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Metarhiziopsis microspora gen. et sp. nov. associated with the elongate hemlock scale

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Abstract: A sporodochial fungus collected from the elongate hemlock scale, *Fiorinia externa* (Ferris) in Coventry, Connecticut, is described. This fungus has characteristics of both *Metarhizium* and *Myrothecium* but develops setae surrounding white to buff sporodochia and dry conidia in chains, a combination of characters found in neither genus. Phylogenetic analyses of the complete small subunit ribosomal DNA (*ssu*), partial *ef1-α*, and complete 5.8S ribosomal DNA and internal transcribed spacers (ITS) 1 and 2 shows that the fungus is allied with a subclade within *Cordyceps* including the species *C. agriota*, which places this fungus in the Hypocreales, Clavicipitaceae *sensu lato* or the newly erected Ophioclavicipitaceae. Morphological observation and molecular analysis indicate that this fungus is sufficiently different from *Metarhizium* and *Myrothecium* to warrant the erection of a new anamorphic genus. Therefore *Metarhiziopsis microspora* gen. et sp. nov. is proposed.

Key words: Clavicipitaceae, entomopathogen, hyphomycete, Hypocreales, *Metarhizium*, *Myrothecium*, Ophioclavicipitaceae

INTRODUCTION

The genus *Myrothecium* Tode (1790) is characterized by cupulate sporodochia or synnemata surrounded by setae, branched and compact conidiophores, verticillate phialides and green to black conidia in a slimy mass (Tode 1790, Tulloch 1972). *Myrothecium* grows in diverse habitats: from soil, as facultative plant pathogens and as saprophytes on plant debris and are mycotoxigenic to cellulolytic (Tulloch 1972, Domsch et al 1980). *Metarhizium* Sorokin (1883)[2] is characterized by dense sporodochia without surrounding setae, aggregated conidiophores with repeated, verticillate branching, phialides in a dense parallel arrangement and subhyaline conidia in long chains, yellow green in mass (Tulloch 1976, Domsch et al 1980). Species of *Metarhizum* are entomopathogenic and soil-dwelling fungi (Domsch et al 1980, Rakotonirainy et al 1994, Pipe et al 1995).

A fungal specimen was collected from foliage of Fraser fir, *Abies fraseri* (Pursh) Poiret in Coventry, Connecticut, in fall 2006. The fungus develops sporodochia around the elongate hemlock scale, *Fiorinia externa* (Ferris) (Homoptera: Diaspididae) (FIG. 1), which is a destructive armored scale insect that feeds on the foliage of hemlock (*Tsuga* spp.) and other conifers in the genera *Abies*, *Cedrus*, *Picea* and *Pseudotsuga* (McClure 1980a). This fungus is different morphologically and phylogenetically from the published descriptions of *Metarhizium* and *Myrothecium* species. It develops sporodochia surrounded by setae (like *Myrothecium*) and white to buff conidia in chains (like *Metarhizium*).

The objectives of this study were to characterize this fungus morphologically and determine its phylogenetic placement by sequence analysis of three gene regions. The fungus herein is proposed as a new genus and species.

MATERIALS AND METHODS

Morphological analysis and culturing.—Specimens of *Metarhiziopsis microspora* with the elongate hemlock scale on foliage of *Abies fraseri* were collected from a mixed forest in Coventry, Connecticut. The fungus was mounted in lactofuchsin (0.1 g acid fuchsin, 100 mL 85% lactic acid). Morphological characters of the fungus including sporodochia, conidiophores, conidiogenous cells and conidia, were observed with a Nomarski differential interference contrast optical system and a scanning electron microscope (SEM). For SEM, specimens were placed in fixative (3% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.2) at 4 C for 24 h. They were washed three times for 20 min each in 0.1 M cacodylate buffer, pH 7.2, and then fixed 24 h with 1% osmium tetroxide in 0.1 M cacodylate buffer. After three 20 min deionized water washes the samples were dehydrated in an ethanol series (30%, 50%,

