, particularly if plentiful water is available. Ventral branches have also been observed in *C. cavernarum*; it is most probable that these may serve for vegetative reproduction when detached from the parent plant.

In addition *C. spruceanum* produces abundant sporophytes. Male and female plants have been observed growing close to each other. Sporophytes have a great number of spores (>400) that are capable of reproducing new male and female gametophytes in the same year when cultured. Nevertheless, spores have not been seen germinating under natural conditions in the same season they are produced. This is most probably due to the dryness of the soil when spores are released at the onset of the dry season rather than a dormancy requirement. Two female morphotypes have been observed in nature. One morphotype ("the normal") produces 5-15 archegonia and has an involucre with lips closely adjacent to each other. The other morphotype produces 15-30 archegonia and the involucre is

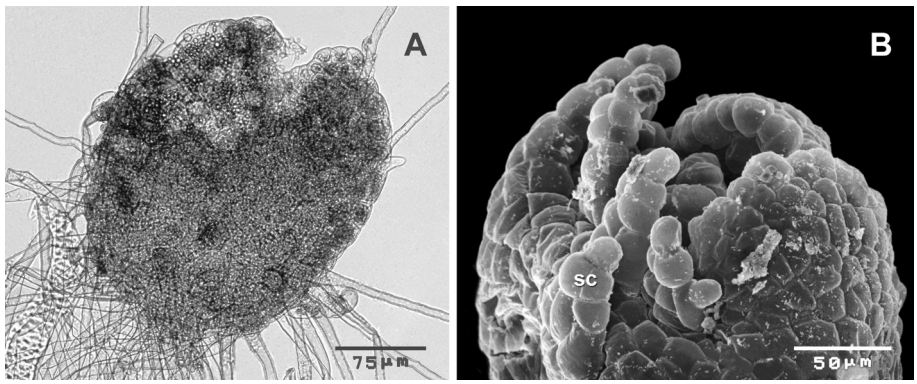

Fig. 1. Tubers. **A-B**. *Cyathodium spruceanum*, sc = ventral scales [From *Salazar Allen et al. 16572*, Panamá].

greatly enlarged, abutting ventrally (Fig. 96 in Salazar Allen, 2005). No sporophytes have been observed in this morphotype. Both morphotypes produce asexual tubers. Spores grown in culture from the first morphotype produce only "normal" gametophytes. Thalli with multiple archegonia can be grown from tubers.

Monoicous species of *Cyathodium* do not produce tubers but produce abundant sporophytes and ventral branches. It is unknown what proportion of sporophytes are produced by autogamy or by cross fertilization.

SPORELING STUDIES

Methodology

Spores of *C. cavernarum, C. foetidissimum* and *C. spruceanum* (Fig. 3) freshly collected were germinated in an environmental growth chamber EGC Q9616 NQ2. The growth medium was Knop (pH 6.0-6.5) in agar (1%), day/night temperatures 22ºC /18ºC and a photoperiod of 12 hours. Light regimes for each species were provided according to observations and light measurements taken in the field. A data logger L1-1000, Li-COR was used to measure light intensity at different levels in the chamber. For *C. spruceanum* and *C. cavernarum* light intensity varied from (6.9-)16 to 21(-25) µmolm^{-2} s^{-1} and for *C. foetidissimum* from 3.7 to 7.3. Capsules were sterilized in 10% commercial Clorox for 1.5-2 min. and washed three times with sterile deionized water. Capsules were opened in a sterile vial containing 500 µml deionized water, 160 µml of the solution containing spores were sown over the agar.

Results

Cyathodium foetidissimum and *C. cavernarum* produced filamentous protonema with abundant chloroplasts (Fig. 2, D). Spores of *C. cavernarum* germinating in distilled water also produced a filamentous protonema (Fig. 2, A-C). The protonema branched after a few cells were produced (Fig. 2B). In one instance, in *C. cavernarum*, a two- branched protonema was observed in early

