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Aurosphaeria, a novel coelomycetous genus

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Abstract — An endophytic pycnidial producing fungus was isolated from *Drosera montana,* growing in the Heath River area of the Bolivian Amazon. Based on morphological characteristics of the pycnidium, the nature of the conidiophores and conidial structure as well surprisingly unique DNA sequence data on this fungus, a new genus, *Aurosphaeria*, is described. The nearest genetic neighbor is *Stemphylium* sp. with a query coverage of 87% and a maximum identity of 89%. This fungus is not morphologically related to *Aurosphaeria.* Morphologically, the most similar fungus is *Hapalosphaeria* sp.; however, *Aurosphaeria* deviates from *Hapalosphaeria* by its unique phialidic conidiophores, the presence of decorative hyphal tufts on the pycnidia, and other features that are herein described. Biochemically, *A. flaviradians* is of interest as it produces copious quantities of brefeldin A, a biologically active compound, in liquid culture.

Key words — rDNA, endophyte

Introduction

Endophytes are a potential source of many novel biologically active compounds (Strobel & Daisy 2003). Defined as microorganisms that live in the interstitial spaces of live plant tissues, endophytes live symbiotically with plants and are not generally considered to be parasitic (Bacon & White 2000). Because these organisms (mostly fungi) are positively associated with plants, there is a great opportunity to find and characterize new endophytes with useful,

non-toxic applications (Strobel & Daisy 2003). Searching for these novel and useful endophytes can be accomplished with a relatively high success rate in unique ecosystems such as the Amazonian rainforest, which possesses the greatest plant diversity on earth (Arnold et al 2001 2007; Mittermeier et al 1999). Associated with this enormous diversity of higher plants is also a diversity of microorganisms. In fact, a recent survey of 150 endophytic fungi from the upper Amazon in Peru and Bolivia showed that about 10% of these endophytes contained rDNA sequences that are wildly divergent from the fungi previously documented in GenBank (Smith et al 2008). These biologically and taxonomically unique endophytes are extremely valuable since they often make useful and novel bioactive metabolites (Strobel & Daisy 2003 Tan & Zou 2001). Additionally, the general biology, host specificity, production of secondary products, host-plant interactions, and endophyte-endophyte interactions of endophytes have scarcely been studied in any plant system, and given the potential benefits of endophytes, these areas are well worth studying.

Isolate P404e was recovered from the inner leaf tissues of a specimen of *Drosera montana* growing in a savannah region located in the Heath River country of the Bolivian Amazon. To capture its prey, *D. montana* has developed unique insect-trapping glandular stalks on its leaves. Previous efforts to isolate endophytes from this plant are not known to the authors. Using molecular techniques, this isolate, P404e, was found to be possessing nrITS sequences that were not similar (<89% similarity) to any taxonomically characterized fungus on record in GenBank. In addition, the specific morphological characteristics of the organism did not match those of any previously described fungus with globose pycnidia, septate conidiophores, and aseptate, hyaline, smooth- walled, globose conidiospores. The fungus was initially of interest due to its ability to inhibit the growth of bacterial test organisms including *Escherichia coli*. Later it was discovered to produce the bioactive compound brefeldin A in copious amounts. Morphological and molecular biology studies indicate that P404e possesses other unique features that are described in this report. The name proposed for this novel endophytic fungus is *Aurosphaeria flaviradians*.

Materials and methods

Fungal isolation

Several intact plants of *Drosera montana* A. St.-Hil. were acquired in the Bolivian Amazon during March of 2007. They were collected in a savanna region adjoining the rainforest at 12° 40' 07" S and 68° 41' 58" W and were transported to Yale University for analysis. Several small pieces from the plant stems including the leaves were cut and placed into 70% ethanol for 30 seconds under a laminar flow hood. Sterile tweezers were used to hold the stems separately in the flame to remove excess alcohol. Then the leaves were scored and the epidermal layer removed. Portions of the inner tissues were then placed onto water agar. After two weeks, a number of fungal isolates were obtained from

this plant. One of these cultures (P404e) was the subject of this study as it produced an unusually yellow metabolite and appeared distinct from the other endophytes. It also possessed antibiotic activity when used in a screening test (Castillo et al 2007).

