Mycologia, 84(6), 1992, pp. 857–862.

THE ANAMORPH OF *OPHIOSTOMA FRANCKE-GROSMANNIAE* IS A *LEPTOGRAPHIUM*

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ABSTRACT

It has been suggested that the anamorph of *Ophiostoma francke-grosmanniae* is a species of *Phialocephala* with phialidic conidium development. This would make it the only species in *Ophiostoma* with a *Phialocephala* anamorph. Light and fluorescence microscopy seemed to confirm the presence of phialidic conidiogenous cells. However, scanning and transmission electron microscope observations revealed tightly packed, yet distinct annellations in the apical region of the conidiogenous cells. This is indicative of the percurrent enteroblastic proliferation that is diagnostic of *Leptographium* anamorphs of *Ophiostoma*. The anamorph of *O. francke-grosmanniae* should, therefore, be disposed in *Leptographium*.

Key Words: conidiogenesis, *Leptographium*, *Ophiostoma*, *Phialocephala*, ultrastructure

The *Leptographium* complex comprises the genera *Leptographium* Lagerberg & Melin and *Phialocephala* Kendrick (Kendrick, **1961**, **1962**, **1963**). In the strict sense, *Leptographium* species are best regarded as anamorphs of *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadhyay & Kendrick (De Hoog and Scheffer, **1984**; Harrington, **1987**; Upadhyay, **1981**). *Verticicladiella* Hughes was formerly a member of the *Leptographium* complex though distinguished from the latter genus by its sympodial, as opposed to annellidic, conidium development (Hughes, **1953**; Kendrick, **1964**). Wingfield (**1985**) reduced *Verticicladiella* to synonymy with *Leptographium* when he found annellidic as well as apparent sympodial development in both these genera.

The genus *Phialocephala* Kendrick was established to accommodate those species in the *Leptographium* complex with phialidic conidium development (Kendrick, **1961**, **1963**; Wingfield et al., **1987**). This genus, however, appears to represent a heterogeneous group consisting of unrelated genera. Wingfield et al. (**1987**) suggested that the genus *Sporendocladia* Arnaud: Nag Raj & Kendrick should be used to accommodate those species of *Phialocephala* with ring wall building conidial development (Minter et al., **1983**). The remaining species of *Phialocephala* are probably still heterogeneous and deserve further consideration (Wingfield et al., **1987**).

There are no known connections between any described species of *Phialocephala* and teleomorph genera. Some ascomycete genera are purported to have unnamed *Phialocephala* conidial states, but no connection has yet been made to *Ophiostoma* (Harrington, **1988**). Species of *Ceratocystis* Ellis & Halst. *s.l.* commonly have *Leptographium* and *Graphium* Corda anamorphs, many of which resemble *Phialocephala* in broad terms. In particular, it has been suggested that the anamorph of *Ophiostoma francke-grosmanniae* Davidson is a species of *Phialocephala* (Davidson, **1971**; Harrington, **1988**; Upadhyay, **1981**).

Ophiostoma francke-grosmanniae and its anamorph occur in the larval galleries of the bark beetle *Hylecoetus dermestoides* (Lymeiylid) in oak trees (*Quercus* spp.) (Upadhyay, **1981**). The fungus was first collected by Dr. Helen Francke-Grosmann and described by Davidson (**1971**). It was formerly known as *Ceratocystis francke-grosmanniae* Davidson but was transferred with 13 other species of *Ceratocystis* to *Ophiostoma*, because of the absence of a *Chalara* (Corda) Rabenh. anamorph, high tolerance to cycloheximide and unique cell wall components (De Hoog and Scheffer, **1984**).

It seems unusual that *O. francke-grosmanniae* should be the only species of *Ophiostoma* to have a *Phialocephala* anamorph. This contradiction has inspired a detailed examination of *O. francke-grosmanniae*. This study centers on conidium development as this characteristic has been crucial in the separation of *Phialocephala*, *Leptographium* and *Verticicladiella*.