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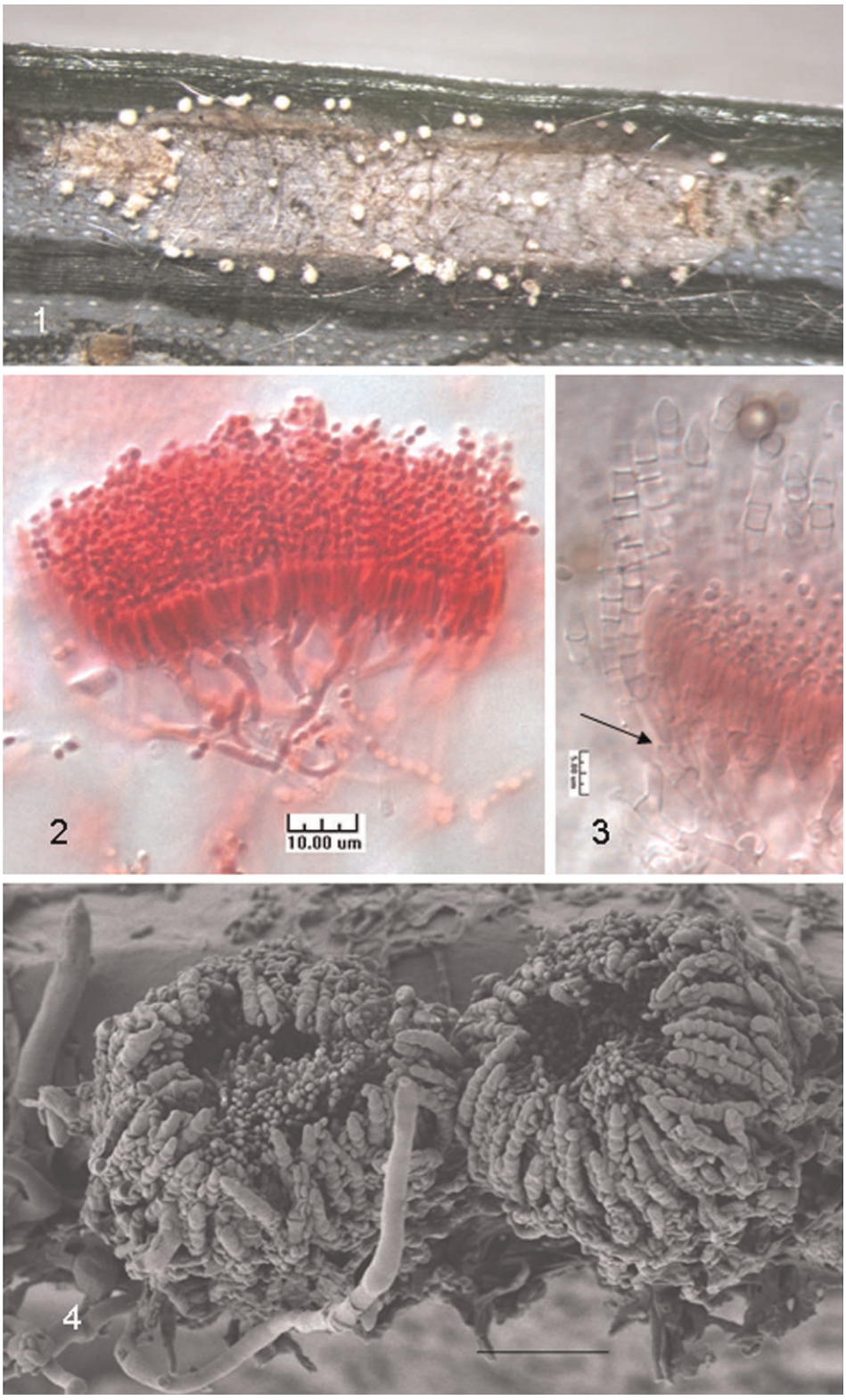
[2] The errors in a widely cited reference for genus *Metarhizium* and *M. anisopliae* (Metschn.) Sorokin (Sorokin N. 1883. Plant parasites of man and animals as causes of infectious diseases 2:268–291) were pointed out in Steinhaus (1975). This erroneous Sorokin reference unfortunately has been regularly cited up to the present.