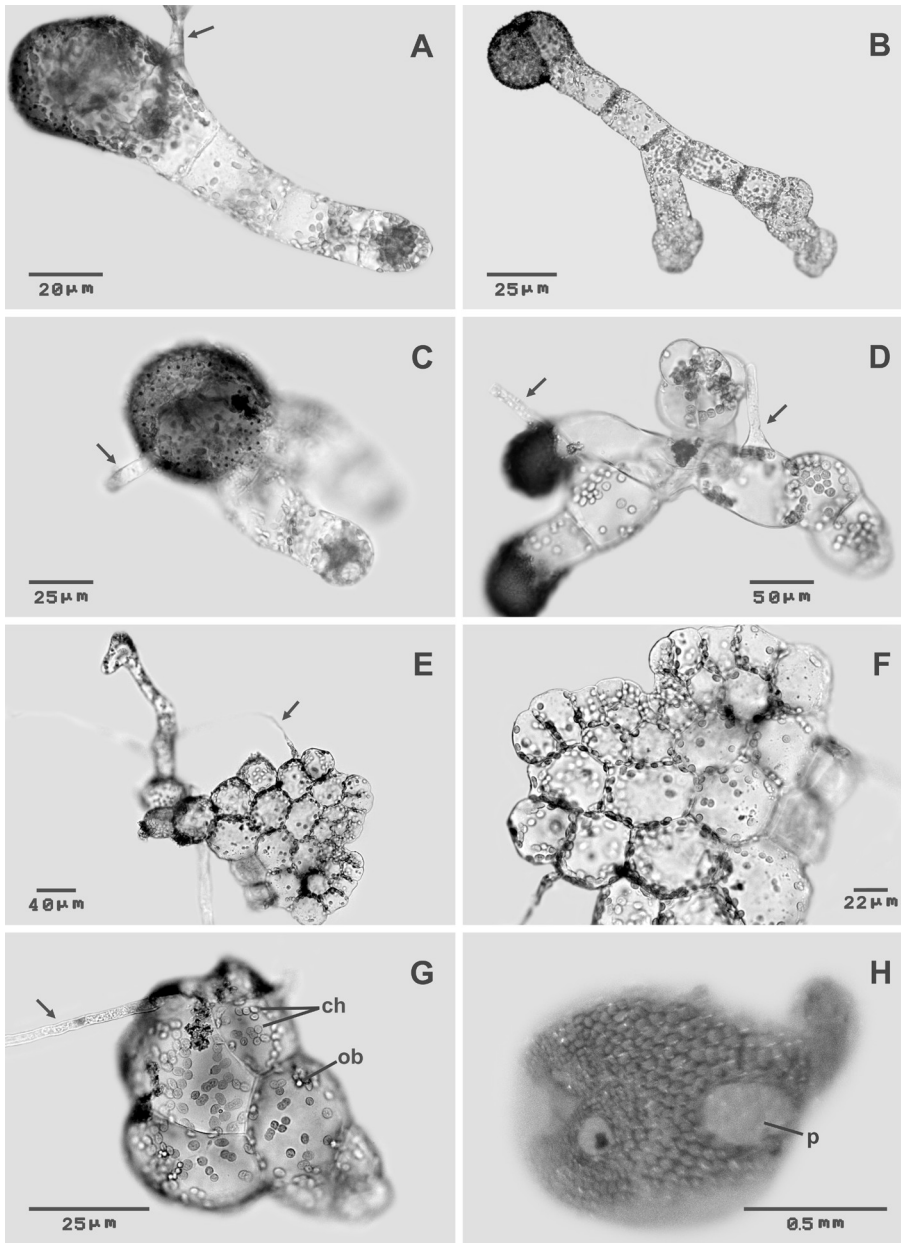

Fig. 2. Germination of spores. **A-C -** *Cyathodium cavernarum:* spores in distilled water, a month after collected. - **D-F -** *Cyathodium foetidissimum:* early stages (**D**); spores after a month germination (**E-F**). **- G-H -** *Cyathodium spruceanum:* early stages (**G**); after 2 months (**H**). [A-C from *Salazar Allen et al. 17005*, Panamá; D-F from *Salazar Allen et al. 17047*, Costa Rica; G-H from *Salazar Allen et al. 16817*, Panamá; all collections at PMA]. Arrows point to rhizoids, ch = chloroplasts, ob = young oil body, p = pore.

germination of spores (Fig. 2C). A multicellular bud-like structure developed at the apex of the filamentous protonema (Fig. 2, B, D). This expanded into a dorsiventral thallus with air cavities and pores. Rhizoids were also produced in early stages of protonemal development (Fig. 2, C-G). Spores of *C. spruceanum* did not seem to have a noticeable uniseriate filamentous phase. Instead, they germinated into short multicellular globose protonemata (Fig. 2G). The dorsiventral thalli soon developed at the apex of the multicellular protonemata producing large air cavities and pores (Fig. 2H). Studies under natural conditions are needed to see whether sporeling patterns are similar or vary from those observed under controlled environmental conditions. Further growth experiments are planned for the three species growing in Panama and for cultures of *C. foetidissimum*.