MATERIALS AND METHODS

An isolate of *Ophiostoma francke-grosmanniae* (ATCC 22061) was grown on 2% malt extract agar (20 g Difco malt agar; 20 g Difco malt extract, 1 L distilled water) and incubated at 20 C for approximately 3 wk until the onset of conidium production. Conidiogenesis was examined using light and fluorescence microscopy, as well as scanning and transmission electron microscopy (SEM, TEM).

Specimens for fluorescence microscopy were mounted on glass slides in a 0.05% w/v solution of cellufluor white M 2R optical brightener in 0.1 M phosphate buffer. Samples were examined with a Zeiss Axioskop fluorescence microscope and photographed using Ilford FP4 film.

Material for SEM and TEM was cut from the agar, fixed in 3% glutaraldehyde and 1% osmium tetroxide (OsO_4) in a 0.1 M phosphate buffer (pH 7) and dehydrated in a graded acetone series (50, 70, 95, and 100%). Specimens for SEM were critical point dried (liquid CO_2), mounted, coated with gold/palladium and viewed with an ISI scanning electron microscope.

Two fixation procedures were used in the preparation of material for TEM. The first technique was the same as that for SEM. In the second technique, material was fixed for 15 min in 0.5% $KMnO_4$, because $KMnO_4$ is known to produce high contrast in the cytoplasmic membranes (Hayat, **1970**). All specimens for TEM were dehydrated in the same graded acetone series used for SEM and embedded in epoxy resin, polymerized at 70 C for 8 h (Spurr, **1969**). Ultrathin sections (60 nm) were cut on an ultramicrotome, mounted on copper grids (200 mesh) and stained for 20 min in uranyl acetate, followed by 10 min in lead citrate (Reynolds, **1963**). Sections were examined with a Philips EM 300 transmission electron microscope.

The differing abilities of taxa in *Ceratocystis s.l.* to tolerate the antibiotic cycloheximide have been used as an important indication of their relatedness to one another (Harrington, **1981**). Therefore, cycloheximide tolerance studies were done by inoculating *O. francke-grosmanniae* on malt extract agar amended with different concentrations of cycloheximide in order to confirm the transfer of *O. francke-grosmanniae* from *Ceratocystis* to *Ophiostoma* as well as to illustrate that its anamorph is a true *Leptographium*. Concentrations of cycloheximide included were 0, 0.05, 0.1, 0.5, 1, 2.5 and 5 g/L. Growth of colonies was calculated after 4 and 8 days by taking the average of two colony diameter measurements for each plate. Three petri dishes were used at each concentration and the experiment was conducted twice at 25 C in the dark. The average colony diameters of the two experiments were calculated and tabulated (TABLE I).

RESULTS

Light microscopy showed hyaline, one-celled conidia that were globose to subglobose, or ovoid to oblong (FIG. 1). Fluorescence and bright field micrographs indicated ampulliform to lageniform conidiogenous cells and small collarettes with apparent periclinal thickening inside and just beneath the collarettes (FIGS. 2, 3). The suggestion that the conidiogenous cell had undergone proliferation could not be resolved in these micrographs and the conidiogenous cells appeared to be phialidic.

From SEM studies, it was clear that conidia had only one attachment scar (FIG. 4). Distinct, yet tightly packed annellations were clearly visible at the apex of the conidiogenous cells (FIGS. 5, 6). Unusually long percurrent proliferations were also observed (FIG. 7). SEM studies also showed the presence of accessory conidiogenous loci on the hyphae. In contrast to those on macronematous conidiophores, these cells clearly proliferated sympodially during conidiation (FIG. 8).

Transmission electron micrographs showed that conidia develop through percurrent proliferation of the conidiogenus cells (FIGS. 5, 6, 9). This would be followed by holoblastic ontogeny. Micrographs of the specimens fixed in potassium permanganate exhibited very high contrast of all the membranous surfaces and walls (FIG. 9), although cytoplasmic membranes were very poorly preserved. Annellations were therefore much more prominent in these micrographs than those of the material fixed in glutaraldehyde and osmium tetroxide (FIG. 10).

