Ecological and molecular evidence suggests that *Leptographium serpens* is a recent introduction to southeastern forests.

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ABSTRACT: *Leptographium* spp. (Ascomycota, Ophiostomatales) have been implicated as important factors in loblolly pine decline in the southeastern United States. Among the species most commonly recovered from roots and root-feeding beetles are *L. procerum*, *L. terebrantis*, and *L. serpens*. While the two former species are widespread and well documented in eastern US forests, *L. serpens* has only recently been reported from North America. Originally described from Italy, *L. serpens* has been reported from several European countries, as well as South Africa. In South Africa, pine hosts and bark beetle vectors are known to be introduced, suggesting that the fungus is introduced as well. While the sexual form, *Grosmannia serpens*, has been reported from several European nations, sexual structures have not been observed in South Africa nor the southeastern United States. Sequence data from three genes (internal transcribed spacer of the ribosomal RNA, beta-tubulin, and transcription elongation factor 1-alpha) also support the hypothesis of nativity to Europe and introduction to South Africa and the southeastern US. Potential exotic beetle vectors for *L. serpens* are discussed, and further molecular evidence, in the form of microsatellite markers, is also being pursued.

Historical Evidence

The mycota of southeastern forests and timber has been studies since the early 20th century. Several species of ophiostomatoid fungi such as *Leptographium* spp. have been described from the southeast, including: *Ophiostoma pluriannulatummm*(Hedgcock 1906), *Ophiostoma multiannulatum*(Davidson 1935), *Leptographium terebrans* (Barras and Perry 1971). *Leptographium procerum*, also hypothesized to be a native fungus, was described from eastern Canada (Kendrick 1962) and is associated with insects known to be native to the southeast. *Leptographium serpens* was originally described from Italy (Goidanich 1936), with subsequent observations in other countries in Europe including France, Poland, Spain, Portugal, and the United Kingdom (Siemaszko 1939, de Ana Magan 1982, Morelet 1988, Wingfield and Gibbs 1991). While the type locality of a described organism doesn't necessitate it being native to that locality, the breadth of the historical reports from Europe, and the lack of historical reports from surveys in the southeastern US support the hypothesis of a recent introduction.

Pathogenicity and vector relationships

There is evidence to suggest that *Leptographium serpens* may be more aggressive than other *Leptographium* species in tree roots (Eckhardt, Jones and Klepzig 2004). Most *Leptographium* spp. are considered only moderately virulent, with the exception of *L. wageneri* causing black stain root disease in the Pacific Northwest(Hansen et al. 1988). This characteristic is consistent with a non-native fungus which has not co-evolved with its hosts.

While *Leptographium* spp. are typically carried on their vectors phoretically (on the body of the vector, not in specialized structures called mycangia), they are typically restricted to a suite of insects with similar niches. In the southeast, *L. serpens* has been reported from a broad range of bark beetles and ambrosia beetles (see table below, from Eckhardt et al. 2007.), suggesting a lack of coevolution with these insects.

Number of insects trapped (2000, 2001, 2002)	*Lt* [1,2]	*Lp*	*Ls*	*Ltr*
Hylastes salebrosus[3] (22, 2013, 1322)	25	30	57	2
Hylastes tenuis[3] (12, 801, 424)	20	25	51	1
Pachylobius picivorus (7, 327, 148)	92	14	—	—
Colopterus unicolor[3,5] (0, 0, 279)	17	15	33	4
Hylobius pales (11, 2, 143)	78	66	—	—
Xyleborinus saxesini[3] (0, 29, 98)	—	—	70	—
Dendroctonus frontalis[4] (8, 33, 0)	17	—	—	—
Ips spp.[4] (3, 24, 0)	95	—	—	—
Xylosandrus crassiusculus[3,6] (0, 9, 12)	—	—	62	—
Pityophagus cephalotes[3] (0, 10, 5)	40	20	55	—
Monarthrum mali[3] (0, 14, 0)	—	—	64	—
Xylosandrus compactus[3,6] (0, 0, 9)	—	—	77	—
Corthylus punctatissimus[3] (0, 7, 0)	—	29	14	—
Dendroctonus terebrans (0, 0, 4)	90	—	—	—
Orthotomicus caelatus[3] (0, 0, 4)	—	—	50	—
Gnathotrichous materiorius[3] (0, 0, 3)	—	—	67	—