Scanning electron microscopy

Scanning electron microscopy was performed on P404e following procedures described by Castillo et al. (2005). Agar pieces and carnation leaf pieces supporting fungal growth were placed in filter paper packets then placed in 2% gluteraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2-7.4) with Triton-X 100 (a wetting agent), aspirated for 5 minutes and then left overnight. The following day, samples were washed six times in 15 min washes of water buffer 1:1, followed by a 15 min wash in 10% ethanol, a 15-min wash in 30% ethanol, a 15 min wash in 50% ethanol, five 15-min washes in 70% ethanol, and then finally left overnight or longer in 70% ethanol. The samples were then rinsed six times for 15 min in 95% ethanol then three 15-min washes in 100% ethanol, followed by three 15 min washes in acetone. The microbial material was then critically point dried, gold sputter coated, and images were recorded with an XL30 ESEM FEG in the high vacuum mode using the Everhart-Thornley detector. Conidiospores and pycnidia were measured using Image J software (available online: http://rsb.info.nih.gov/ij/).

Fungal growth and storage

Several methods were used to store the isolated fungus as a pure culture. The fungus was grown on potato dextrose agar (PDA) for two weeks, and then it was cut into small squares which were placed into vials containing 15% glycerol and stored at –70° C. However, the most effective method of storage was on sterile barley seeds that had been colonized by the fungus for at least 10 days and these were air dried and then stored at –70° C. The fungus has been designated as isolate 2349 in the living culture collection of Montana State University.

Fungal DNA isolation and acquisition of ITS- 5.8S rDNA sequence information

P404e was grown on potato dextrose broth for 7 days, after which the mycelium was harvested and the genomic nucleic acid (DNA) extracted using DNeasy Plant and Fungi Mini Kit (Qiagen), according to the manufacturer's directions. The ITS regions of the fungus were amplified using PCR with the universal ITS primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'). All other procedures were carried out as previously described by Ezra *et al*., 2004. The DNA was sequenced at the W.M. Keck Facility at Yale University, and the sequences were submitted to GenBank on the NCBI web site: http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the GenBank database using the BLAST software on the NCBI web site: http://www.ncbi.nlm.nih.gov/BLAST/).

The sequence data of this fungus are deposited in GenBank as EU977279. A phylogenetic tree showing the relationship of P404e to other fungal organisms was constructed (Edgar, 2004; Ronquist and Huelsenbeck, 2003; Smith et al., 2008).

Isolation and characterization of brefeldin A

Brefeldin A, an ER-golgi transport inhibitor (Dinter & Berger 1998), was recovered from a 100 ml, 5-week-old potato dextrose (PD) broth culture of isolate P404e. After

growth of the culture according to these parameters, the resulting broth was filtered through several layers of cheesecloth to remove the fungal mycelia. The filtrate was then extracted with an equal volume of dichloromethane (CH_2Cl_2), after which the solvent was evaporated to dryness. The resulting material was stored in a minimal volume of methanol at 4°C until further analysis was performed. Given that this crude extract showed bioactivity in an immunomodulatory context, silica column chromatography was performed to separate the mixture of compounds present in the crude extract. A fraction eluting with a solvent system of 93:7 v/v of CH_2Cl_2:CH_3OH formed crystals when evaporated to dryness. The crystals were re-dissolved in a minimal volume of CH_2Cl_2 then the solvent was re-evaporated slowly at 4°C to prepare crystals suitable for x-ray crystallography. Once the larger crystals were formed, they were subjected to standard x-ray crystallographic procedures, which unequivocally determined the molecule to be brefeldin A.

Taxonomy

Aurosphaeria Sun J. Lee, Strobel, Eisenman, B. Geary, P.N. Vargas & S.A. Strobel, **gen. nov.**

MycoBank 512382

Aurosphaeria *deviare de* Hapalosphaeria *sollicitus phialidi conidiphoras et habemus ornamenti hyphae indumentum in pycnidia.*

Type species: *Aurosphaeria flaviradians.*

Etymology: The genus name, *Aurosphaeria* is taken from the Latin word *auro,* meaning gold, and *sphaeria,* meaning sphere-like, to describe the golden, nearly spherical pycnidia.