MOLECULAR MEASURES OF SPECIES RELATIONSHIPS

Selected morphological characters and their states

The five neotropical species of *Cyathodium* are distinguished not only by distinctive spore wall architectures (Fig. 3), but also by a suite of gametophytic and sporophytic characters as summarized in Table 1. Detailed species descriptions, illustrations and discussions can be found in Salazar Allen (2005).

To address the relationships among the *Cyathodium* species at the molecular level and to investigate the level of variation within species, nucleotide sequence variation in the nuclear ribosomal DNA region ITS1 - 5.8S rRNA - ITS2 was analyzed.

Methodology

DNA was extracted from freshly collected gametophytes of *C. bischlerianum, C. cavernarum, C. foetidissimum* and *C. spruceanum* using DNeasy Plant Mini Kits (QIAGEN Inc.). The samples used in the final phylogenetic analysis are listed in Table 2. All vouchers are deposited at PMA. The sequencing of *C. bischlerianum* failed and, consequently, the species is not included in the analysis. The sequenced DNA templates were obtained by PCR using the primer pair ITS-Bryo: 5'-GGA AGG AGA AGT CGT AAC AAG G-3' and ITS-4R: 5'-TCC TCC GCT TAT TGA TAT GC-3'. The amplified region covered the whole distance from the end of the 18S rRNA gene to the beginning of the 26S rRNA gene, including the region ITS1 - 5.8S rRNA ITS2. The 20-µl PCR-amplification reactions contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer, about 10-20 ng of genomic DNA and 1.2 units of DynaZyme II DNA polymerase (Finnzymes). The thermocycler (MJ Research, Inc., model PTC-200) was programmed for 4 min denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 90 s. An additional 8-min elongation followed the last cycle. Amplification products were extracted from a stained 1% agarose gel and purified using a QIAquick Gel Extraction Kit (QIAGEN, Inc.) before sequencing was conducted using an automated sequencer (ABI Prism 377 XL, PE Applied Biosystems) with the PCR primers. Sequencing was conducted to both directions. The sequence analysis was based on data from both ITS1 and ITS2. The sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and later hand-edited. The primary sequence data were bootstrapped with SEQBOOT 100 times, after which pairwise

