A new *Ophiostoma* species in the *O. pluriannulatum* complex from loblolly pine roots.

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Abstract: Various Ophiostomatoid fungi have been implicated as contributing factors to the decline of pines in the southeastern USA. During a survey for these fungi in loblolly pine (*Pinus taeda*) roots at Fort Benning, GA, we encountered a species of *Ophiostoma* with a *Sporothrix* anamorph, morphologically similar to *O. pluriannulatum*. This species has not been reported from pine roots in this region. Moreover, a closely related congener, *O. subannulatum*, is reported to infect conifer roots, and we sought to identify this fungus based on morphology, as well as ITS and beta-tubulin sequence comparisons. Isolates observed were grossly similar to those of *O. pluriannulatum*, with unusually long perithecial necks, but different in culture morphology. Sequences of the ITS rDNA were identical to those of *O. pluriannulatum*, and similar to *O.multiannulatum* and *O. subannulatum*. Sequence data from the beta-tubulin gene region revealed the absence of intron 4 and presence of intron 5, similar to the latter two species, but distinct from *O. pluriannulatum*, which has intron 4 and not intron 5. Phylogenetic analyses of beta-tubulin sequences showed that all of our isolates group together in a clade distinct from *O. multiannulatum* and *O. subannulatum*. Given the arrangement of introns, we believe that our isolates represent a novel species. This new fungus is currently being described, and its pathogenicity, biology and ecology are also being studied.

Detail of the ostiolar hyphae

Introduction

Decline of southern yellow pines has been of concern to the region's foresters for a number of years. While many abiotic and biotic factors are likely to be involved in the decline, the role of Ophiostomatoid fungi has been considered and studied in some detail (Nevill et al. 1995, Otrosina et al. 1999, Eckhardt et al. 2007). Many of these fungi are known as blue-stain fungi, for their mainly cosmetic effects on timber post-harvest, or in trees which have been infested by scolytid bark beetles, which they rely upon as vectors. Some of these fungi have been demonstrated to be effective pathogens of trees (e.g. *Ophiostoma ulmi*, and *O. novo-ulmi* causing Dutch elm disease), and animals (e.g. *Sporothrix schenkii* causing sporotrichosis). The taxonomy of this group has been debated by mycologists at the ordinal, familial, generic and species levels for many years, though evidence from molecular data has fortified many taxonomic entities and reshuffled others in recent years. A benefit of these types of analyses has been the ability to resolve species complexes and to identify cryptic species, morphologically similar species that are in fact separated by the fixation of alleles into distinct monophyletic lineages.

Since the description of *Ophiostoma pluriannulatum* (Hedgc.) H. and P. Sydow (Hedgcock 1906) from blue stained wood of *Quercus rubra*, other similar species have been described, e.g. *O. subannulatum* Livingston & Davidson (Livingston and Davidson 1987) and *O. multiannulatum* (Hedgc. & Davidson) N. Fries (Davidson 1935). All these species have *Sporothrix* anamorphs, and perithecia with long necks and typically with rings along the necks (annuli or annulae). Another species, very different in morphology but part of the complex based on molecular data, was described recently as *O. carpenteri* (Hausner et al. 2003, Zipfel et al 2006).

We report on a species of *Ophiostoma* collected from loblolly pine (*Pinus taeda* L.) roots at Fort Benning, GA, which bears superficial morphological similarity to members of the *O. pluriannulatum* complex (OPC), but which we suspect is an undescribed species.

Methods and Materials

Root excavations

The new *Ophiostoma* sp. was isolated from roots excavated during a survey for root-inhabiting Ophiostomatoid fungi at Fort Benning, GA. Loblolly pines (*Pinus taeda* L.) were excavated using a modified two-lateral root sampling as described by Eckhardt et al (2007). Roots were processed by surface sterilization in a solution of ethanol, commercial bleach, and distilled water, prior to rinsing in tap water and plating on malt extract agar (MEA) and MEA amended with cycloheximide and streptomycin (CSMA). Ophiostomatoid fungi were identified by their characteristic perithecia or conidiophores and transferred to fresh CSMA or MEA depending on the composition of the donor plate. Isolates of the new species were sent to the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria for subsequent morphological and molecular analyses.