Unique Characteristics: The genus is differentiated from *Hapalosphaeria* in possessing unique phialidic conidiophores, decorative hyphal tufts on the pycnidia, and globose erumpent pycnidia.

Aurosphaeria flaviradians Sun J. Lee, Strobel, Eisenman, B. Geary, P.N. Vargas & S.A. Strobel, **sp. nov.** Figs. 1–3.

MycoBank 512383; GenBank EU977279

Fungus in natura est consociatis cum Drosera montana *Pycnidia aurum, est in 2-7 verticillata, globosae, membrana angosta, cum textura angularis, ornamento indumento pubescentia dematiacea hyphae, et 148± 5μ. Conidiophoras translucida, ramis in apice, formis in celula membrana pycnidial, 8-15 μ longis. Conidiogenous celula enteroblastis, phialidiae, determinativus, translucida, lisa. Conidia globosae, hyalinis, lisa, membrana angosta, egluttulata, 2.5± 0.1 μ.*

Etymology: The organism produces a bright yellow metabolite in culture, and thus from the Latin *–flaviradians* or 'radiating yellow'.

Type: Bolivia, Amazon, Heath River Forest, March 14, 2007, K. Eisenman & P.N. Vargas (MONT 2349, **holotype**).

Living culture collection: isolated from the same *Drosera montana* plant as the dried type collection and maintained in Montana State University mycological culture

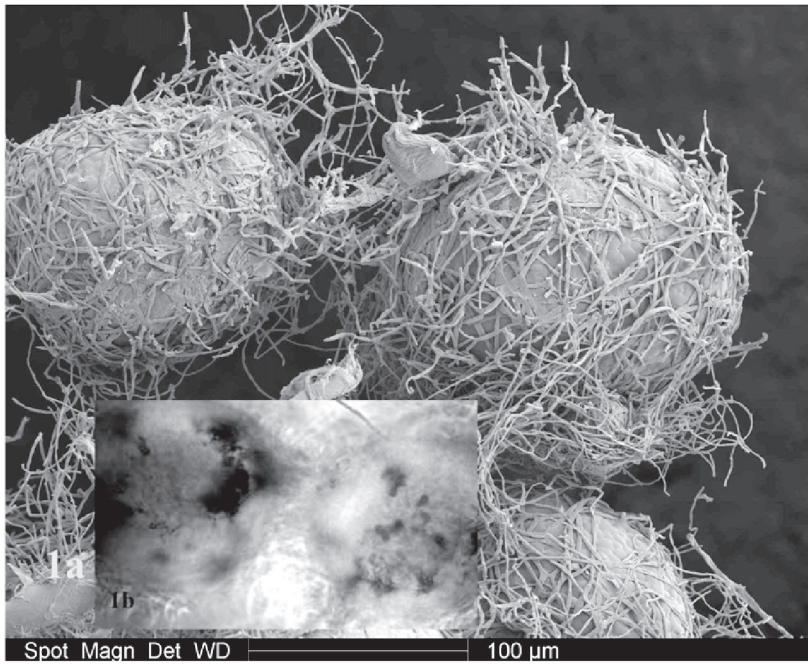

Fig.1a Two globose pycnidia observed by SEM. Tufts of hyphae decorate each pycnidium. These structures possess no ostiole and thus spore release is via some disruptive event leading to cleavage of the pycnidial wall. The bar represents 100µ. Fig 1b (inset). Light microscopy reveals the characteristic dark hyphal tufts that decorate each of two pycnidia. The size of the pycnidia is noted in the legend to Fig 1a.

collection; **Bolivia**, Amazon — Heath River Forest: March 14, 2007, K. Eisenman & P.N. Vargas MONT 2349 (6/21/2008).

