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A new *Leptographium* species from the roots of declining *Pinus sylvestris* in Switzerland

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Summary

Scots pine (*Pinus sylvestris*) trees have been declining in the Rhône valley, Switzerland, for almost three decades. In an assessment of the role of fungi in this syndrome, the dominant fungus isolated from stained roots was a *Leptographium* species, morphologically similar to the asexual state of *Grosmannia serpens*. We examined isolates of this fungus based on DNA sequences of four protein-coding genes including actin, β-tubulin, calmodulin and translation elongation factor-1 alpha. The results showed that they were of a distinct, undescribed taxon related to species in the *Grosmannia serpens* and *G. wageneri* complexes. The fungus, described here as *Leptographium rhodanense* sp. nov., resembles other species in the two species complexes morphologically, and most probably ecologically, as is suggested by the fact that it was isolated from stained pine roots.

1 | INTRODUCTION

Since the early 1990s, a serious decline of *Pinus sylvestris* (Scots pine) resulting in high levels of mortality has occurred in the inner alpine Rhône valley (canton Valais) of Switzerland (Rigling & Cherubini, 1999; Rigling et al., 2006). Various aspects of both abiotic and biotic stresses have been investigated as possible factors contributing to the decline (Wermelinger, Rigling, Schneider Mathis, & Dobbertin, 2008). However, little is known about the possible roles of pathogenic fungi other than a few incidences of localized damage, which were not regarded as significant (Engesser, Forster, Meier, & Odermatt, 2000). Heiniger, Theile, Rigling, and Rigling (2011) consequently considered the occurrence and possible role of pathogenic fungi in the decline.

The study of Heiniger et al. (2011) was conducted at two study sites, Salgesch and Stalden, in the inner alpine Rhône valley. More than 200 Scots pine trees ranging from 50 to 180 years old were sampled over a period of 5 years from May 2001 to February 2005. Many blue-stain fungi were isolated from various parts of the trees. Among these, a species with *Leptographium*-like conidiophores and dark, serpentine vegetative hyphae in culture, resembling those of *Grosmannia serpens*, was the most common fungus isolated from the roots (Heiniger et al., 2011). The aim of this study was to determine the identity and phylogenetic relationships of these isolates, and to consider their biology together with those of closely related species.

The taxonomy of the genera *Leptographium* and *Grosmannia* remains to be resolved in view of the one fungus = one name (1F1N) principles adopted in the Melbourne Code (Hawksworth, 2011; Hawksworth et al., 2011). Traditionally *Leptographium* was used for asexual species or the anamorphic state of "holomorphic" species, while *Grosmannia* was used to refer to species with known sexual states. Under the new Code, the older name *Leptographium* (Lagerberg, Lundberg, & Melin, 1927) will take priority over *Grosmannia* (Goidànich, 1936). However, the type species of these two genera group in different species