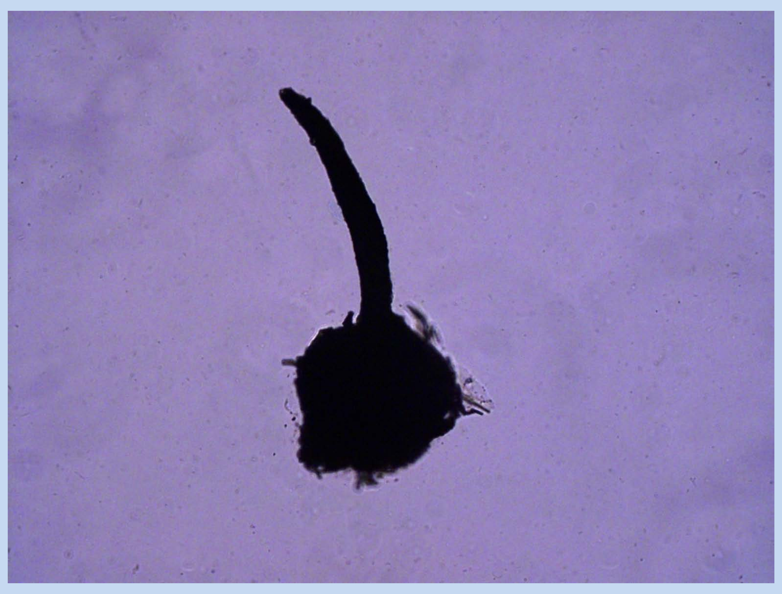
[1] *Lt* = *L. terebrantis*, *Lp* = *L. procerum*, *Ls* = *L. serpens*, *Ltr* = *L. truncatum*.
[2] Some insects had associations with more than one species of *Leptographium*.
[3] Not previously associated with a *Leptographium* species.
[4] Only found on plot C2, which was adjacent to a southern pine beetle (SPB) spot that was active 2000 and 2001. Spot was cut and trees removed 3 wks before trapping in 2002.
[5] Seventy-eight percent of these insects also carried an undescribed *Leptographium* species that was recovered from hardwood roots.
[6] Not known or reported to utilize conifers as a primary host.

How did it get here?

In South Africa, *L. serpens* is almost certainly introduced. The pine hosts are introduced, and the bark beetle vectors are introduced (Wingfield and Marasas 1980, Zhou et al. 2001). In Europe, *L. serpens* has been associated with native pines and native insects. In the southeastern United States, *L. serpens* has been reported from loblolly pine, and from several native insects and some introduced ambrosia beetles (Eckhardt et al. 2007).

One introduced insect that may have brought *L. serpens* into the US is *Hylastes opacus (Haack 2006)*. This bark beetle has been reported from the northeastern United States, and may be present in the southeast as well. This beetle is likely rare in the southeast, as a similar native species, *Hylastes tenuis,* is abundant, which may compete with *H. opacus* (R. Rabaglia, personal communication). However, the fungi vectored by *H. opacus* may have been transferred to *H. tenuis*.

Sexual or Asexual?



In the Ascomycota, fungi may be homothallic (self-fertile) allowing for production of sexual structures in single cultures, or heterothallic (self-sterile), requiring two isolates of opposite mating types to produce sexual structures. In the Ophiostomatales (including *Grosmannia* and *Leptographium*), the sexual structures are perithecia (pictured above). Perithecia have been reported from cultures from Spain, France, and Italy, (Goidainich 1936, de Ana Magan 1985, Morelet 1986)but not from South Africa or the southeastern United States, suggesting that: 1. *L. serpens* is heterothallic, 2. Both mating types are present in Europe, and 3. Only one mating type has been introduced into South Africa and the United States.

Conclusions

Based on the historical evidence, genetic evidence from protein coding genes, and ecology, *Leptographium serpens* is likely to be a recent introduction into southeastern forests. We propose *Hylastes opacus* as the original vector, although this beetle has not been confirmed in the southeast. The fungus seems to have shifted hosts (to our native pines) and vectors (to an array of bark and ambrosia beetles and weevils). This fungus may play an important role in loblolly pine decline, as management strategies for exotic pathogens are different from strategies for native fungi.

