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Chlorostroma subcubisporum gen. et sp. nov. and notes on the systematic position of *Thuemenella cubispora*

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Chlorostroma subcubisporum is a new genus and species erected to accommodate a taxon featuring a green stroma bearing perithecia, asci with an apex that does not become blue in iodine, and subcubical brown ascospores with a prominent germination slit. The two extant collections are inhabitants of *Hypoxylon* stromata from North Carolina. Although this taxon seems to be a member of family Xylariaceae (Xylariales, Ascomycota), its position is equivocal owing to anomalies in cultures and molecular data. *Thuemenella cubispora*, which *C. subcubisporum* resembles in general ascus and ascospore morphology, was tentatively considered by others to be a member of family Xylariaceae primarily on the basis of its *Nodulisporium* – like anamorph. Molecular data presented herein supports this taxonomic disposition.

Keywords: ascomycetes, pyrenomycetes, Great Smoky Mountains National Park, LSU.

In the course of a survey of the mycobiota of the Great Smoky Mountains National Park in North Carolina we collected a pyrenomycetous fungus inhabiting stromata of *Hypoxylon perforatum* (Schwein.:Fr.) Fr. that we believe to represent a previously undescribed genus and species. In some respects this new taxon resembles *Thuemenella cubispora* (Ellis & Holway) Boedijn, an enigmatic species that has been well-studied by Samuels and Rossman (1992) and others who are cited and referenced in their paper. We herein describe the new taxon and discuss its possible taxonomic disposition. We likewise supply molecular data that unequivocally supports the disposition of *T. cubispora* in family Xylariaceae.

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Materials and Methods

Morphological and Cultural Procedures

Attempts were made to culture the new taxon to be described from ascospores and stromatal material. However, the cultures always had the characteristics of the host fungus, *Hypoxylon perforatum* (see later) and, thus, we could not use cultures for either morphological or molecular studies. Attempts were made to use stromatal and perithecial material for molecular studies, but the results were equivocal. However, we took the opportunity to conduct a molecular study of *Thuemenella cubispora* to assess its putative systematic position.

Thirty-five ascospores and ten asci of the new taxon were measured in distilled water using a Reichert brightfield microscope. The range of ascospore dimensions is given, rounded to the nearest full or half micron, with exceptional dimensions in parentheses. Means and standard deviations of length and width were computed from unrounded measurements. The amyloid reaction in ascus apices of water-mounted material was tested with Melzer's iodine reagent. Photography was accomplished with an Olympus differential interference contrast microscope and a Nikon digital camera. Color standards are from Rayner (1970).

Taxon sampling

GenBank accession numbers for all taxa are listed in Table 1. Molecular studies were accomplished on two cultures of *Thuemenella cubispora* kindly furnished by Amy Rossman, USDA National Fungus Collections, Beltsville, Maryland USA. These were the cultures used in the study of Samuels and Rossman (1992). Since the taxonomic placement of *T. cubispora* has been questioned, representatives from several orders throughout the Sordariomycetes were included in these analyses. Two loculoascomycetes, *Botryosphaeria ribis* and *Capronia mansonii*, were used as outgroup taxa. All voucher specimens are deposited in the Illinois Natural History Survey Mycological Collections (ILLS) or Washington State University fungal herbarium (WSP).

DNA extraction, PCR amplification, sequencing and sequence alignment

A DNeasy Mini Plant extraction kit (Qiagen) was used for extracting DNA from either dried ascomata or cultures grown in potato dextrose broth (Difco) following the manufacturers protocols

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Table 1. Taxa used in this study.