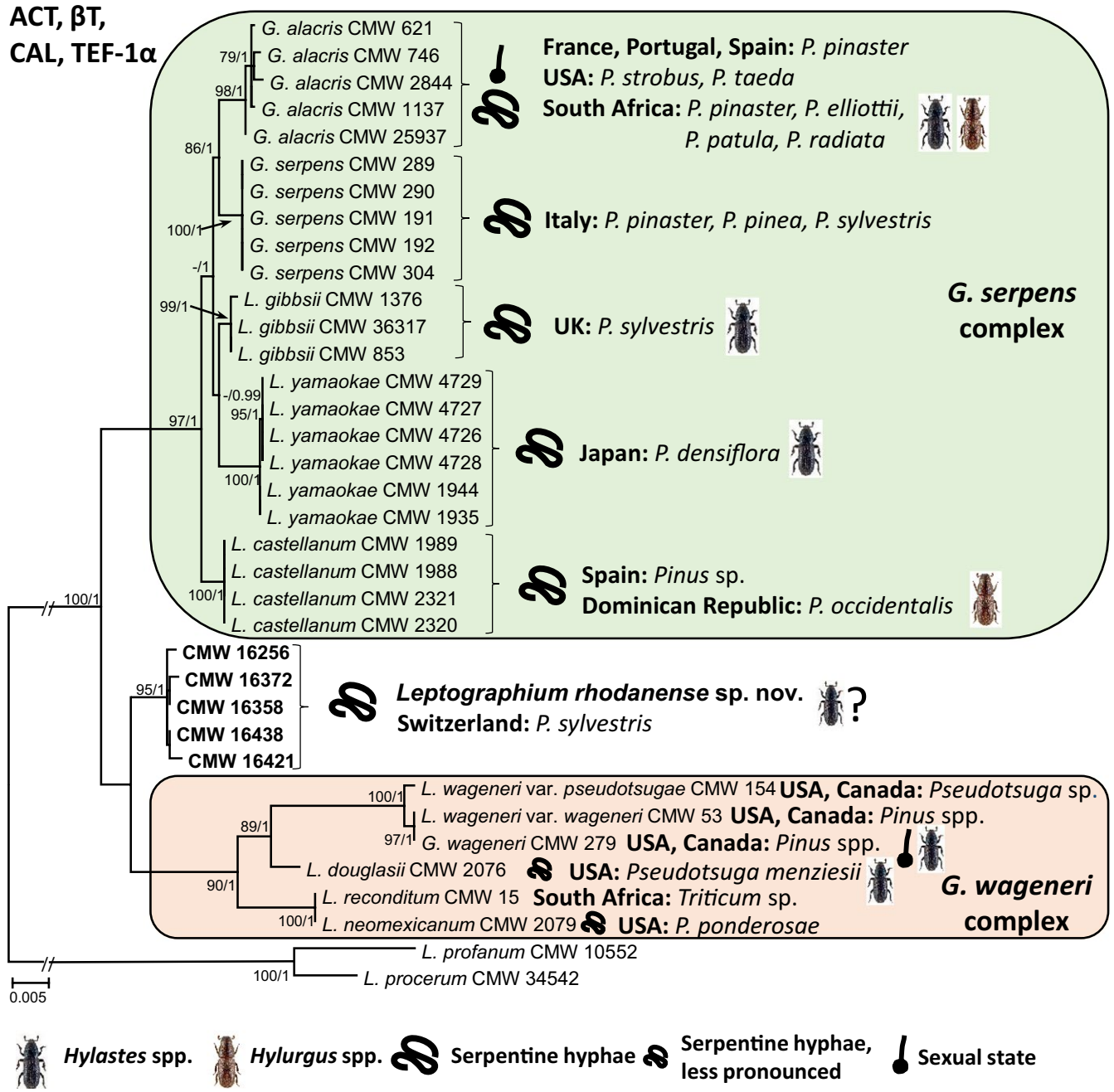

FIGURE 1 Maximum likelihood tree derived from analyses of concatanated dataset of ACT, βT, CAL and TEF-1α gene regions. Statistical supports (ML/BI) are indicated at nodes

complexes, the generic boundaries of which need to be clarified with a thorough multigene analyses (De Beer & Wingfield, 2013; Jacobs & Wingfield, 2013). De Beer and Wingfield (2013) thus cautioned against unnecessary name changes that could result from the application of 1F1N principles before the generic boundaries have been resolved through a thorough and comprehensive multigene analyses. In this paper, we thus follow the recommendations by De Beer and Wingfield (2013) to ensure nomenclatural stability for the interim until the taxonomy on this group of fungi is clarified. The recommendations include that the names of all known species in *Leptographium* and *Grosmannia* are maintained and used as before and that new species outside the *Grosmannia penicillata* complex are described in *Leptographium*.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates and morphology

In total, 18 *Grosmannia serpens*-like isolates were collected by Heiniger et al. (2011) and these were included in the present study. Isolates have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