Steinhaus EA. 1975. Disease in a minor chord: being a semihistorical and semibiographical account of a period in science when one could be happily yet seriously concerned with the diseases of lowly animals without backbones, especially the insects. Columbus: Ohio State University. 488 p.



FIGS. 1–4. *Metarhiziopsis microspora* on elongate hemlock scale. 1. Sporodochia surrounding elongate hemlock scale. 2. Sporodochium, conidiophores, phialides, conidia and branching system. 3. Partial sporodochium, setae, conidiophores, phialides and conidia. Arrow points to location where setae are connected to the sporodochium. 4. Sporodochia, setae and conidia. Bars: 2, 3 = 10 μm, 4 = 20 μm.

70%, 95%, and 100%, 20 min each, except 100% had three changes, two for 20 min and one overnight). The dehydrated samples were critical point dried (Polaron E3000) (Bozzola and Russell 1991). All specimens were attached to aluminum mounts on carbon tape, sputter coated with AuPd (Polaron E5100) and observed in the FESEM (Zeiss DSM982 Gemini Field Emission Scanning Electron Microscope).

The fungus was placed aseptically on malt extract agar (MEA) made with 15 g malt extract broth (Difco), 15 g agar (Oxoid), 0.075 g chloramphenicol (Fisher), 750 mL distilled water, 0.75 ml trace metal solution (1 g $ZnSO_4 \cdot 7H_2O$, 0.5 g of $CuSO_4 \cdot 5H_2O$, 100 mL distilled water) and 1 mL 1N NaOH and on cornmeal agar (CMA) made with 12.75 g cornmeal agar (Difco), 0.075 g chloramphenicol (Fisher), 750 mL distilled water. The plates were incubated at 25 C for 15 d.

DNA extraction, sequencing and phylogenetic analysis.—A small amount of fungal tissue was scraped from an agar plate and placed in 100 µL STE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer with 150 mg of 425–600 µm acid washed glass beads and placed in a Mini-Beadbeater (Biospec Products) for 40 s. Two microliters of the resulting STE solution were used in a standard 100 µL PCR reaction (QIAGEN Tac PCR Core Kit) incubated at 94 C for 3 min followed by 35 cycles of 94 C for 45 s, 45 C for 30 s and 72 C for 2 min. Two primers were designed to amplify and sequence each of three regions of the fungal genome as follows: The fungal ITS region, 16SF-FNG (TGATATGCTTAAGTTCAGT) and 28SR-FNG (ACAAGGTCTCCGTTGGTGAAC) primers were used for amplification and sequencing. The small subunit rDNA primers NS1 (GTAGTCATATGCTTGTCTC) and NS8 (TCCGCAGGTTCACCTACGGA) were used for amplification and sequencing and primers NS2 (GGCTGCTGGCACCAGACTTGC), NS3 (GCAAGTCTGGTGCCAGCAGCC), and SR10R (TTTGACTCAACACGGG) for sequencing. Sequencing of the *ef1-α* region was accomplished in two pieces. The first 600 nucleotides were amplified and sequenced with primers EFA2VOSS (TGATCTACMAGTGCGGTGGT) and EFARVOSS (CATCCTTGGAGATACCAGC). The last 1000 nucleotides were amplified and sequenced with primers 983F (GCYCCYGGHCAYCGTGAYTTYAT) and 2218R (ATGACACCRACRGCRACRGTYTG). Primers 1567RINTB (ACHGTRCCRATACCACCRAT), 2212R (CCRAACRGCRACRGTYYGTCTCAT) and 997F (CARGAYGTBTACAAGATYGGTGG) were used for sequencing the last portion of this gene. PCR products were eluted from a QIAGEN PCR purification column and submitted for sequencing. Sequence products were assembled with Chromas Pro version 1.34 software (Technelysium Pty Ltd., Tewantin, Queensland, Australia).