Ophiostoma francke-grosmanniae tolerated the presence of cycloheximide in the growth medium (TABLE I), except at extremely high concentrations, and low concentrations even appeared to stimulate growth.

DISCUSSION

SEM and TEM studies revealed distinct and tightly packed annellations at the apices of conidiogenous cells in *O. francke-grosmanniae*. This

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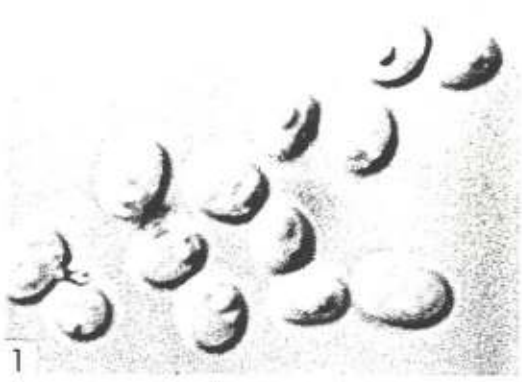
FIGS. 1–6. Conidia and conidiogenous cells of the anamorph of *Ophiostoma francke-grosmanniae*. 1. Light micrograph showing somewhat rounded conidia, ×4600. 2. Fluorescence micrograph of conidiogenous cell showing apparent phialides, ×3500. 3. Light micrograph of conidiogenous cells showing apparent periclinal thickening towards the inside of the conidiogenous cells (arrows) suggestive of phialidic conidial development, ×2500. 4. Scanning electron micrograph of conidia showing single attachment scars (arrows), ×5000. 5. Scanning electron micrograph revealing tightly packed annellations at the apices of conidiogenous cells (arrows), ×4200. 6. Transmission electron micrograph showing annellated apices (arrows) of the conidiogenous cells. $KMnO_4$ fixation, ×17,000.



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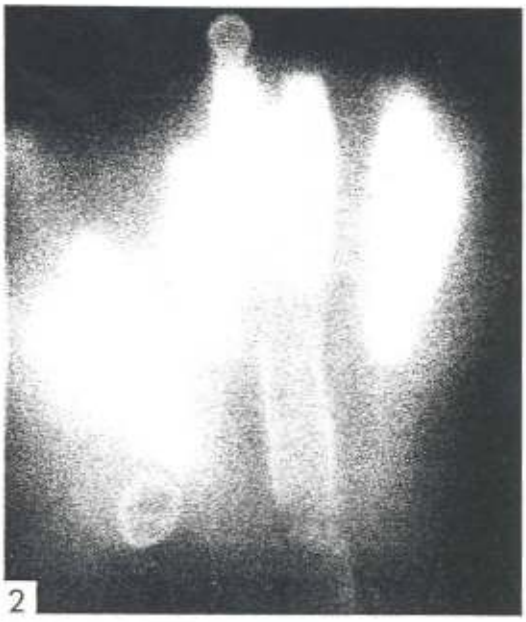

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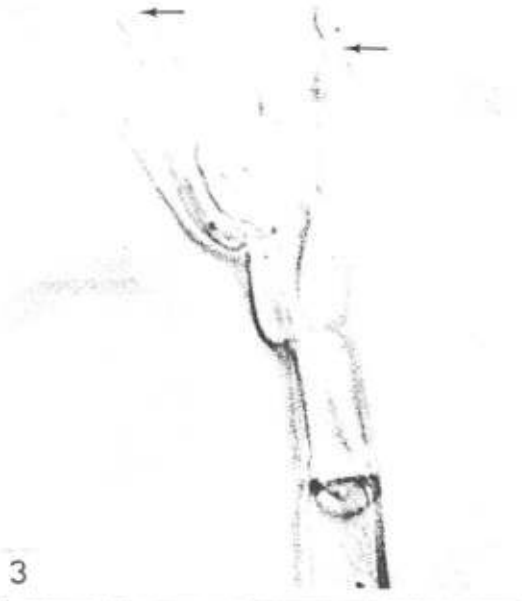