TABLE 1 Isolates of *Leptographium rhodanense* and isolates representing *Grosmannia wageneri* complex sequenced in this study

Species	Isolate No.	GeneBank Accession No.				
		ITS2-LSU	ACT	βT	CAL	TEF-1α
Leptographium douglasii	CMW 2076		KY424502	KY424512	KY424522	KY424532
Leptographium neomexicanum	CMW 2079	*AY553382*	KY424503	KY424513	KY424523	KY424533
Leptographium reconditum	CMW 15	*AF343690*		*AY534931*		*AY536177*
Leptographium rhodanense[b]	CMW 16256 = CBS 138286	=KY424539	KY424504	KY424514	KY424524	KY424534
Leptographium rhodanense[a]	CMW 16358 = CBS 138285	=KY424539	KY424505	KY424515	KY424525	KY424535
Leptographium rhodanense[b]	CMW 16372	=KY424539	KY424506	KY424516	KY424526	KY424536
Leptographium rhodanense[a]	CMW 16421	=KY424539	KY424507	KY424517	KY424527	KY424537
Leptographium rhodanense[b]	[T]CMW 16438 = CBS 138284	KY424539	KY424508	KY424518	KY424528	KY424538
Grosmannia wageneri	CMW 279		KY424500	KY424510	KY424520	KY424530
Leptographium wageneri var. *pseudotsugae*	CMW 154	*AF343706*	KY424499	KY424509	KY424519	KY424529
Leptographium wageneri var. *wageneri*	CMW 53		KY424501	KY424511	KY424521	KY424531

[a]mating-type *MAT1-1*.
[b]mating-type *MAT1-2*.
[T]ex-holotype.
CMW, the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS=CBS-KNAW, Utrecht, the Netherlands.
GenBank numbers in italic indicate sequences were not produced in this study.
Additional isolates and their mating type genes (confirmed to be *L. rhodanense* based on βT sequences but not included in phylogenetic analysis) = CMW 16284[a], 16278, 16306[b], 16311, 16313[a], 16327[a], 16336, 16350, 16369[a], 16371[b], 16409, 16418, 16455.

Ex-type isolates were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and the dried cultures in the National Collection of Fungi (PREM), Roodeplaat, South Africa.

In order to describe morphological characteristics and to make comparisons with other species, isolates were grown on sterilized pine twigs in 2% water agar medium. Microscopy, growth in culture and tests for sensitivity to cycloheximide applied the methods described by Marincowitz, Duong, De Beer, and Wingfield (2015). Micromorphological features were described based on the standards suggested for *Leptographium* species in the monograph of Jacobs and Wingfield (2001).

2.2 | DNA extraction, PCR, DNA sequencing and phylogenetic analyses

For initial identification, sequences of the internal transcribed spacer region 2 and partial large subunit (ITS2-LSU) were determined for all 18 isolates included in the study. To enable comparisons with the sequence data sets for the *G. serpens* complex in the study of Duong, De Beer, Wingfield, and Wingfield (2012), sequences of an additional four protein-coding genes, including parts of the actin (ACT), the β-tubulin (βT), the translation elongation factor-1 alpha (TEF-1α) and the calmodulin (CAL) genes, were amplified and sequenced for a subset of five of the isolates. DNA extraction, primers, PCR and sequencing protocols were the same as that described by Duong et al. (2012).

Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed on the aligned sequence data sets of the five gene regions (data not shown). A concatenated data set including sequences of the four protein-coding gene regions were then compiled. In both ML and BI analyses of these data sets, the general time reversible model with a gamma distribution of rates across sites (GRT+G) was used. Maximum likelihood was conducted using raxmlGUI v1.3 (Silvestro & Michalak, 2012). Ten runs of maximum likelihood searches followed by 1000 bootstrap replicates were conducted. Bayesian inference analyses were conducted using MrBayes 3.2 (Ronquist et al., 2012). Ten parallel runs with five million generations were conducted, trees were sampled every 10th generation, and 25% of sampled trees were discarded as burn-in phase. Bayesian posterior probability values were calculated from the remaining trees.

