Molecular characterization of the entomopathogenic fungi *Lecanicillium* spp. (Deuteromycota: Hyphomycetes) isolated from white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae), in British Columbia

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Abstract—The entomopathogenic fungal genus *Lecanicillium* Gams and Zare includes species that are highly pathogenic to many genera of insects. Three species, *Lecanicillium longisporum* (Petch) Zare and W. Gams, *L. muscarium* (Petch) Zare and W. Gams, and *L. pissodis* Kope and Leal, were found to be entomopathogens of adult white pine weevils, *Pissodes strobi* (Peck), in coastal British Columbia. Morphological characteristics were used to identify these species, but variation in conidial shape and size made it difficult to classify some of the isolates into the correct species of *Lecanicillium*. To confirm the identity of these *Lecanicillium* species, we used molecular tools such as polymerase chain reaction – restriction fragment length polymorphism and DNA sequencing.

Résumé—Le genre de champignons entomopathogènes *Lecanicillium* Gams et Zare contient des espèces qui sont fortement pathogènes pour de nombreuses catégories d'insectes. Trois espèces, *Lecanicillium longisporum* (Petch) Zare et W. Gams, *L. muscarium* (Petch) Zare et W. Gams et *L. pissodis* Kope et Leal, sont des entomopathogènes des adultes du charançon du pin blanc, *Pissodes strobi* (Peck), dans la région côtière de la Colombie-Britannique. Les caractéristiques morphologiques nous ont servi à identifier ces espèces, mais la variabilité de la forme et de la taille des conidies rend difficile le rattachement de certains de ces isolats à la bonne espèce de *Lecanicillium*. Afin de confirmer l'identité de ces espèces de *Lecanicillium*, nous avons utilisé des outils moléculaires, tels que la réaction de polymérisation en chaîne – polymorphisme de la longueur des fragments de restriction et le séquençage de l'ADN.

[Traduit par la Rédaction]

The white pine weevil, *Pissodes strobi* (Peck) (Coleoptera: Curculionidae), can severely impact the regeneration of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), Engelmann spruce (*P. engelmanni* Parry), white spruce (*P. glauca* (Moench) Voss) (Pinaceae), and their hybrids in British Columbia (B.C.), Canada. In B.C., adult weevils overwinter in the forest duff, usually near the tree from which they emerged in the fall. Early in the spring, the weevils mate and lay eggs under the bark of the previous year's terminal leader and the larvae mine downward, consuming the phloem and killing the leader (Alfaro 1994). Subsequent terminal-leader growth

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Table 1. Polymerase chain reaction – restriction fragment length polymorphism patterns of *Lecanicillium muscarium, L. longisporum, L, attenuatum*, and *Lecanicillium* spp. isolates in this study.

		ITS region			β-tubulin gene				Mitochondrial DNA
Strain	Species	*Hae*III	*Hinf* I	*Msp*1	*Alu*I	*Cfo*I	*Hinf* I	*Hae*III	*Hae*III
IMI 246427*	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
CBS 317.70B	*L. longisporum*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	400,140	6.8, 4, 3.6, 3.5, 3, 2.6
CBS 170.76	*L. attenuatum*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360, 180	360,180	6.2, 5, 4.4, 3
PFC 17	*L. pissodis*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	240,180,140	360,180	6.5, 4.2, 3.8, 2.8, 2.3
PFC 18	*L. pissodis*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	240,180,140	360,180	6.5, 4.2, 3.8, 2.8, 2.3
PFC 19	*L. pissodis*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	240,180,140	360,180	6.5, 4.2, 3.8, 2.8, 2.3
PFC 3	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 4	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 5	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 6	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 9	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 10	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 11	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 12	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 14	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 15	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
PFC 16	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
AFS 2	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
AFS 3	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
AFS 4	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8
AFS 6	*L. muscarium*	300,160,90,70	300,140,100,90	290,140,110,60	450,90	280,200	360,180	360,180	6.1, 6, 4.2, 2.8

*From Zare and Gams (2001).