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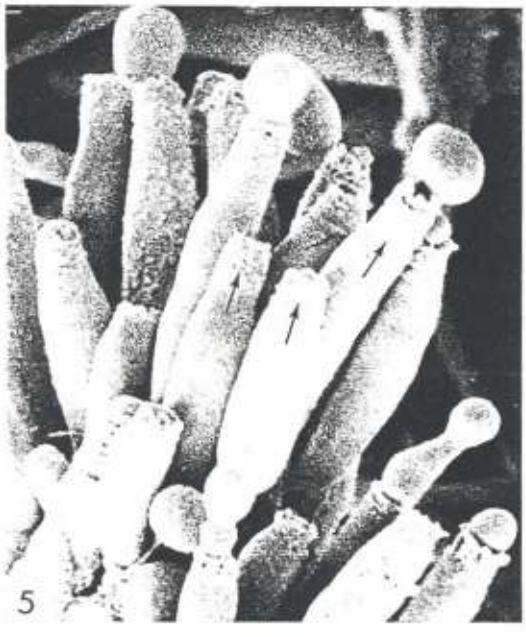

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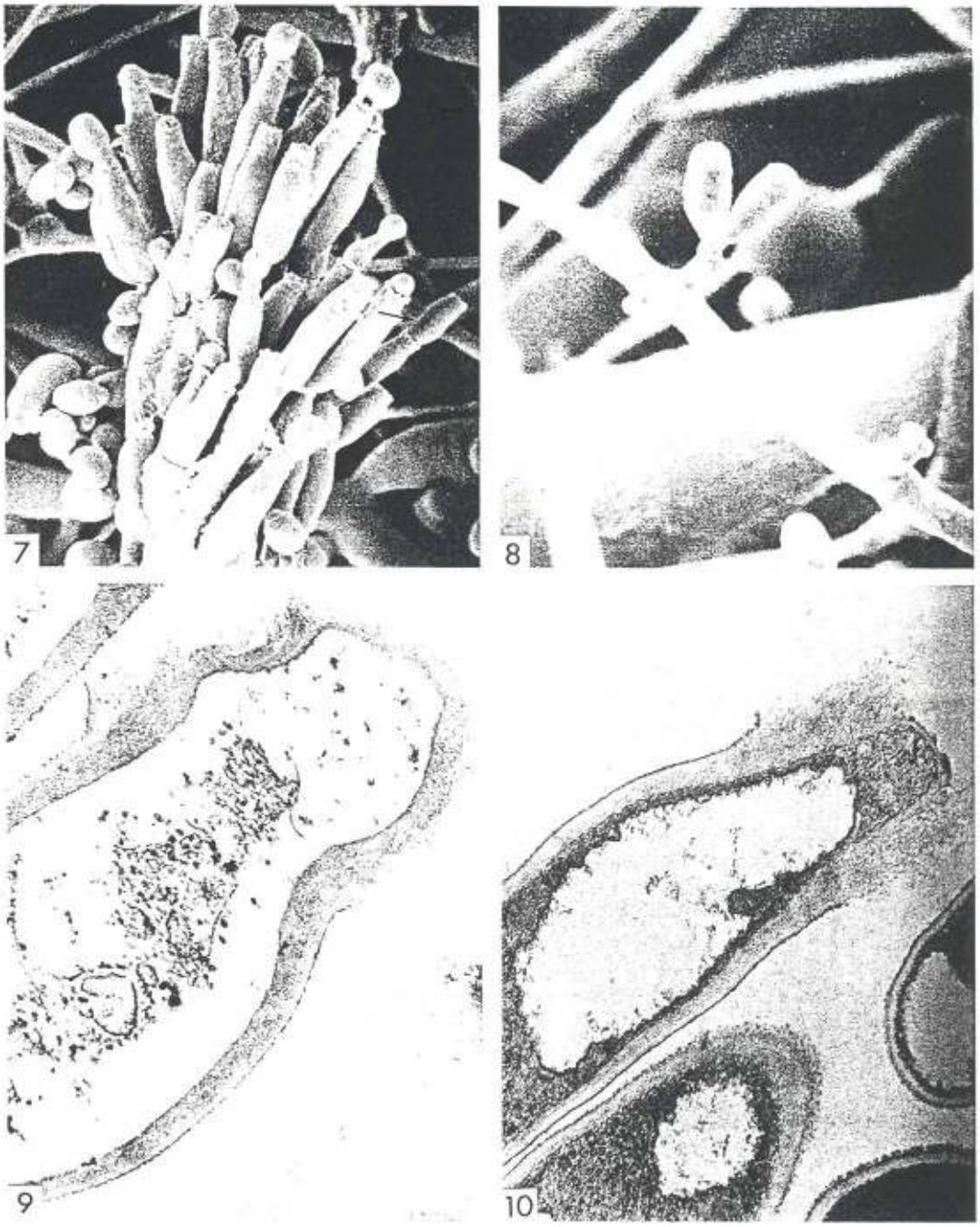