Teleomorph: The teleomorph of this fungus is unknown. The molecular data from the 18S rDNA gene sequences of *A. flaviradians* provide no hint as to the fungal family relationship that this organism may possess (Bruns et al., 1991; Reynolds and Taylor 1993; Mitchell et al., 1995; Guarro et al., 1999; Taylor et al., 1999).

Aurosphaeria flaviradians is associated in nature with *Drosera montana.* It was not recovered from other plants growing in the same topographical area. This fungus is characterized by having whitish colonies on PDA (top side) and a yellow undulating grooved mycelia mat on the reverse side. The erumpent pycnidia are golden, usually occurring in clusters of 2 to 7, and are globose with no apparent ostiole. The pycnidia are also thin-walled, made of textura angularis, and are decorated with tufts of dematiaceous hyphae (148 ± 5 µm).

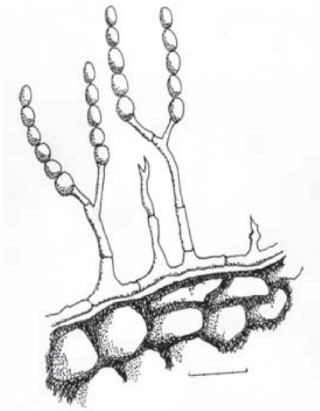

Fig. 2 (left).Masses of conidiospores associated with matrix material as observed by SEM. The bar is equivalent to 10µ. Fig. 3 (right). An artist's rendition of conidiospore production within the pycnidium. The bar is equivalent to 7.5µ.

Thus, this fungus is a coelomycete. Conidiophores, which line the inside pycnidial walls, are hyaline, branched at the tips, and formed from inner cells of the pycnidial wall. They range in length from 8 to 15 µm. The conidiogenous cells are enteroblastic, phialidic, determinate, hyaline, and smooth. Conidia (2.5± 0.1 µm) are globose, hyaline, smooth, thin-walled, eguttulate, and borne in chains (Fig. 2). This fungus produces brefeldin A and related compounds.

Comments on the biology of *Aurosphaeria flaviaradians*

Aurosphaeria flaviradians produces a whitish-tan mycelium on PDA. After a week in culture, numerous erumpent, globose, and golden pycnidia are produced in a concentric fashion on both 1/10 and PDA, resulting in a yellowish-tan, roughened surface almost resembling sand paper. This roughened surface is due to the numerous clumps of protruding pycnidia. The clumps contain 2 to 7 or 8 pycnidia. On the reverse side of the PDA plate, the mycelium is yellow and undulates at the center and periphery of the culture. The pycnidia are superficial on the agar surface and possess no ostiole. The tufts of interwoven dematiaceous hyphae that decorate the surface of each pycnidium are randomly arranged (Fig.1a,b). The pycnidia dehisce to disseminate spores. These conidial characteristics are completely unlike those of the nearest phylogenetic relatives, *Stemphylium* sp., *Berkleasmium* sp. and *Lophiostoma* sp., all of which possess darkened, multi-cellular conidia (Irwin et al. 1986, Moore 1959, Tanaka & Harada 2003). The closest morphological relative of this fungus appears to be *Haplosphaeria deformans*, which *A. flaviradians* resembles in some respects,

but *H. deformans* lacks conidiophores and erumpent pycnidia decorated with hyphal tufts (Fig. 1a, b).

Aurosphaeria flaviradians has only been collected from the Heath River area in Bolivia. It exists as an endophyte in an insectivorous plant, *Drosera montana.* It was not possible to isolate it from any other plant species in proximity to its original host. No symptoms or signs of the fungus were evident on the host supporting it, and its role in the plant is currently unknown. However, given the products that it makes, one may infer that a complex biology governs fungus+plant interactions.

The secondary metabolites of this organism were also studied. After 5 weeks of culture in PD broth, *A. flaviradians* produced a variety of pigmented and non-pigmented compounds, some with distinct odors, which were separated via silica column chromatography. Of these, one compound crystallized and the structure was solved by x-ray crystallography. The findings, which were additionally supported by mass spectrometry data, concluded that the compound was the Golgi-disruptor brefeldin A (Fujiwara et al., 1988). None of the phylogenetic relatives of this fungus mentioned above have been reported to produce brefeldin A.