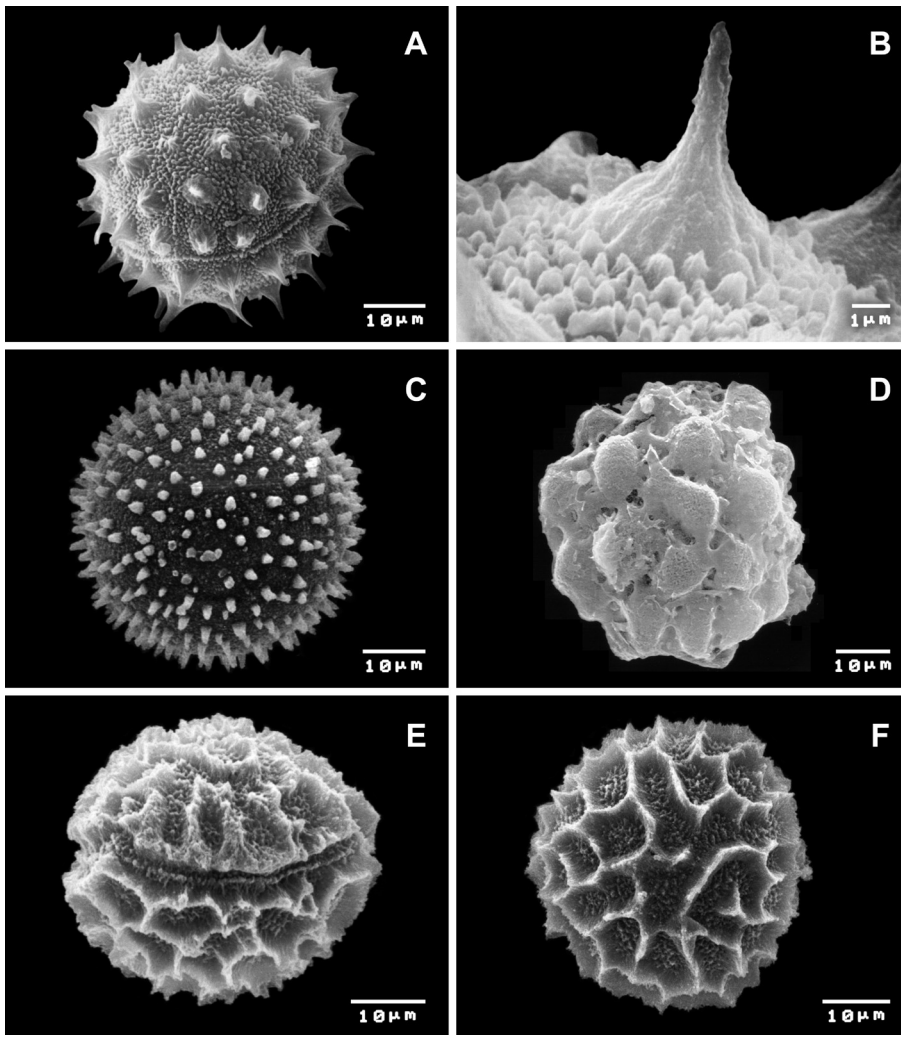

Fig. 3. Spores. **A-B -** *Cyathodium bischlerianum:* echinate spore; **A**, proximal face with guirdle and faint trilete mark; **B**, detail of spine and exine. – **C -** *Cyathodium cavernarum:* spore baculate-spinose, distal face. – **D -** *Cyathodium foetidissimum:* spore verrucose tuberculate, distal face. **- E-F -** *Cyathodium spruceanum:* spores lamellate with close reticulum, **E**, view of cingulum, **F**, distal face. [A-B from *Salazar Allen et al. 16612*, Panamá; C from *Salazar Allen et al. 16692*, Panamá; D, from *Salazar Allen et al. 17049*, Costa Rica; E-F from *M. Rodríguez 25*, Panamá; all collections at PMA].

genetic distances, based on the F84 model (Kishino & Hasegawa, 1989; Felsenstein & Churchill, 1996), were computed using DNADIST, both programs from the PHYLIP 3.6 software package (Felsenstein, 2004). Genetic distances were then clustered using KITSCH, carrying out the Fitch-Margoliash method (Fitch & Margoliash, 1967), while the program CONSENSE was used to find the consensus tree, and DRAWGRAM was used to plot a rooted tree, all programs from the

Table 1. Distinguishing characters among species of Neotropical *Cyathodium* and current geographic distribution.

Characters	*Cyathodium foetidissimum*	*Cyathodium cavernarum*	*Cyathodium bischlerianum*	*Cyathodium steerei*	*Cyathodium spruceanum*
			GAMETOPHYTIC		
Thallus habit	Elongated (4.0-17.0 × 1.0-6.0 mm), dichotomously branched	Rosettes or fan-shaped (3.8-9.0 × 0.7-2.2 mm)	Rosettes or fan-shaped (3.5-6.5 × 1.8-2.7 mm)	Elongated (2-5 × 1.5-3.0 mm), dichotomously branched	Elongated, large (13-20 × 1.5-8.0 mm), dichoto mously branched
Ventral protuberance	Prominent at midthallus	Absent	Absent	In tubers and near antheridial receptacles	Few cells at midthallus
Oil cells in thallus	Present	Present	Present	Present	Only in border cells
Oil bodies in cells with chloroplasts	Absent	Absent	Absent	Absent	Present
Position of pores	In two or more rows	In two or more rows	One or two pores near apex	In two or more rows	In two or more rows
Rhizoids	Smooth and strongly tuberculate	Undulate and smooth	Undulate and smooth	Undulate and tuberculate	Undulate and tuberculate
Asexual diaspores	Absent	Absent	Absent	Tubers	Tubers
Sexual condition	Monoicous. Few thalli with only one type of receptacle (male)	Monoicous	Monoicous	Dioicous	Dioicous
Position of male receptacles	Terminal	Lateral	Lateral	Lateral	Terminal, dorsal
Male receptacles	Sessile	Sessile	Sessile	Very short pedicellate	Short pedicellate
Shape of involucre	Laminar or sheet- like	bivalvate	bivalvate	bivalvate	bivalvate
Number of archegonia/ involucre	6-8 in one or two groups	2-5 in one group	1-2 in one group	5-10 in one group	5-30 in one group
			SPOROPHYTIC		
Sporophytes per involucre	1-4 (in two groups)	1-3 in one group	1-2 in one group	1	1-2 in one group
Spore ornamentation	Verrucose-tuberculate	Baculate-spinose	Echinate	Irregular lamellae with open reticulum	Lamellae forming a closed reticulum
			DISTRIBUTION		
Geographic region	Paleotropical, Neotropical (Costa Rica, probably Ecuador)	Paleotropical, Neotropical	Neotropical (Panama)	Neotropical (Argentina)	Neotropical

Table 2. *Cyathodium* samples used in molecular studies. All samples were collected by N. Salazar Allen *et al.* and are deposited at PMA.