DNA preparation, PCR, sequencing and alignment

To determine the relationship of the new species within the genus *Ophiostoma*, DNA was prepared for PCR by scraping mycelium from axenic MEA cultures, and adding 150 mL of PrepMan Ultra (Applied Biosystems Inc.), and a trace of autoclaved sand to facilitate maceration with a sterile pestle. The macerated material was then incubated at 95 C for 10 minutes, the centrifuged for 10 minutes at 13,000 rpm (room temperature). Approximately 75 mL of the supernatant was transferred to a clean Eppendorf tube and was then used for PCR. Portions of the ribosomal DNA were amplified using the primers ITS1F and ITS4 (Gardes and Bruns 1993), and the b-tubulin gene region using the primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995).

Amplifications were performed under the following conditions: an initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 60 sec, 50 °C annealing for 60 sec, and 72 °C extension for 60 sec, and a final extension period at 72 °C for 7 minutes. Reaction mixtures, 50 mL total volume, consisted of 5 mL 10× PCR reaction buffer (JMR Holdings, USA), 2.5 mM MgCl2, 10 mM dNTPs, 10 mM of each primer, 2.5 U Supertherm Taq polymerase (JMR Holdings, USA) and 4 mL of genomic DNA, corresponding to a typical mass of 500 ng DNA. Purification of successfully amplified PCR products was achieved using the Roche High Pure PCR Purification Kit according to the manufacturer's protocol. Amplicons were then sequenced using the Big DyeTM Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA) with the primers noted above in forward and reverse directions, and analyzed on an ABI PRISIMTM 377 or ABI PRISIMTM 3100 Genetic Analyzer (Applied Biosystems).

Crude sequences were then visualized using MEGA 3.1 (Kumar et al 2004), with consensus sequences derived from the forward and reverse reads. Alignments created using this same program's ClustalW function, or aligned manually in cases where the arrangement of introns precluded alignment.

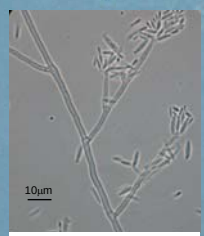
Morphological data

Isolates of the new species were grown on pine twig agar (PTA, water agar 15 g/L with twice-autoclaved *Pinus patula* twigs embedded), as well as oatmeal agar (OA) and 2% malt extract agar (MEA) or to assess characters of the mycelium, perithecia, and conidia. Specimens were mounted in lactophenol with cotton blue. Measurements were made using a Zeiss Axioskop2 using brightfield and phase contrast microscopy, and measurements were made using the software package AxioVision Release 4.4.1.0. Fifty measurements of each structure parameter were measured and minima, maxima, and average structure parameters recorded.

On pine twig agar, abundant perithecia are formed

On Oatmeal Agar, zonate growth may occur, with copious perithecia and conidia

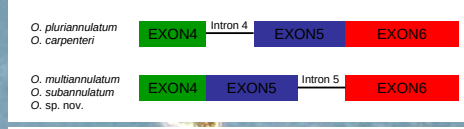
On MEA. The appearance of the *Sporothrix* stage of the fungus is lanose-floccose

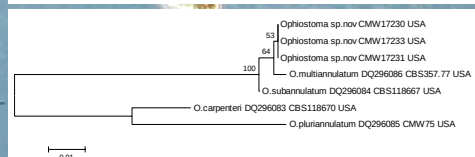


Detail of the *Sporothrix* anamorph, showing primary and secondary conidia

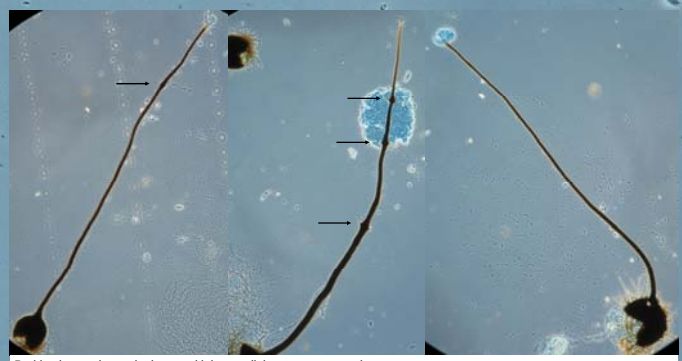
Ascospores of the new *Ophiostoma* sp.