The partial DNA sequence transcribing the ribosomal spacer region (3′ end of the 18S (*ssu*), the 5′ end of the 28S ribosomal RNA units and the complete sequences for the 5.8S ribosomal RNA and internal transcribed spacers (ITS) 1 and 2 were submitted through the nucleotide MegaBLAST procedure (Zhang et al 2000) via the NBCI Website (www.ncbi.nlm.nih.gov/blast/), using the nonhuman, nonmouse database. This preliminary analysis indicated relationships might exist within the Clavicipitaceae.

Nucleotide sequences for the *ssu* ribosomal DNA, ribosomal spacer region and *ef1-α* genes for *Cordyceps* and related clavicipitaceous fungi representative of Clades A, B and C in a recent revision of Clavicipitaceae (Sung et al 2007) and homologous sequences from *Myrothecium verrucaria* and *M. inundatum* were obtained from GenBank (TABLE I). Only species for which all three regions were available were used in our analyses. The three sequence regions from each of 51 taxa were concatenated, then aligned with each other using MEGA version 4 (Tamura et al 2007). Phylogenetic analyses were accomplished with PAUP version 4.0b software (Swofford 1998). Our analyses included maximum parsimony analysis with the heuristic search method, maximum likelihood analysis with the heuristic search method, neighbor joining analysis and bootstrap analysis set for distance using neighbor-joining/UPGMA search parameters (1000 replicates). Bootstrap confidence intervals were set at 50%.

These DNA sequences have been placed in the GenBank database: the partial sequences transcribing the 18S and 28S ribosomal RNA units and the complete sequences for the 5.8S ribosomal RNA and internal transcribed spacers (ITS) 1 and 2 (EF543262); the sequence transcribing the entire 18S subunit ribosomal RNA (EU420126); and the sequence transcribing the *ef1-α* gene (EU420127).

TAXONOMY

Metarhiziopsis D.W. Li, Cowles, & Vossbrinck gen. nov.

Mycobank registration No. MB 511393.

Fungi mitosporici, Hyphomycetes.

Sporodochia primum subalba, deinde sublutea, cupuliformia, circumvallata septatis setis; septa setarum fusca, prope apicem attenuata. Conidiophora determinata, macronemata, fasciculata, erecta, hyalina et levia, ramosa. Cellulae conidiogenae phialidicae, determinatae, discretae, cylindricae, leves, hyalinae. Conidia unicellularia, hyalina, catenulata, connexa columnas.

Typus generis: *Metarhiziopsis microspora* D.W. Li, Cowles, & Vossbrinck

Etymology: Resembling *Metarhizium*.

Sporodochia white to buff, cupulate, formed from closely compacted conidiophores, surrounded by differentiated septate setae, septa of setae dark, with tapering tips. Conidiophores determinate, macronematous, in groups, erect, hyaline and smooth, repeatedly branched. Phialides determinate, discrete, cylindrical, smooth, hyaline, unicellular, in groups forming a concave and dense palisade layer. Conidia unicellular, hyaline, catenulate, forming columns.

Metarhiziopsis microspora D.W. Li, Cowles, & Vossbrinck sp. nov. FIGS. 1–8

Coloniae in MEA, 34–36 mm diam in 15 diebus ad 25 C, subalbae vel subluteae, luteae infra, in CMA 51–53 mm diam.