FIGS. 7–10. Conidiophores and conidiogenous cells of the anamorph of *O. francke-grosmanniae*. 7. Scanning electron microscopy of conidiogenous cells with unusually long proliferations (arrows), ×2400. 8. Conidiogenous loci on hyphae showing sympodial proliferation as in species of *Sporothrix*, ×4300. 9. Transmission electron microscopy after $KMnO_4$ fixation revealing distinct annellations at apices of conidiogenous cells, ×29,000. 10. Transmission electron micrograph of conidiogenous cell after glutaraldehyde and osmium tetroxide fixation with indistinct annellations, ×28,000.

TABLE I

GROWTH OF *OPHIOSTOMA FRANCKE-GROSMANNIAE* ON MALT EXTRACT AGAR AMENDED WITH INCREASING AMOUNTS OF CYCLOHEXIMIDE

Cycloheximide concentration (g/L)	Average colony diameter (mm)[a] Days	
	4	8
0	10.0	19.5
0.05	10.9	20.9
0.1	9.9	20.4
0.5	10.2	20.0
1.0	9.5	19.0
2.5	9.6	18.1
5.0	8.5	16.6

[a] Averages of colony diam were calculated from two colony diam on each of three petri dishes incubated at 25 C in darkness.

indicates that a succession of conidia develops through repeated percurrent proliferation of these cells, which thus gradually elongate (Minter et al., **1982**) (FIG. 11).

Light microscopy can be misleading where annellations are tightly packed and remnants of outer wall layers are well developed. A distinct, yet false, impression can even be gained of the presence of well developed periclinal thickening. SEM and TEM, therefore, appear to be essential for critical examination of such anamorphs.

Conidium development in *O. francke-grosmanniae* involves an apical wall building process. This can be deduced from the single attachment scar on conidia (Minter et al., **1982**, **1983**). Apical wall building is a common feature among anamorphs of *Ophiostoma* and *Ceratocystiopsis* (Minter et al., **1983**) and is in contrast to anamorphs of *Ceratocystis s.s.* where conidia develop through ring wall building (Minter et al., **1982**, **1983**; Wingfield et al., **1988**).

It was particularly interesting to find a second kind of micronematous conidiophore giving rise to conidia through sympodial proliferation typical of *Sporothrix* Hekt. & Perkins. Species of *Graphium*, characterized by synnematous conidiomata, commonly possess *Sporothrix* synanamorphs (De Hoog, **1974**). It is therefore surprising that this feature has rarely been reported in the *Leptographium* anamorphs of *Ophiostoma*, though such a state is apparently also present in the *Leptographium* anamorph of *Europhium aurea* (Robins.-Jeff. & Davids.) Upadhyay (Upadhyay, **1981**). The structures observed in *O.*