Taxon[a]	Source[b]	GenBank Accession No.
Anthostomella sp.	SMH 3101	AY780050
Apiospora sinensis	HKUCC 3143	AY083831
Botryosphaeria ribis	GenBank	AY004336
Cainia graminis	CBS 136.62	AF431949
Camarops petersii	JM 1655	AY346265
Camarops ustulinoides	SMH 1988	AY346267
Capronia mansonii	CBS 101.67	AY004338
Ceratocystis virescens	C69	U47824
Chaetosphaerella phaeostroma	SMH 4585	AY346274
Chaetosphaeria innumera	SMH 2748	AY017375
Clypeosphaeria uniseptata	HKUCC 6349	AY083830
Coniochaeta ligniaria	SMH 2569	AY346275
Coniochaetidium savoryi	TRTC 51980	AY346276
Daldinia childiae	**ILLS 58258**	**EF562505**
Daldinia concentrica	ATCC 36659	U47828
Diaporthe phaseolorum	FAU 458	AY346279
Diatrype disciformis	CBS 197.49	DQ470964
Eutypa sp.	SMH 3580	AY346280
Graphostroma platystoma	CBS 270.87	AY083827
Hypomyces luteovirens	TAA 169835	AF160237
Hypoxylon fragiforme	HKUCC 1022	AY083829
Lasiobertia sp.	SMH2065	AY346288
Lasiosphaeria ovina	SMH 1538	AF064643
Microascus trigonosporus	RSA 1942	U47835
Nectriopsis violacea	MUCL 40056	AF193242
Neurospora crassa	MUCL 19026	AF286411
Nitschkia grevillei	SMH 4663	AY346294
Petriella setifera	ATCC 26490	U48421
Plectosphaerella cucumerina	FAU 508	U17399
Poroconiochaeta discoidea	SANK 12878	AY346297
Pyrenomyxa picea	**ILLS 58257**	**EF562506**
Pyrenomyxa picea* as *Pulveria porrecta	**TRTC 47274**	**EF562507**
Rosellinia necatrix	HKUCC 9037	AY083824
Schizoparme botrytidis	AR 3504; SMH 1354	AF408383
Sordaria macrospora	Buck s.n.	AY346301
Thuemenella cubispora	**AR 2669; CBS 119807**	**EF562508**
Thuemenella cubispora	**GJS 88-41; ATCC 76480**	**EF562509**
Valsa ceratosperma	AR 3426	AF408387
Xylaria acuta	ATCC 56487	AY544676
Xylaria hypoxylon	ATCC 42768	U47841
Zignoëlla ovoidea	SMH 2605	AF064641

[a] Taxa sequenced in this study are in **bold**.

[b] ANM, Andrew N. Miller; AR, Amy Rossman; ATCC, American Type Culture Collection; Buck, William Buck; C, culture collection of T.C. Harrington; CBS, Centraalbureau voor Schimmelcultures, Netherlands; FAU, Francis A. Uecker; GJS, Gary J. Samuels; HKUCC, University of Hong Kong Culture Collection; JM, Jack Murphy; MUCL, Mycotheque de l'Universite catholique de Louvain, Belgium; RSA, Rancho Santa Anna Culture Collection; SANK, Sankyo Research Laboratories, Tukuba, Japan; SMH, Sabine M. Huhndorf; TAA, Institute of Zoology and Botany, Tartu, Estonia; TRTC, Royal Ontario Museum, Toronto, Canada; UAMH, University of Alberta Microfungus Collection and Herbarium.

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except tissues were ground in 50–100 μL of AP1 buffer instead of liquid nitrogen. The relative quantity of total genomic DNA was observed on a 1% TBE agarose gel stained with ethidium bromide. The first 1100 bp of 28S large subunit (LSU) was PCR-amplified on a MJ Research PTC 200 thermal cycler using PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare) and primers LROR and LR6 (Vilgalys & Hester, 1990; Rehner & Samuels, 1995). The following thermocycling parameters were used: initial denaturation at 95 C for 5 min followed by 35–40 cycles of 95 C for 30 s, 50 C for 15 s, and 72 C for 1 min with a final extension step of 72 C for 10 min. PCR products were purified using a QIAquick PCR purification kit (Qiagen). A BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used to sequence both strands using a combination of the following primers: LROR, LRAM1, LR3, LR3R, LR5, LR6 (Vilgalys & Hester, 1990; Rehner & Samuels, 1995; Huhndorf *et al.*, 2004). Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer. Each sequence fragment was subjected to an individual blast search to verify its identity. Sequences were assembled and aligned with Sequencher 4.5 (Gene Codes), optimized by eye, and manually corrected when necessary.