The role of brefeldin A and its derivatives made by this fungus in the context of host-fungus biology is unknown. However, the discovery of this novel organism nicely illustrates a main tenant of this report—that areas of the world housing vast plant diversity also could possess comparable microbial diversity. Such unusual microbial diversity is likely to lead to products and processes that have implications for a wide variety of biotechnical applications (Strobel & Daisy, 2003 Strobel 2006).

Phylogenetic results

Given the highly conserved nature of fungal rDNA sequences, the ITS (internal transcribed spacer) region of the rDNA is particularly useful in determining both the identity of any given specimen as well as its nearest phylogenetic relatives (Mitchell et al., 1995). Sequences of the P404e rDNA ITS1 and ITS2 regions were compared to the GenBank database using BLAST. The most similar organisms were unnamed and uncultured fungi with query coverage and maximum identity of the top hit being 93% (AY699698.1). Out of the taxonomically characterized organisms, the closest genetic relative was a *Stemphylium* sp. with a query coverage of 87% and a maximum identity of 89% (EU339369.1). In order to view the data in a wider phylogenetic context, a tree was constructed using methods of Smith et al., 2008. Phyutility was initially used to retrieve the top-ranking 100 BLAST sequences; however, this number was then reduced to 40 to remove more distant redundancies (Smith & Dunn, 2008). From the resulting list, sequences from such organisms without

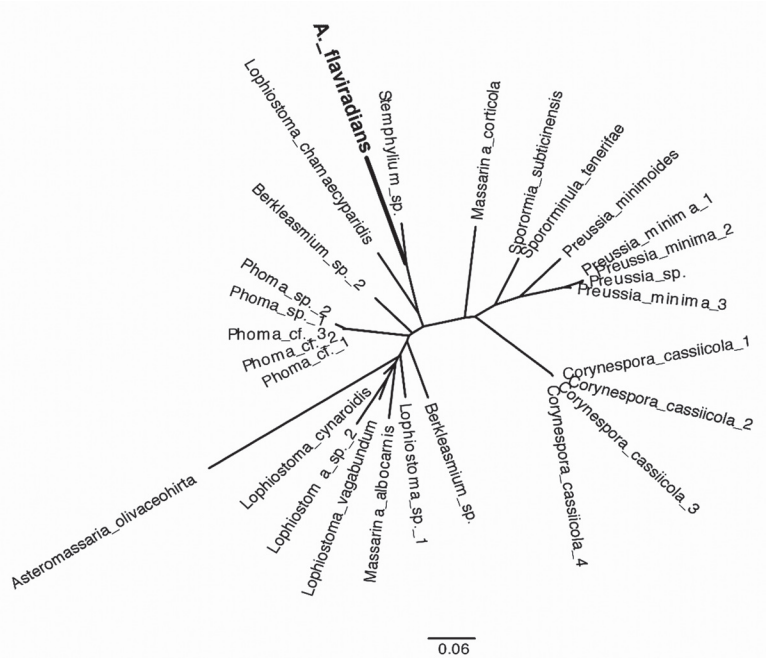

Fig. 4. Unrooted radial tree detailing the phylogenetic location and evolutionary distances of *Aurosphaeria flaviradians* to the 27 most similar ITS sequences in Genbank, omitting such entries as "uncultured fungi". Figtree v1.1.2 was used to visualize the tree.

taxonomic characterization (such as those named "Uncultured fungus" and "Fungal sp.") were excluded, and the resulting list of 27 sequences was then aligned using MUSCLE (Edgar 2004), with sites missing >50% of the data excluded from analysis (Smith et al. 2008). Bayesian analyses were then performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003 Smith et al. 2008). Figtree v1.1.2 was used to visualize the analyzed data in an unrooted radial tree (Fig. 4). The molecular biological analysis supports and confirms the conclusion that *A. flaviradians* is a unique fungus.

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