Sporodochia primum subalba, deinde sublutea, cuplifor-

TABLE I. GenBank accession numbers for regions used for phylogenetic analyses

Species	ITS	ef1-α	ssu
Aphysiostroma stercorarium	AY894979	AF543782	AF543769
Aschersonia badia	EF190278	DQ522317	DQ522573
Balansia henningsiana	U57404	AY489610	AY545723
Beauveria caledonica	AY245625	EF469057	AF339570
Claviceps fusiformis	AJ133392	DQ522320	DQ522538
Claviceps purpurea	AB099508	AF543778	AF543765
Cordyceps agriota	AY245626	DQ522322	DQ522540
Cordyceps bifusispora	AY245627	EF468746	EF468952
Cordyceps capitata	EF530933	AY489615	AY489689
Cordyceps cardinalis	AB237660	DQ522325	AY184973
Cordyceps chlamydosporia	AB100362	DQ522327	DQ522544
Cordyceps entomorrhiza	AJ786561	EF468749	EF468954
Cordyceps gracilis	AJ786564	EF468751	EF468956
Cordyceps gunnii	AJ536551	AY489616	AF339572
Cordyceps heteropoda	AB084157	EF468752	EF468957
Cordyceps irangiensis	AY646400	DQ522329	DQ522546
Cordyceps kyusyuensis	AY781661	EF468754	EF468960
Cordyceps militaris	EU326220	DQ522332	AY184977
Cordyceps nutans	AJ786583	DQ522333	DQ522549
Cordyceps ochraceostromata	AY245646	EF468759	EF468964
Cordyceps ophioglossoides	AJ786588	AY489618	AY489691
Cordyceps pruinosa	AB044635	EF468760	EF468965
Cordyceps scarabaeicola	AF199592	DQ522335	AF339574
Cordyceps sinensis	EF555097	EF468767	EF468971
Cordyceps sphecocephala	AY646402	DQ522336	DQ522551
Cordyceps takaomontana	EF495105	EF468778	EF468984
Cordyceps unilateralis	AY494596	DQ522339	DQ522554
Epichloë typhina	AB105953	AF543777	U32405
Glomerella cingulata	EU326204	AF543772	U48427
Haptocillium balanoides	EF546660	DQ522342	AF339588
Hydropisphaera erubescens	AF422977	DQ522344	AY545722
Hypocrea lutea	AB027384	AF543781	AF543768
Isaria farinosa	DQ888729	DQ522348	DQ522558
Isaria tenuipes	EU149928	DQ522349	DQ522559
Lecanicillium attenuatum	EF192939	EF468782	AF339614
Lecanicillium fusisporum	EU284721	EF468783	AF339598
Lecanicillium psalliotae	AD160994	EF469066	EF469128
Leuconectria clusiae	AF220976	AY489627	AY489700
Metarhiziopsis microspora	EF543262	EU420127	EU420126
Metarhizium album	AF137167	DQ522352	DQ522560
Metarhizium anisopliae	EU307931	AF543774	AF339579
Myrothecium inundatum	AY254152	AY489626	AY489699
Myrothecium verrucaria	EF211127	AY489608	AY489681
Nomuraea atypicola	EF029230	EF468786	EF468987
Nomuraea rileyi	AB100361	EF468787	AY624205
Paecilomyces lilacinus	AY213668	EF468792	AY624189
Phytocordyceps ninchukispora	AY245642	EF468795	EF468991
Pochonia bulbillosa	DQ132810	EF468796	AF339591
Pochonia chlamydosporia	AY555965	EF469069	AF339593
Pochonia rubescens	DQ516078	EF468797	AF339615
Verticillium dahliae	EU109532	AY489632	AY489705

mia, 34–128 μm diam (Med. = 71 ± 23, n = 30), circumvallata septatis setis; setae 24–61 μm longae (Med. = 41 ± 8, n = 30); septa setarum fusca, prope apicem attenuata. Conidiophora determinata, macronemata, fasciculata, erecta, hyalina et levia, ramosa. Cellulae conidiogenae phialidicae, determinatae, discretae, cylindricae, leves, hyalinae, (5.6–)6.2–8.5(–9.9) (Med. = 7.3 ± 1.2, n = 30) × (1.5–)1.6–2.0(–2.4) (Med. = 1.8 ± 0.2) μm.