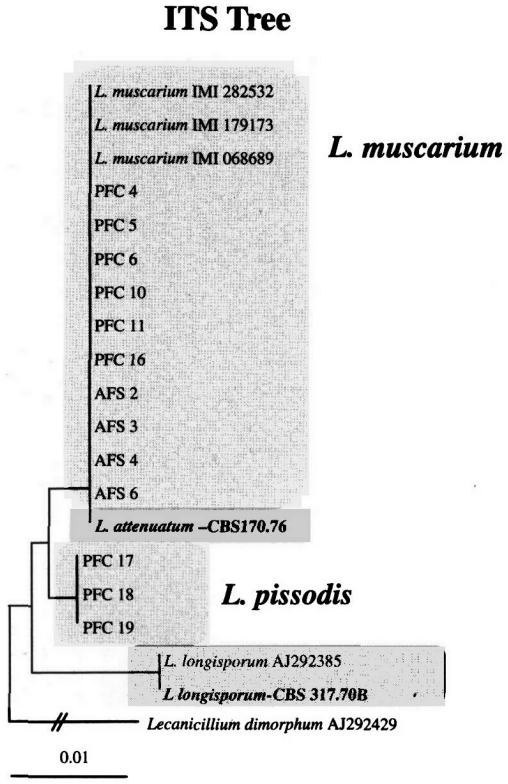
results in crooks and forks, which reduce wood quality. Depending on infestation levels, weevil attacks can reduce the yield of host trees by as much as 40% (Alfaro *et al.* 1995).

The newly described genus *Lecanicillium* Gams and Zare (formerly *Verticillium* Nees) (Deuteromycota: Hyphomycetes) includes entomogenous species that are pathogenic on a wide range of insect hosts and have a cosmopolitan distribution (Zare and Gams 2001). Alfaro *et al.* (1985) proposed several methods and control agents to regulate *P. strobi* populations, including an integrated pest management strategy using entomopathogenic fungi. Kope *et al.* (2000) isolated *Lecanicillium* (identified as *Verticillium lecanii* (Zimmerman) Viégas) from dead adult *P. strobi* and from soil, and fulfilled Koch's postulates demonstrating their entomopathogenic nature. In 2006, Kope *et al.* reported on the collection of additional isolates of *Lecanicillium* spp. from cadavers of *P. strobi* and again fulfilled Koch's postulates. The intent of their research was to identify isolates that could be used as biocontrol agents for *P. strobi*. The occurrence of these entomopathogenic fungi in the natural habitat of *P. strobi* and their demonstrated efficacy make them a promising candidate for development as a biocontrol agent for *P. strobi*. However, little is known about these *Lecanicillium* species, and to properly use them to control *P. strobi*, their exact identification must be confirmed.

The work of Kope *et al.* (2006) was based on 27 *Lecanicillium* spp. isolates from several sites in B.C. that were identified as either *L. longisporum*, *L. muscarium*, or simply *Lecanicillium* spp., using morphological traits. These authors used similarities and differences in conidial shape and size to identify the *Lecanicillium* spp. isolates. In taxonomy, however, the use of morphological traits alone is often not sufficient for separating cryptic species that are morphologically similar. Therefore, the aim of this study was to clarify the identity of these indigenous *Lecanicillium* spp. isolates using molecular tools such as polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing.

Fourteen isolates were collected from adult *P. strobi* at the Pacific Forestry Centre (PFC), Victoria, B.C., and 4 isolates from soil at the Applied Forest Science (AFS), Victoria, B.C. One isolate each of *L. attenuatum* (CBS 170.76), *L. muscarium* (IMI 246427), and *L. longisporum* (CBS 317.70B) were used as reference species for comparison