Phylogenetic analyses

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10 (Swofford, 2002). Portions of the 5' and 3' ends of LSU were excluded from all analyses due to missing data in most taxa. Introns in one specimen of *Pyrenomyxa picea* (ILLS 58257) and both specimens of *Thuemenella cubispora* were also excluded from all analyses. Nine ambiguously aligned regions were delimited and a portion of the phylogenetic signal was recovered from eight of these nine regions by recoding them using INAASE 2.3b (Lutzoni *et al.*, 2000). The remaining unambiguously aligned characters were subjected to a symmetrical stepmatrix to differentially weight nucleotide transformations using STMatrix ver. 2.2 (François Lutzoni and Stefan Zoller, Biology Dept., Duke University), which calculates the costs for changes among character states based on the negative natural logarithm of the percentages of reciprocal changes between any two character states. Unequally weighted MP analyses were performed with 10 000 random addition heuristic searches, TBR branch-swapping, MULTREES option in effect, zero-length branches collapsed, constant characters excluded, and gaps treated as missing. Branch support was estimated by performing 1000 bootstrap replicates (Felsenstein, 1985) each consisting of 100 random addition heuristic searches as above.

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MODELTEST 3.7 (Posada & Crandall, 1998) was used to determine the best-fit model of evolution for LSU. A ML analysis was performed using this model with 100 stepwise random addition replicates and TBR branch-swapping with a reconnection limit of twelve. Constant characters were included and ambiguously aligned characters were excluded from the ML analysis. Bayesian analyses were performed using MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) as an additional means of assessing branch support. Constant characters were included, the above model of evolution was implemented, and 10 million generations were sampled every 1000th generation resulting in 10 000 total trees. The Markov chain always achieved stationarity after the first 10 000 generations (= 10 trees), so the first 1000 trees, which extended well beyond the burn-in phase of each analysis, were discarded. Posterior probabilities were determined from a consensus tree generated using the remaining 9000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were ultimately being sampled during each analysis.

Taxonomy

Chlorostroma A. N. Mill., Lar. N. Vassiljeva & J. D. Rogers, **gen. nov.**

Stromata viridia cum peritheciis inclusa. Asci cum parte apicali in liquore iodato Melzeri non cyanescente. Ascosporae brunneae, plus minusve cubicae, rima germinativa praeditae. Typus generis: – *Chlorostroma subcubisporum* A. N. Mill., Lar. N. Vassiljeva & J. D. Rogers

Stromata green with perithecia embedded. Asci with apical part not bluing in Melzer's iodine reagent. Ascospores brown, more or less cubical, with a germination slit.

Etymology: – Chloro = green + stroma = cushion

Chlorostroma subcubisporum A. N. Mill., Lar. N. Vassiljeva & J. D. Rogers, **sp. nov.** Figs. 1–7.

Stromata pulvinata vel plus minusve hemisphaerica, 2–4 mm diam, 1–1.5 mm crassa, externe viridia vel atrovirentia, interne ochracea; cum coloribus in KOH dissolutis aurantiacis vel nullis; mollia et friabilia. Perithecia 0.2–0.3 mm diam. Ostiola umbilicata, interdum in disco exiguo praedita. Asci octospori, 75–85 µm longitudine tota, ca. 8 µm crassi, partibus sporiferis 60–70 µm longitudine, parte apicali in liquore iodato Melzeri non cyanescente. Ascosporae brunneae, unicellulares, plus minusve cubicae vel interdum discoideae, cum sporae summis et ultimis persaepe plus minusve conicis, 7.5–9(10.5) × 6–7.5 µm, rima germinativa recta longa praeditae. Paraphyses sparsae.

Holotypus. – U. S. A., North Carolina, Great Smoky Mountains National Park, Haywood Co., Big Creek, Baxter Creek Trail, 35° 45' 4.8" N, 83° 6' 34.7" W,

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518 m. elev., on stromata of *Hypoxylon perforatum*, 9 Sept 2005, leg. George Mugambi, WSP 71238.

Stromata pulvinate to more or less hemispheric, 2–4 mm diam, 1–1.5 mm thick, externally dull green (70) to dark green (21), internally ochreous (44); yielding luteous (12) to orange (7) pigment or no color in 10 % KOH; soft and friable. Perithecia 0.2–0.3 mm diam. Ostioles umbilicate, sometimes in a vague disc. Asci 8-spored, 75–85 µm total length, ca. 8 µm broad, the spore-bearing part 60–70 µm long, with apical part not bluing in Melzer's iodine reagent. Ascospores brown, one-celled, more or less cuboid or sometimes discoid, the first and last spore in the ascus often somewhat conic, 7.5–9 (10.5) × 6–7.5 µm (mean length 8.00 µm, SD ± 0.75 µm; mean width 6.60 µm, SD ± 0.63 µm) (n = 35), with long germ slit. Few paraphyses present.

Etymology. – For the more or less cuboid shape of the ascospores.