2.3 | Mating-type PCR and ability to mate in culture

Mating types of three *L. wageneri* varieties, *G. alacris* and Swiss *Leptographium* spp. were determined by PCR using mating-type primers and the PCR protocol developed for species of *Leptographium sensu lato* by Duong, De Beer, Wingfield, Eckhardt, and Wingfield (2015). In an attempt to obtain sexual structures, isolates were paired with each other as described by Grobbelaar, De Beer, Bloomer, Wingfield, and Wingfield (2010), but using pine twigs rather than hardwood twigs added to culture media.

3 | RESULTS

Analyses of the β-tubulin region showed that the 18 Swiss isolates considered in this study belonged to a single taxon that together with species in the *G. serpens* and *G. wageneri* complexes formed a monophyletic clade in *Leptographium sensu lato* (data not shown). ML and

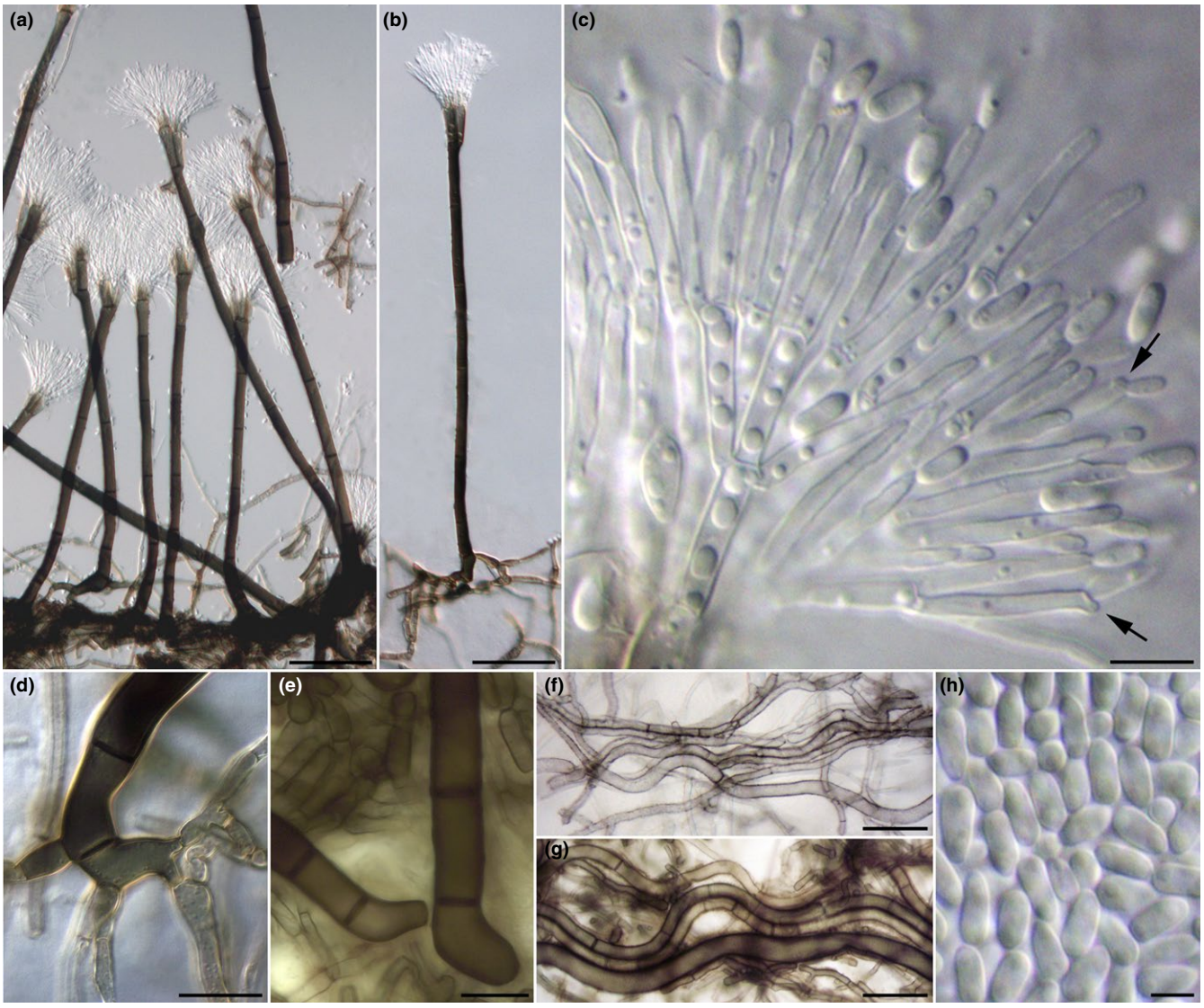

FIGURE 2 Microscopic images of *Leptographium rhodanense* (ex-holotype CBS 138284 = CMW 16438) (a, b) Conidiophores on 2% MEA. (c) Conidiogenous apparatus showing blastic conidiogenesis (arrows). (d, e) Foot cells. (f) Young serpentine hyphae. (g) Aged serpentine hyphae. (h) Conidia. Scale bars: a, b = 50 μm; c, d = 10 μm; e, h = 5 μm; f, g = 20 μm

BI analyses of the concatenated data set of ACT, βT, CAL and TEF-1α gene regions resulted in trees with identical topologies in which the Swiss isolates formed a well-supported monophyletic clade, clearly distinct from all known species in the *G. serpens* and *G. wageneri* complexes (Figure 1).

Based on mating-type PCRs, Swiss isolates either contained the *MAT1-1* or *MAT1-2* idiomorph, but never both (Table 1). However, laboratory crosses with isolates of opposite mating type failed to produce the sexual state of the fungus.