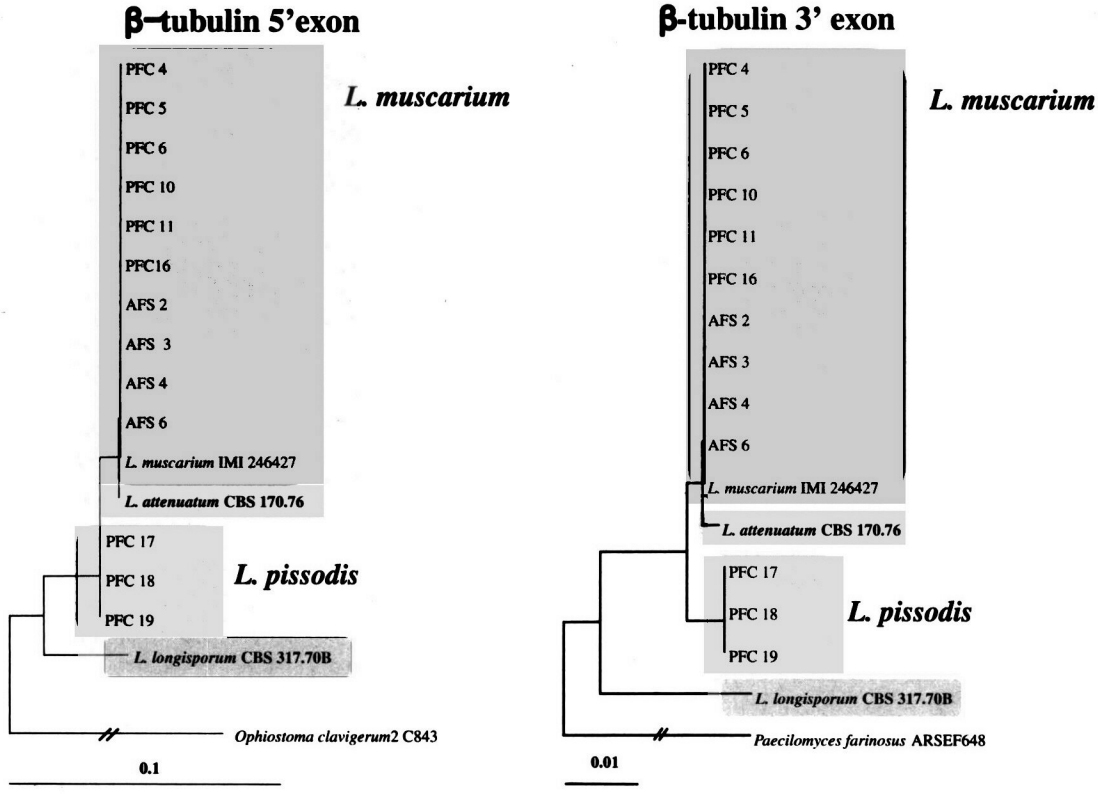
Fig. 1. Neighbour-joining tree for *Lecanicillium* ITS sequences rooted with *Lecanicillium dimorphum* as an outgroup species, showing the relationship between isolates PFC-4, -5, -6, -10, -11, and 16, AFS-2, -3, -4, and -6, and *L. muscarium*, and the position of this clade among *Lecanicillium* species.



with AFS and PFC isolates. To carry out PCR-RFLP and DNA sequencing, the ribosomal internal transcribed spacer (ITS) region, the 5′-exon and 3′-exon regions of the β-tubulin gene, and total mitochondrial DNA of PFC and AFS isolates were prepared as described by Kope and Leal (2005). The ITS region was amplified with the primer set ITS-4/ITS-5 (White *et al.* 1990) and the 5′-exon and 3′-exon regions of the β-tubulin gene were amplified using the primer sets Bt2E/Bt12 (Kim *et al.* 2003) and Bt1a/Bt1b (Glass and Donaldson 1995), respectively.

Zare and Gams (2001) carried out PCR-RFLP on the ITS region, the β-tubulin gene, and total mitochondrial DNA to identify *Lecanicillium* species. One disadvantage with PCR-RFLP assays compared with DNA sequencing is the possibility that not all of the polymorphisms of interest will affect a restriction site, and thus some may not be detected.

Fig. 2. Neighbour-joining tree for *Lecanicillium* β-tubulin sequences rooted with *Ophiostoma clavigerum* and *Paecilomyces farinosus*, showing the relationship between PFC-4, -5, -6, -10, -11, and -16, AFS-2, -3, -4, and -6, and *L. muscarium*, and the position of this clade among *Lecanicillium* species. *Ophiostoma clavigerum* and *P. farinosus* were chosen as outgroup species because they are closely related to *Lecanicillium* spp.