Distribution. – Known only from stromata of *Hypoxylon perforatum* in North Carolina, USA.

Material examined. – U. S. A., North Carolina, Great Smoky Mountains National Park, Haywood Co., Cataloochee, Rough Fork Trail, 35° 36' 59.3" N, 83° 7' 15.2" W, 869 m. elev., on stromata of *Hypoxylon perforatum*, 23 May 2006, leg. L. N. Vasilyeva, WSP 71239.

Results of Phylogenetic analyses

The final alignment of 41 LSU sequences was 1708 bp in length and contained 259 parsimony-informative characters. The best-fit model of evolution determined by MODELTEST was the General Time-Reversible model (Rodríguez *et al.*, 1990). MP and ML analyses each produced a single tree, which did not differ significantly in topology. The single MP tree is shown in Fig. 8.

Discussion

Chlorostroma subcubisporum has a great resemblance to *Thuemenella cubispora* in general morphology of stromata, asci, and ascospores. Stromata of *Thuemenella*, however, are pale brownish and the ascospores are greenish or yellowish and lack a discernible germ slit. It has not been recorded as an inhabitant of *Hypoxylon* stromata. Interestingly, it has been recorded from Great Smoky Mountains National Park from two locations in Tennessee (Samuels & Rossman, 1992).

The systematic position of *Chlorostroma* is currently unclear. It appears to be a pyrenomycete (Class Sordariomycetes, Subclass

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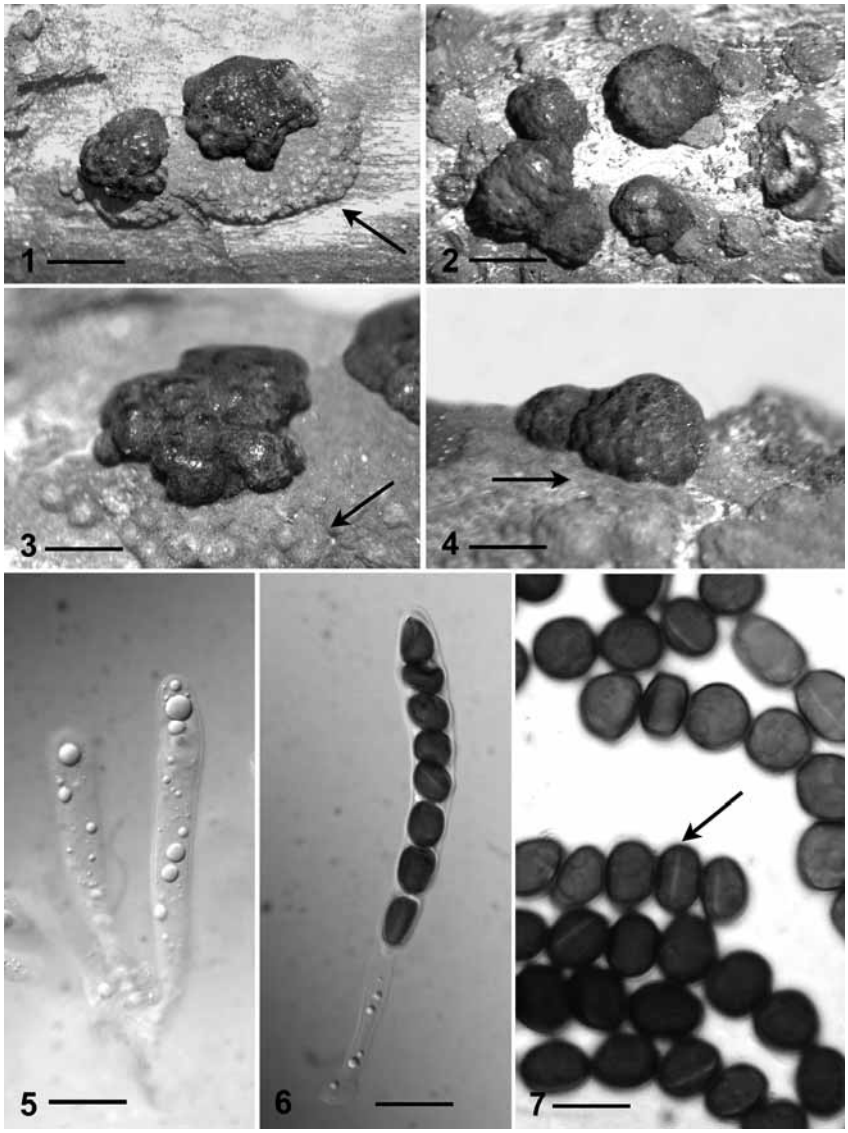