Species	*Location*	*Voucher*	*Accession number*
C. cavernarum	Parque Nal. Altos de Campana, Panamá	17005	DQ229910
	Costa Rica	20549	DQ229909
	Costa Rica	20555	DQ229912
	Costa Rica	20568	DQ229911
	Costa Rica	20680	DQ229908
	Costa Rica	20917	DQ229913
	Costa Rica	20918	DQ229914
	Costa Rica	20921	DQ229915
C. foetidissimum	Parque Nal. Tapantí, Costa Rica	20618	DQ229905
	Parque Nal. Tapantí, Costa Rica	20619	DQ229907
	Parque Nal. Tapantí, Costa Rica	20627	DQ229906
	Parque Nal. Tapantí, Costa Rica	20635	DQ229903
	Parque Nal. Tapantí, Costa Rica	20636	DQ229904
C. spruceanum	Parque Nal. Altos de Campana, Panamá	16817	DQ229920
	Parque Nal. Altos de Campana, Panamá	16863	DQ229926
	Camino a Canopy Tower, Panamá	16927	DQ229927
	Camino a Canopy Tower, Panamá	16955	DQ229924
	Parque Nal. Altos de Campana, Panamá	16986	DQ229921
	El Valle de Antón, Coclé, Panamá	7007	DQ229925
	Costa Rica	20564	DQ229917
	Costa Rica	20565	DQ229918
	Costa Rica	20569	DQ229919
	Costa Rica	20570	DQ229916
	Costa Rica	20914	DQ229922
	Costa Rica	20916	DQ229923