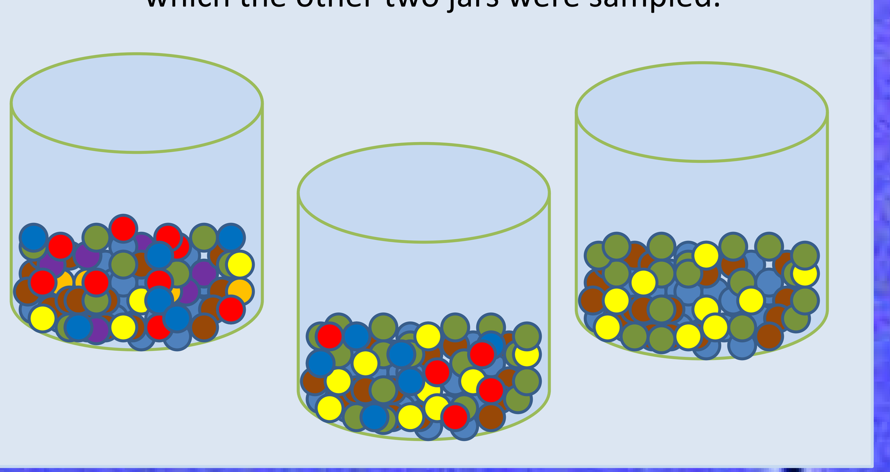
Genetic Evidence, Haplotypes

An organism's genes change over time, by nucleotides being inserted, deleted, or mutated. Haplotypes are unique forms of a gene. Private alleles are haplotypes that are only found in a single region or area, in other words, regionally unique haplotypes. One way to assess relationships between distant populations is by looking at haplotypes. Sequences were resolved from two gene regions for isolates from Europe, South Africa, and the southeastern United States: β-tubulin, and Translation Elongation Factor 1-a. These tables show the results.

Provenance	β-tubulin		Elongation factor 1α	
	Haplotypes	Private alleles	Haplotypes	Private alleles
Europe	5	3	5	3
South Africa	2	0	1	0
Southeastern US	2	1	3	1

Haplotypes Simplified

A simplified analogy of how haplotype analysis works is this: suppose each population is a jar of marbles. If you are given 3 jars of marbles, and told that two of the jars of marbles are randomly sampled from the third, how do you know which one is the source and which are the samples? Now, if you are told that one jar contains 10 colors of marbles, another jar contains 6 marble colors, and the third jar contained five marble colors, one would logically assume that the five color and six color jars could not be the source, as they could have a greater number of colors in the sample than in the source. The four colors represented in the source jar that are absent in the sample jars are analogous to 'private alleles', as they are only present in the parent jar. Also, of the two sample jars have different colors, but both are subsets of the source jar, then one can suppose that the two samples were independent, and not a sample of a sample.
In the image below, the jar on the left is the source from which the other two jars were sampled.



Next Step, Microsatellites

Microsatellites, or Simple Tandem Repeats, are rapidly evolving DNA markers that are useful for tracing the genetic history of populations within species. Microsatellite loci are defined as short (1-6) nucleotide units that are repeated for at least three units.
For example, a simple dinucleotide repeat:

Individual 1: NNNNNNNNATATATATATATATATATATATNNNNNNNNNNNNNN
Individual 2: NNNNNNNNATATATATATNNNNNNNNNNNNNNNNNNNNNN
Individual 3: NNNNNNNNATATATATATATATATATATATATATNNNNNNNNNNN

Because they are rapidly evolving, it is necessary to develop new primers for the microsatellite loci for each new species sampled, and at least 10 independent loci are typically required to accurately catalog population structure. The Forest Health Dynamics Laboratory is working on developing microsatellite primers for *L. serpens* in cooperation with the Forest and Agricultural Biotechnology Institute at the University of Pretoria, South Africa. It is hypothesized that these polymorphic loci will be less diverse within the range of *L. serpens* in the southeastern United States, relative to isolates collected from Europe. This method is the current state-of-the-art technology for supporting hypothesized introductions.

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