Figs. 1–7. *Chlorostroma subcubisporum*. **1–4.** Stromata of *C. subcubisporum* on stromata of *Hypoxylon perforatum* (arrows in figs. 1, 3, 4) (bar = 2 mm). **5.** Two immature asci (bar = 15 μm). **6.** Ascus with eight ascospores (bar = 15 μm). **7.** Ascospores, some of which show a germ slit. Arrow points to one germ slit (bar = 10 μm). Figs. 5-7 from water mounts. All photos from fresh air-dried material.

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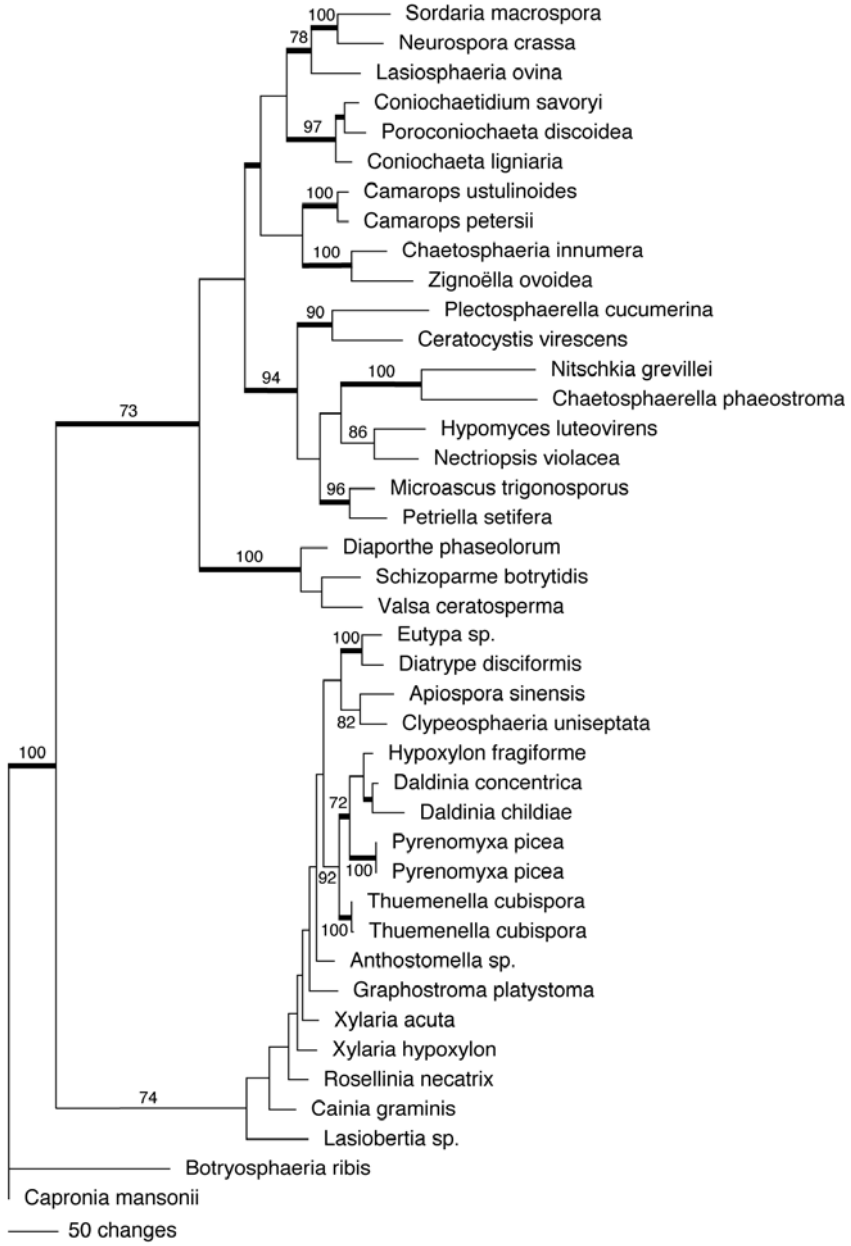

Fig. 8. The single most parsimonious tree generated from a maximum parsimony analysis of 41 LSU sequences. Bootstrap values ⩾70% are shown above or below branches; thickened branches indicate Bayesian posterior probabilities ⩾95%.