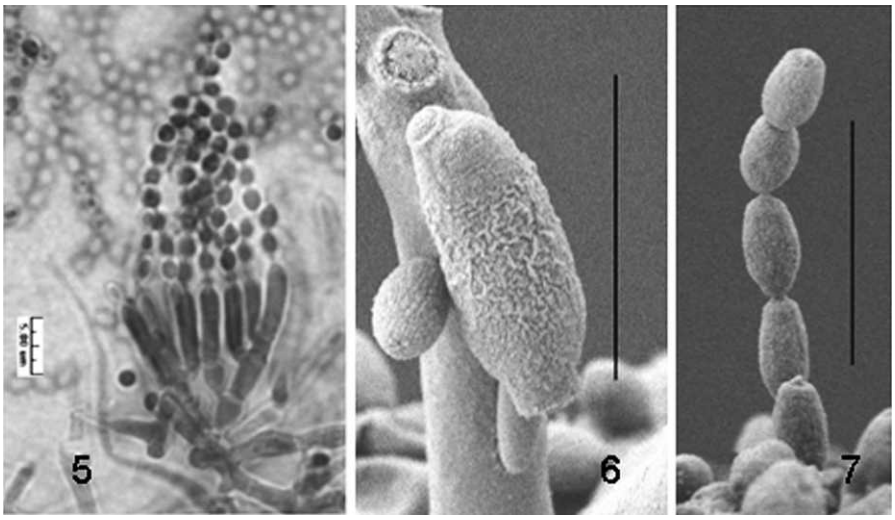


FIG. 5–7. *Metarhiziopsis microspora* in vivo. 5. Phialides and conidia. 6. Phialide. 7. Conidia. Bars = 5 µm.

Conidia unicellularia, elliptica vel oblonga, hyalina et levia, (1.5–)1.7–1.9(–2.2) (Med. = 1.8 ± 0.1, n = 30) × (1.3–)1.4–1.5(–1.6) (Med. = 1.4 ± 0.1, n = 30) µm, longa/crassa 1–1.4 (Med = 1.2), catenulata, connexa columnas rectas solitarias vel multifidas.

Teleomorphosis ignota.

Holotypus: USA. CONNECTICUT: Coventry, 41°47.25′N, 72°21.5′W, isolatus de *Fiorinia externa* (Ferris) in foliis *Abiei fraseri* (Pursh) Poiret, Oct 2006, *R. Cowles.* (BPI 878276) Viva cultura sustentata apud ARSEF (ARSEF 8676) et UAMH (UAMH 10901).

Colonies 34–36 mm diam in 15 d at 25 C on MEA, white to buff, reverse yellow, little sporulation; 51–53 mm diam on CMA. Abundant sporulation.

Sporodochia in vivo white to buff, cupulate, formed from closely compacted conidiophores, 34–128 µm diam (mean = 71 ± 23, n = 30), surrounded by differentiated septate setae; setae 24–61 µm long (mean = 41 ± 8, n = 30) with tapering tips; septa of setae dark.

Conidiophores determinate, macronematous, in groups, erect, hyaline, smooth, repeatedly branched, with 2–3 branches from each node.