β-Tubulin Intron Arrangement in the OPC





An unrooted neighbor-joining tree derived from alignment of partial β-tubulin sequence showing the relationship of the new species to other species in the OPC



Perithecia may have single or multiple annuli, but most commonly none are present.

Results and discussion

Evidence from the ITS rDNA suggests that isolates of the putative new species does belong to the OPC. Given the poor resolution of ITS sequence data for Ophiostomatoid taxa at fine scales (e.g. Chung et al. 2006), we also sought sequence data from part of the b-tubulin gene. Based on b-tubulin data, all of our isolates group together in a clade distinct from other species in the OPC. A notable feature is the arrangement of introns in the b-tubulin gene. While *O. pluriannulatum* sensu stricto and *O. carpenteri* have intron 5 only (no intron 4), *O. multiannulatum*, *O. subannulatum* and our isolates have intron 4 but not intron 5 (Zipfel et al 2006).

The new species is similar to other members of the OPC in having a *Sporothrix* anamorph, perithecia with long necks, and association with pines. Our isolates, particularly those grown on PTA, seldom produce annuli, and those that do have fewer than do congeners of the OPC.

Many *Ophiostoma* spp. and particularly members of the OPC have been found throughout pine forests in North America and beyond. The ecology and pathology of members of *Ophiostoma* in general and this species complex more specifically are highly variable. Some species are highly virulent to their hosts, such as *O. ulmi* and *O. novo-ulmi* in the Dutch elm disease pathosystem. Other species are more commonly associated with timber post-harvest, and one member of the genus (albeit in the mitosporic form, *Sporothrix schenkii*) is a human pathogen. The species here described has been found in association with roots of living pine trees in an area where decline of southern pines has been observed, but the degree of association (i.e. facultative vs. obligate) is still not known, and also the primary vector between trees is unclear. We have collected a similar fungus from root feeding beetles of the genus *Hylastes*, although the frequency with which we collect *Ophiostoma* spp. suggests only a casual relationship with these insects. While *O. subannulatum* was originally described as a fungus pathogenic to grand fir (*Abies grandis*), *O. multiannulatum* and *O. pluriannulatum* were originally described as blue-stain fungi, infecting both hardwoods and conifers. As such, the pathogenicity, biology, and ecology of this fungus are still very much of interest.

Taxonomy

Owing to the infrequency with which this species produces rings on the perithecial necks, we propose the following taxonomic entity, to be formally described in the near future:

Ophiostoma sparsiannulatum Zanzot, DeBeer and Wingfield nom. prov.

Aerial perithecia on pine twig agar (PTA) with long necks (average 1100 mm, range 500-1850 mm, SD± 300mm). Some submerged perithecia observed on PTA had much longer necks, averaging 4500 mm and as long as 8600 mm. Perithecial bases dark brown-black, average diameter 135 mm (54-201, SD±31) . Average diameter of the neck is 13.3 (7.1-21.0 mm, SD±3.3) at the apex and at the base 37.2 (13.0-65.0 mm, SD±10.7). Ostiolar hyphae present, average length 46.9 mm (17.6-80.1 mm, SD±15.3) Asci not observed. Ascospores, 3.06 mm (2.21-3.97 mm, SD±0.33) by 1.02 mm (0.66-1.58 mm, SD±0.14), hyaline, allantoid. Anamorph of form-genus *Sporothrix*, typically growing abundantly amongst the perithecia, in white fluffy masses. Conidiophores variable in length, average length 30.3 mm, (12.5-150.3 mm, SD±21.7), denticles 1.02 mm long, (0.68-1.64 mm, SD±0.26). Conidia average length 5.25 (2.68-9.20, SD±1.48) by width 1.4 mm, (0.75-2.33 mm, SD±0.38) with secondary conidia occasionally forming acropetally.

Mycelium hyaline at first, darkening with age and with copious white floccose aerial growth of the anamorph. Perithecia produced typically within 8 days. Optimum temperature 25 °C, with no growth at 35 °C.

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