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Xylariomycetidae) of order Xylariales, family Xylariaceae. Samuels and Rossman (1992) placed *Thuemenella cubispora* in family Xylariaceae primarily on the *Nodulisporium* – like anamorph. This disposition is strongly supported by our molecular study that places it among undoubted members of the Xylariaceae as a clade near *Pyrenomyxa*, *Daldinia*, and *Hypoxylon* (Fig. 8). Because of the striking resemblance of *Chlorostroma* to *Thuemenella* and especially because of the brown ascospores with germ slits of the former, we suspect that it, likewise, is a member of the Xylariaceae. Deep green stromatal color, however, is uncommon in the Xylariaceae. Among hypoxyloid species *Hypoxylon aeruginosum* J. H. Miller and its variety *macrosporum* J. D. Rogers are noteworthy, but are actually greenish blue, as the species epithet suggests (Ju & Rogers, 1992). *Hypoxylon musceum* J. D. Rogers and a few other species feature green tones (Ju & Rogers, 1992). Among *Hypoxylon* species only *H. rectangulosporum* Ju, J. D. Rogers & Samuels has ascospores approaching the shape of those of *C. subcubisporum* and those of the former are ornamented with ridges and apparently lack a germ slit (Rogers *et al*., 1992). We have considered the possibility that *Chlorostroma* is, in fact, a loculoascomycete (members of Subclass Dothideomycetidae) based on the asci that lack a definite apical structure. However, Luttrell (1973) in discussing ascospores in the Loculoascomycetes wrote, "If the ascospores are unicellular the odds are perhaps nine to one that the genus belongs in the Euascomycetes and are even higher if the spores are globose or are provided with germ slits or pores or are conspicuously ornamented." Our cultural and molecular data, however, are equivocal. Repeated attempts to obtain unambiguous cultures of *Chlorostroma* have yielded cultures of the host substrate, *Hypoxylon perforatum*. Indeed, it appears that the *Hypoxylon* has invaded stromata of *Chlorostroma subcubisporum*. Cases of xylariaceous fungi parasitizing other xylariaceous fungi are few, but given the propensity of xylariaceous fungi to be endophytic, mycoparasitism among them would not be surprising. Preliminary molecular studies utilizing cultures and stromatal material from the two collections have yielded inconsistent and even conflicting results. We thus await fresh material of *C. subcubisporum* to continue our investigation of its systematic position.

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References

Felsenstein, J. (1985). Confidence intervals on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Huelsenbeck, J. P., Ronquist, F. R. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Huhndorf, S. M., Miller, A. N., Fernández, F. A. (2004). Molecular systematics of the Sordariales: the order and the family Lasiosphaeriaceae redefined. *Mycologia* **96**: 368–387.

Ju, Y.-M., Rogers, J. D. (1992). *A revision of the genus Hypoxylon*. APS Press (St. Paul, U. S. A.).

Luttrell, E. S. (1973). *Loculoascomycetes*. In: *The Fungi*, Vol. IVA. *A Taxonomic Review with Keys: Ascomycetes and Fungi Imperfecti* (eds. Ainsworth, G. C., Sparrow , F. K. Sussman, A. S.), Academic Press, New York: 135–219.

Lutzoni, F., Wagner P., Reeb, V., Zoller, S. (2000). Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. *Systematic Biology* **49**: 628–651.

Posada, D., Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **49**: 817–818.

Rayner, R. W. (1970). *A mycological colour chart*. Commonwealth Mycological Institute, Kew, Surrey, UK.

Rehner, S. A., Samuels, G. J. (1995). Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73** (Suppl 1): S816–S823.

Rodríguez, F., Oliver, J. F., Marín, A., Medina, J. R. (1990). The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142**: 485–501.

Rogers, J. D., Ju, Y.-M., Hemmes, D. E. (1992). *Hypoxylon rectangulosporum* sp. nov. *Xylaria psidii* sp. nov., and comments on taxa of *Podosordaria* and *Stromatoneurospora*. *Mycologia* **84**: 166–172.

Ronquist, F., Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Samuels, G. J., Rossman, A. Y. (1992). *Thuemenella* and *Sarawakus*. *Mycologia* **84**: 26–40.

Swofford, D. L. (2002). PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer Associates, Sunderland, MA

Vilgalys, R., Hester, M. (1990). Rapid identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

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