3.1 | Taxonomy

Leptographium rhodanense Marinc., T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. Figure 2. Mycobank MB819506.

Etymology: Name reflects the Rhône river that runs through the valley of Valais in Switzerland and where the samples were collected.

Description: Conidiophores single, upright, 270–500 μm high from basal cell to conidiogenous cells. *Stipes* smooth, uniformly pigmented throughout, brown, slightly bulging at apex, 185–380 μm long, 7.8–13 μm wide, with 5–9 septa. *Basal cells* rhizoid to foot-like. *Conidiogenous apparatus* branched in 2–3(–4) tiers: *primary branches* in a whorl of 2–8, 14–27.5 × 3.5–7.5 μm, *secondary branches* 9–20 × 2–4 μm, *tertiary branches* 8.5–15 × 2–9 μm, *quaternary branches* divergent. *Conidiogenous cells* in a whorl of 3–5, cylindrical, gradually tapering towards the apex, often slightly constricted at the base, hyaline, smooth, tip showing sympodial growth, (10–)14–15(–19) × (1–)2 μm. *Conidia* blastic, ellipsoidal, gradually tapering to truncated base, hyaline, (4–)5(–7) × 2(–3) μm. *Serpentine hyphae* darker and wider than ordinary vegetative hyphae, 7.5–17 μm wide. *Vegetative hyphae* constricted at septum, pale brown becoming dark pigmented with age, 2.5–4 μm wide, foremost hyphae growing curved, not straight. *Colonies* with optimum growth at 20–25°C in the dark, reaching 78 mm in 4 days at 20°C,

translucent, inner circle 40 mm olivaceous brown, mycelium flat, rarely aerial, edges nearly smooth, no effect on medium, fertile. The addition of cycloheximide showed 27% reduction in growth area at 20°C.