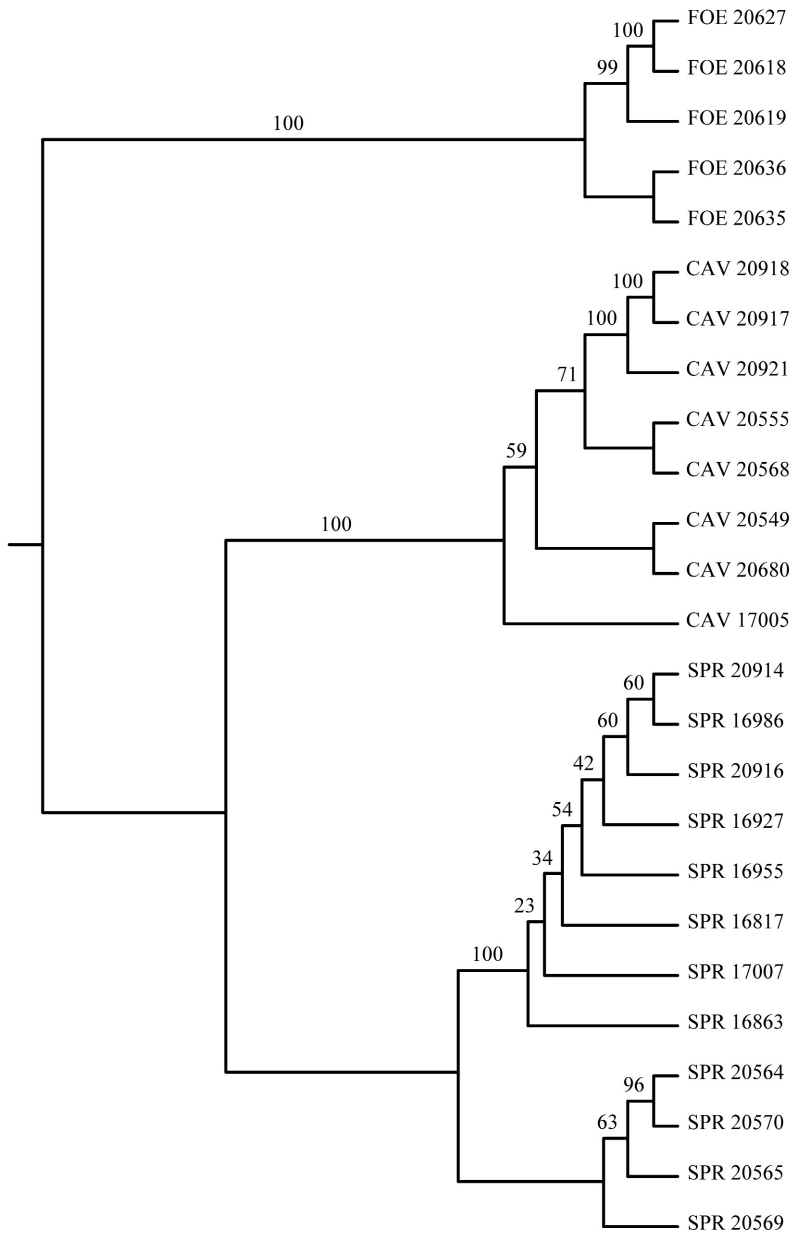

Fig. 4. A phenogram obtained from the analysis of nuclear-encoded ITS showing phylogenetic relationships among samples belonging to the species *Cyathodium foetidissimum* (FOE), *C. cavernarum* (CAV) and *C. spruceanum* (SPR). Collection numbers are given for each sample. All vouchers are at PMA. Bootstrap percentages are shown above the branches.

PHYLIP package. To test the relationship between the genetic and geographical distances between sample pairs, a Mantel test (Mantel, 1967) was conducted for *C. cavernarum* and *C. spruceanum*, both species included samples from a relatively wide geographical area.

Results

Among within-species samples, the lengths of ITS1 and ITS2 of *C. cavernarum* varied between 1217-1253 bp and 264-268 bp, respectively, while the length of ITS1 of *C. spruceanum* varied between 977-996 bp, but the length of ITS2 always equaled 270 bp. In the case of *C. foetidissimum*, only the first 462-463 bp of the sequence of ITS1 were of sufficient quality to be included in the phylogenetic analysis (as seen in Fig. 4), each species is genetically clearly distinct. Within *C. spruceanum*, four samples from Costa Rica (20564, 20565, 20569 and 20570) formed a distinct cluster. The average genetic distances (F84 model) and standard deviations between samples within the species of *C. cavernarum, C. foetidissimum* and *C. spruceanum* equalled 0.020 ± 0.009, 0.017 ± 0.011 and 0.014 ± 0.012, respectively. However, the distances between the species pairs CAV-FOE, CAV-SPR and FOE-SPR were high, equalling 0.572 ± 0.018, 1.036 ± 0.024 and 0.531 ± 0.011, respectively. The differences between *C. foetidissimum* and *C. spruceanum* are large enough to cause problems with the alignment of the ITS sequences in places. However, our result is clear despite the lack of a complete ITS sequence in *C. foetidissimum*. The Matel test revealed significant positive correlations between genetic and geographic distances in the species tested; the correlation coefficients equaling 0.480 ($P< 0.05$) and 0.350 ($P< 0.001$) in *C. cavernarum* and *C. spruceanum* respectively.

DISCUSSION

The molecular analysis supports the species segregation derived from morphology, at least for the three species studied. Among the samples of *C. cavernarum* and *C. spruceanum* collected from different sites in Costa Rica and Panama, the samples originating from close geographical areas were genetically more closely related than the geographically more distant samples. The relationships between Neotropical *Cyathodium* and their Asiatic relatives remain to be determined.

Acknowledgments. Sincere thanks to Mr. Denis Lamy for inviting us to present a paper on Neotropical *Cyathodium*, a taxonomic research proposed to N. Salazar Allen by the late Dr. H. Bischler. Special acknowledgment to Laura Forrest and P. Regan and for revision of the English and helpful suggestions, to S. Moss for use of his equipment, to C. Chung, my technician at STRI, and J. C. Villarreal, who have helped with collecting, processing and culturing *Cyathodium*. Special acknowledgment to B. Crandall-Stotler for critical revision of the manuscript and for helpful suggestions to improve it. Thanks to A. Espinosa and E. Lépiz & J. De Gracia for help in the field. Special thanks to STRI, J. Cevallos for SEM micrographs, L. González of the Digital Imaging Laboratory for plates, Dr. N. Gómez and N. Rivas for technical support with phytochemistry, to University of Panama, A. Soler for identification of diatoms, R. Cambra for identification of Coleoptera, to library personnel at STRI for help with literature searching, to Maria Leone (STRI) for help with logistics related to collecting permits and to A.N.A.M authorities and rangers for their helpful cooperation. Financial support for research from STRI to N. Salazar Allen and from the Academy of Finland to H. Korpelainen (project 50525) is gracefully acknowledged.

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