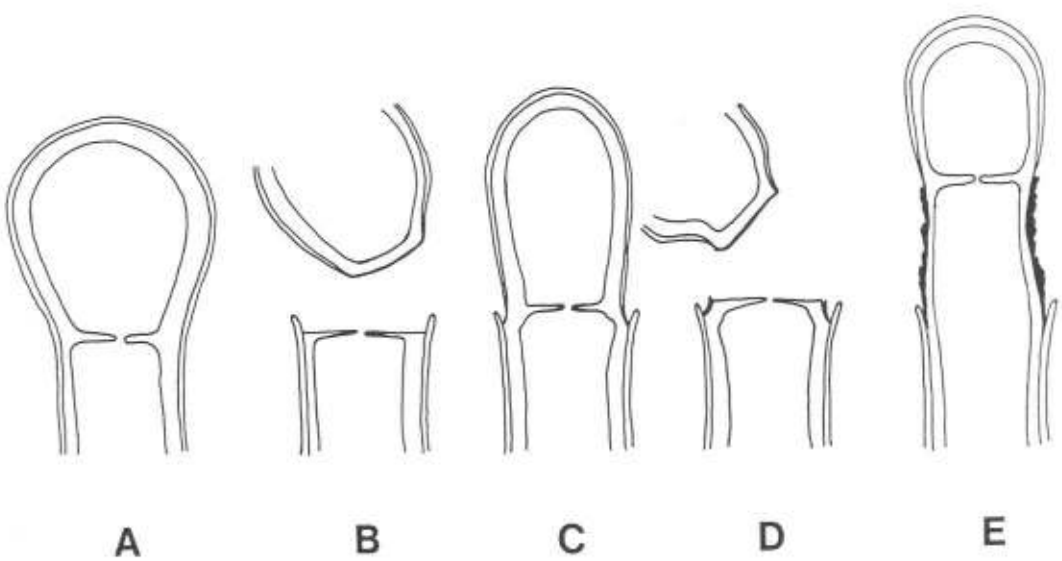

FIG. 11. Conidium development in the anamorph of *O. francke-grosmanniae*. A. The first conidium is formed holoblastically and is delimited. B. Conidial secession. C. Subsequent conidium is formed by percurrent enteroblastic proliferation and holoblastic ontogeny and is delimited. D. Conidial secession. E. Successive conidia are formed by percurrent enteroblastic proliferation and holoblastic ontogeny, leaving tightly packed, yet distinct annellations at the apices of the conidiogenous cells. Using light microscopy, these can appear to be phialides with periclinal thickening.

francke-grosmanniae represent a *Sporothrix* synanamorph. Similar inconspicuous synanamorphs might also be found associated with the *Leptographium* anamorphs of other *Ophiostoma* species.

Glutaraldehyde and osmium tetroxide fixation for TEM did not permit observation of the tightly packed annellations seen in scanning electron micrographs of *O. francke-grosmanniae*. Although potassium permanganate is a poor fixative, staining with this compound instead of osmium tetroxide fixation emphasized these characteristics (Hayat, **1970**). Unlike osmium tetroxide, potassium permanganate is insoluble in oils and fats. Material stained by potassium permanganate therefore exhibits high contrast in membranous surfaces due to metal deposits on the membranes in the form of a fine, dense precipitate (Hayat, **1970**). Our results suggest that this staining method might provide a useful tool for the examination of cell walls during conidium development in fungi.

It is well known that species of *Ophiostoma* can tolerate high concentrations of cycloheximide. In contrast, species of *Ceratocystsis s.s.* are extremely sensitive to this antibiotic (Harrington, **1981**, **1987**; Wingfield et al., **1988**). Growth of *O. francke-grosmanniae* was not inhibited by the presence of cycloheximide and even appeared to be slightly enhanced at low concentrations. Growth was inhibited only at extremely high concentrations such as 5 g/L. These results suggest that *O. francke-grosmanniae* is indeed a member of the genus *Ophiostoma* rather than *Ceratocystis s.s.* They also tend to confirm the conclusions drawn from our study of conidium development.

The anamorph of *O. francke-grosmanniae* would best be placed in *Leptographium* and not *Phialocephala*. It is, therefore, not exceptional amongst the anamorphs of *Ophiostoma* other than the fact that it has closely packed annellations. Such minor differences do not justify generic separation although they can be misleading when dependence is placed on light microscopy. For the present we have chosen not to provide a species epithet for the anamorph of *O. francke-grosmanniae*, as we feel that this should await a complete taxonomic reevaluation of *Leptographium*.

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Accepted for publication July 30, 1992