Habitat: roots of *Pinus sylvestris*.

Distribution area: Switzerland.

Holotype: Switzerland, canton Valais, Stalden, root of *Pinus sylvestris*, May 2004, U. Heiniger, holotype PREM 61102 (dried culture of CBS 138284), ex-holotype CBS 138284 = CMW 16438.

Additional specimens examined: Switzerland, canton Valais, Stalden, root of *Pinus sylvestris*, Feb 2004, U. Heiniger, CBS 138285 = CMW 16358; Salgesch, root of *Pinus sylvestris*, May 2002, U. Heiniger, CBS 138286 = CMW 16256.

4 | DISCUSSION

Results of this study have shown that a *Leptographium* sp. consistently isolated from the roots of dying *P. sylvestris* trees in Switzerland represents a new taxon described here as *L. rhodanense*. The distinctive serpentine hyphae produced by this species in culture resemble those of species in the *G. serpens* complex and they were less similar to those found in some species in the related *G. wageneri* complex which have less pronounced serpentine hyphae. While some *Leptographium* spp. can be identified using the dimensions of their conidiogenous apparatuses and conidia (Jacobs & Wingfield, 2001), this is not possible for species in the *G. serpens* complex. DNA sequence comparisons are thus necessary to distinguish species in this group (Duong et al., 2012) and these showed clearly that the Swiss isolates represent a novel taxon.

Mating-type PCRs confirmed the presence of both mating types among isolates of *L. rhodanense* and this shows that the species is heterothallic. It was, however, not possible to confirm this based on laboratory tests in culture. A heterothallic state in *L. rhodanense* is consistent with the fact that members of the *G. serpens* and *G. wageneri* complexes are known to be heterothallic (Duong, De Beer, Wingfield, & Wingfield, 2016).

All species in the *G. serpens* complex and the majority of those in the *G. wageneri* complex occur on *Pinus* spp. and this is consistent with the ecology of *L. rhodanense*. The only exceptions are *L. wageneri* var. *pseudotsugae* and *L. douglasii* that occur on *Pseudotsuga* (Wingfield, Harrington, & Crous, 1994; Zambino & Harrington, 1989) and *L. reconditum* that was described from the rhizosphere of wheat (Jooste, 1978). *Leptographium rhodanense* was isolated from stained and diseased roots. From the same trees that were the source of isolates for this study, *Hylastes* spp. were the most common insects isolated from roots (unpublished data, Heiniger et al., 2011). Although no attempts were made to isolate fungi from these insects nor their galleries (Heiniger, pers. comm.), it seems probable that a species in this genus is a vector of the fungus. This would be consistent with the fact that many root-infecting *Leptographium* spp. are associated with *Hylastes* spp. (Goheen & Cobb, 1978; Kirisits, 2004; Masuya, Yamaoka, Kaneko, & Yamaura, 2009; Wingfield, Capretti, & MacKenzie, 1988; Wingfield & Knox-Davies, 1980). However, isolations of this fungus from the insects as well as from their galleries should be undertaken to establish the nature of this relationship with confidence.

Heiniger et al. (2011) observed that the occurrence of blue-stain fungi was positively related to the crown transparency of trees, which was used as a measure of tree health. They also noted that a high number of the roots of moderately healthy trees were infected with *L. rhodanense*. From these observations, they suggested the blue-stain fungi including *L. rhodanense* could, together with their insect vectors, play a role in the decline of Scots pine in Rhône Valley. It is unlikely that *L. rhodanense* is a primary pathogen as is the case with the three varieties of *L. wageneri* varieties (Harrington & Cobb, 1988). However, the fungus could contribute to the decline of the trees where its insect vectors have infested the roots and as has been shown for *G. serpens* (Goidànich, 1936) and *G. alacris* (Wingfield & Knox-Davies, 1980; Wingfield & Marasas, 1980; Zhou, De Beer, Wingfield, & Wingfield, 2002).

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