However, PCR-RFLP has the advantage of being more cost-effective than sequencing. When we carried out PCR-RFLP analysis, the patterns obtained for the ITS region, β-tubulin gene, and total mitochondrial DNA allowed the isolates to be divided into two groups. RLFP patterns for isolates PFC-3, -4, -5, -6, -9, -10, -11, -12, -14, -15, and -16 and isolates AFS-2, -3, -4, and -6 corresponded to *L. muscarium* (Table 1). Isolates PFC-17, -18, and -19 displayed distinct RFLP patterns for β-tubulin (*Hin*fI) and mtDNA (*Hae*III; Table 1) and have been reported as a new species, *L. pissodis* Kope and Leal (Kope and Leal 2005).

To confirm identification at the species level obtained by means of PCR-RFLP, we sequenced the ribosomal ITS region and the β-tubulin gene for a subset of the isolates (PFC-4, -5, -6, -10, -11, and -16 and AFS-2, -3, -4, and -6) and the reference species. The ITS region is highly conserved within species but generally exhibits greater variation among species. It can be very useful for determining relationships between fungal genera and species (Bruns *et al.* 1992), but it does not allow differentiation of closely related taxa (Hermosa *et al.* 2000; Harrington *et al.* 2001; Jacobs *et al.* 2001). Sequence information for conserved genes such as β-tubulin is frequently used for fungal diagnostics to complement ITS information (McKay *et al.* 1999; Fraaije *et al.* 2001; Kim *et al.* 2004). The β-tubulin gene can be amplified easily and is suitable for developing taxon-specific diagnostics.

The results of neighbour-joining analysis of the ITS sequence data were consistent with those obtained using PCR-RFLP, and placed isolates PFC-4, -5, -6, -10, -11, and -16 (accession Nos. EF679158, EF679159, EF679160, EF679161, EF679162, and EF679163, respectively) and AFS-2, -3, -4, and -6 (accession Nos. EF679154, EF679155, EF679156, and EF679157, respectively) in a branch with the reference isolate, *L. muscarium* (Fig. 1). Their

sequence similarity with *L. muscarium* was 100%. The sequence analysis of the partial 5′-exon and 3′-exon regions of the β-tubulin gene confirmed the identity of isolates PFC-4, -5, -6, -10, -11, and -16 (accession Nos. EF679170, EF679171, EF679172, EF679173, EF679174, and EF679175, respectively) and AFS-2, -3, -4, and 6 (accession Nos. EF679166, EF679167, EF679168, and EF679169, respectively) as *L. muscarium* (Fig. 2). The β-tubulin gene sequence of the reference cultures *L. attenuatum* (CBS 170.76), *L. longisporum* (CBS 317.70B), and *L. muscarium* (IMI 246427) were registered in the database of the National Center for Biotechnology Information (NCBI) as EF679176, EF679177, and EU000248, respectively. The ITS sequence for the reference cultures *L. attenuatum* (CBS 170.76) and *L. longisporum* (CBS 317.70B) were registered in the NCBI database as EF679164 and EF679165, respectively.

In conclusion, the PCR-RFLP results and sequencing of both the ITS region and the 5′-exon and 3′-exon regions of the β-tubulin gene of PFC and AFS isolates showed that the isolates examined in this study are *L. muscarium* rather than *L. longisporum*. Kope *et al.* (2006) had previously reported isolates PFC-3, -5, -10, and -16 and AFS-6 as *L. longisporum* and isolates PFC-6 and -12 as belonging to the genus *Lecanicillium*, but had not classified them at the species level. Their earlier observations of interspecific differences in pathogenicity must therefore be ascribed to differences among isolates of *L. muscarium*. The results of the current study demonstrate the utility of using molecular methods to identify *Lecanicillium* species. Molecular characterization based on PCR and DNA sequencing is now extensively used in the taxonomic classification of organisms, especially when identification based solely on morphology may be ambiguous. The identification of these entomopathogenic *Lecanicillium* species indigenous to B.C. is important for their potential commercialization as biocontrol agents in the future.

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