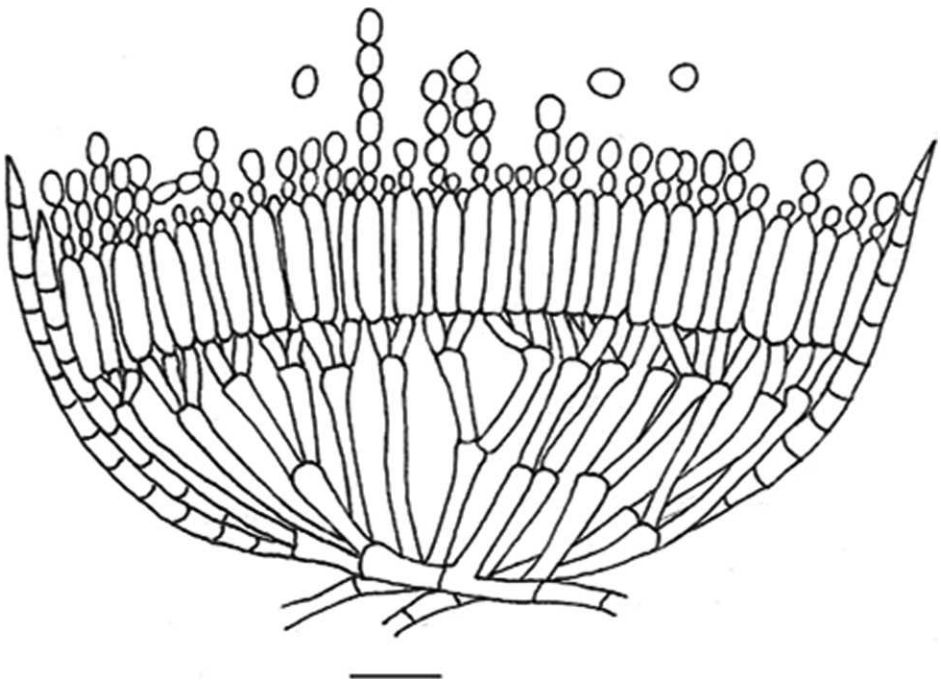


FIG. 8. Line drawing of sporodochium of *Metarhiziopsis microspora.* Bar = 5 µm.

Phialides determinate, discrete, cylindrical, smooth, hyaline, slightly curved when developed close to the edge of the sporodochia, (5.6–)6.2–8.5(–9.9) (mean = 7.3 ± 1.2, n = 30) × (1.5–)1.6–2.0(–2.4) (mean = 1.8 ± 0.2) µm, without conspicuous collarettes, unicellular, in groups forming a concave and dense palisade layer.

Conidia unicellular, ellipsoid or oblong, hyaline and smooth, (1.5–)1.7–1.9(–2.2) (mean = 1.8 ± 0.1, n = 30) × (1.3–)1.4–1.5(–1.6) (mean = 1.4 ± 0.1, n = 30) µm, ratio of length/width 1–1.4 (mean = 1.2), catenulate, in single or split columns; conidial columns whitish to yellowish.

Teleomorph unknown.

Holotype: USA. CONNECTICUT. Coventry, 41°47.25′N, 72°21.5′W, associated with *Fiorinia externa* (Ferris) on foliage of *Abies fraseri* (Pursh) Poiret, Oct 2006, R. Cowles (BPI 878276). Living cultures maintained at ARSEF (ARSEF 8676) and UAMH (UAMH 10901).

Etymology: The specific epithet is chosen to indicate the small size of the spores.

Distribution: Connecticut, USA.

Habitat: on conifer foliage, growing from elongate hemlock scale, *Fiorinia externa*.

Additional specimens examined: USA. CONNECTICUT: Torrington, Burr Pond State Park, on *Tsuga canadensis* (L.) Carrière foliage, 25 Feb 2007, *Carole Cheah* (BPI 878277).

Phylogenetic analyses.—Bootstrap analyses with the combined *ssu*, *ef1-α* and the ribosomal spacer region (listed as ITS in TABLE I) placed *M. microspora* as the sister taxon of *Cordyceps agriota* A. Kavam., (= *Ophiocordyceps agriotidis* [A. Kawam.] G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora) with a 98% bootstrap confidence interval. Our bootstrap analyses also show a close relationship (94%) between these two species and *Cordyceps entomorrhiza* (Dicks.) Fr. (= *Ophiocordyceps entomorrhiza* [Dicks.] G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora) and *Cordyceps gracilis* (Grev.) Durien & Mont. (= *Ophiocordyceps gracilis* [Grev.] G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora). In addition all three analyses (neighbor joining, maximum parsimony and maximum likelihood) include *Cordyceps unilateralis* (Tul.) Sacc. (= *Ophiocordyceps unilateralis* (Tul.) Petch) either as the sister group of the above four taxa or as the sister taxon of *Cordyceps entomorrhiza* (FIG. 9). In none of the analyses did *Metarhiziopsis microspora* show a close relationship to either *Metarhizium* or *Myrothecium* species, the two genera with which it shows morphological similarities. Our analyses clearly place *M. microspora* within the Clavicipitaceae and more specifically within Clade B of Sung et al (2007); whereas *Metarhizium* has been placed in Clade A, and *Myrothecium* has been placed as outgroup to Clavicipitaceae (Castelbury et al 2004). Therefore, we can conclude that *Metarhiziopsis* belongs to neither *Metarhizium* nor *Myrothecium*.

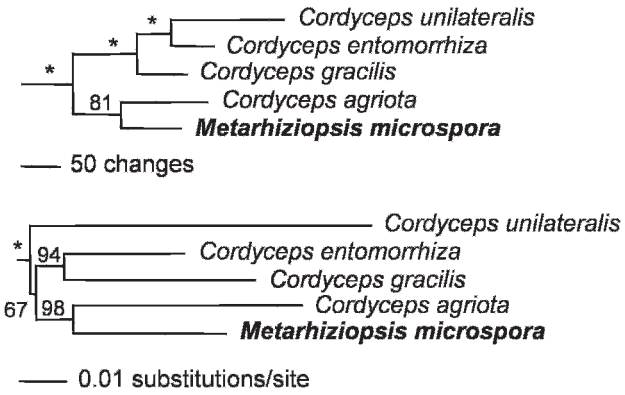


FIG. 9. Maximum parsimony (top) and neighbor joining phylogenies (bottom) of *Metarhiziopsis* to related fungi, based on three concatenated sequences (see methods), with superimposed bootstrap values. Other taxa for which bootstrap values did not give significant support are not shown.

DISCUSSION

The sporodochia of *Metarhiziopsis microspora* share some similarities with *Metarhizium* and *Myrothecium* in the morphological characters of phialides and conidiophores. *Metarhiziopsis microspora* conidia are in dry chains like those of *Metarhizium* but with setae surrounding sporodochia as in *Myrothecium*, thus giving *Metarhiziopsis microspora* characteristics of both genera. Its small conidia in whitish to buff columns are distinct, both in size and color, from species of *Metarhizium* and *Myrothecium*. Furthermore DNA sequence data and phylogenetic analyses showed that *M. microspora* is different from *Metarhizium* and *Myrothecium* and clearly places *Metarhiziopsis microspora* with other species of entomopathogenic fungi within Clade B of Clavicipitaceae, Hypocreales.

Myrothecium and the morphologically similar anamorph genera form a paraphyletic group basal to the Hypocreaceae/Clavicipitaceae (Rossman et al 2001). *Myrothecium* was placed tentatively placed in the Bionectriaceae (Rossman et al 2001). In a later study analysis of DNA sequences from four nuclear and one mitochondrial gene showed that species of *Stachybotrys*, species of *Myrothecium* and two other tropical hypocrealean species form a previously unknown monophyletic lineage within the Hypocreales (Castlebury et al 2004). Their results suggested that *Myrothecium* and *Stachybotrys* are closely related and belong to an undescribed family.

Studies have shown that *Cordyceps* is polyphyletic (Artjariyasripong et al 2001, Stensrud, Hywel-Jones,

Schumacher 2005, Yokoyama et al 2006, Sung et al 2007). *M. microspora* is related to the *C. unilateralis* subclade, within Clade B of Clavicipitaceae, for which Sung et al (2007) has suggested the erection of the family Ophiocordycipitaceae. The strong support for relation to *C. agriota* more specifically places *M. microspora* within the *C. unilateralis* subclade and suggests that, if a teleomorph was found for this fungus, it would be classified within the newly erected *Ophiocordyceps* genus (Sung et al 2007).

The analysis of the full set of 51 taxa shows a number of unresolved taxa in the form of polychotomies. (Because relationships to a number of species could not be definitely determined, they are not shown in FIG. 9.) However clear relationships among *M. microspora* and the four species of *Cordyceps* within Clade B of Clavicipitaceae (Sung et al 2007) are shown. Phylogenetic resolution for *M. microspora* was achieved with the analysis of three genetic regions, but the polychotomies arising at the generic and higher level demonstrated why five or more genes are necessary to provide sufficient parsimony informative characters to resolve these relationships (Sung et al 2007).

M. microspora is a pathogen of elongate hemlock scale, as determined by completing Koch's postulates (JAP Marcelino, University of Vermont, 2007, pers comm). The fungus develops sporodochia surrounding dead scales on conifer foliage and often is found to coexist with *Lecanicillium lecanii*, *Cladosporium oxysporum* and *Tripospermum* sp., for which more studies will be needed to determine their ecological relationships. Elongate hemlock scale is an introduced species from Japan (McClure 1980b), but it is not clear whether *M. microspora* is a native or introduced species.

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