Whole Genome Sequencing Resource of the European Larch Canker Pathogen *Lachnellula willkommii* for Molecular Diagnostic Marker Development

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Abstract

The filamentous ascomycete fungus *Lachnellula willkommii* is the causal agent of European larch canker (ELC), one of the most destructive diseases of larch in Europe and a regulated plant pathogen of quarantine significance in Canada and the United States. *L. willkommii* was first detected in Massachusetts, North America in 1927 on a larch plantation cultivated with nursery stock imported from Great Britain. Despite the decades of practices aimed at eliminating the pathogen, it has reappeared in coastal areas of Canada and the United States. There is concern ELC could spread throughout the range of eastern larch, a transcontinental species typical of the Boreal forest that spans the North American landscape. There is geographic range overlap between several nonpathogenic indigenous *Lachnellula* species and the reported distribution of *L. willkommii* in North America. Morphological and biological methods to distinguish *L. willkommii* are often inadequate as the fungus does not always produce the phenotypic structures that distinguish it from these other saprophytic *Lachnellula* species. Whole genome sequencing technologies were used to obtain the draft genome sequences of *L. willkommii* and six other *Lachnellula* species. Molecular markers identified from the genomic data may be used to discriminate *L. willkommii* from its nonpathogenic relatives.

Lachnellula species are filamentous ascomycete fungi within the family Hyaloscyphaceae (Dharm 1965). Most *Lachnellula* species are saprophytic or weakly parasitic, but some cause cankers on conifers with a narrow host range (Hahn and Ayers 1943). Within the genus *Lachnellula*, *L. willkommii* is the most pathogenic species and causes European larch canker (ELC) of *Larix* and *Pseudolarix* species. Young trees infected with this pathogen may be killed or become girdled and permanent cankers on the trunks of older trees cause stem distortion and breakage, with significant impact on natural or planted stands of native larch and on plantations of exotic larch species (Lohwag 1963). *L. willkommii* appears to have spread from Asia to across Europe, infecting various species of larch and devastating larch plantations (Yde-Andersen 1979). *L. willkommii* was first reported in North America in 1927 in Massachusetts by members of the Harvard Forest School who identified the pathogen on Japanese and European larch nursery stock imported from Scotland in the early 1900s (Spaulding and Siggers 1927). Since its introduction to North America, *L. willkommii* has been a fungal disease of plant quarantine significance and a regulated pathogen in Canada and the United States (Canadian Food Inspection Agency 2018; USDA/APHIS 2012). Decades of sanitation efforts involving quarantine and the destruction of infected trees and branches were believed to have eradicated the disease from the area; however, the disease has since reappeared in coastal areas in Maine, and has been detected in Canada in the Maritimes on Prince Edward Island, Nova Scotia, and New Brunswick and remains under official control and quarantine in these provinces (Brandt 1997; Magasi and Pond 1982; Miller-Weeks and Stark 1983; Ostaff 1985; Simpson and

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Keywords

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Harrison 1993). While ELC caused by *L. willkommii* appears to be restricted to the mild-wintered Maritime regions, there is the potential for ELC to spread throughout the range of eastern larch, which is a transcontinental species typical of the Boreal forest that spans the North American landscape. Several saprophytic *Lachnellula* species native to North America share overlapping geographical distributions with *L. willkommii*. *L. willkommii* is almost indistinguishable morphologically from its nonpathogenic sister species, *L. occidentalis*, and the two are often found together on dead plant debris (Baral 1984; Hahn and Ayers 1934). Because samples sent to diagnostic labs for testing are often taken from dead tree material, culturing the fungi for morphological identification isn't possible. When live samples are available, the two fungi do not always produce the morphological structures that may distinguish them when cultured in the lab. Genomic data obtained from whole genome sequencing is a valuable resource for developing molecular diagnostic testing tools. Comparative genomic analysis of *L. willkommii* and its close relatives will allow researchers to develop molecular markers that can distinguish it from its nonpathogenic sister species. In this study, we performed whole genome sequencing, de novo assembly, and annotation of *L. willkommii* (CBS 172.35) and six other *Lachnellula* species: *L. arida* (CBS 203.66), *L. cervina* (CBS 625.97), *L. hyalina* (CBS 185.66), *L. occidentalis* (CBS 160.35), *L. subtilissima* (CBS 197.66), and *L. suecica* (CBS 268.59).

Materials and Methods

Agar-culture plugs preserved in glycerol were retrieved from liquid nitrogen storage and transferred as hyphal masses onto 2% potato dextrose agar (PDA) plates and incubated in the dark for 2 weeks at 20°C. Thereafter, agar plugs with hyphal tip were taken and transferred to fresh PDA plates overlain with single sheets of porous cellophane (ThermoFisher Scientific) and incubated two more weeks. Mat tissue was scraped off of the cellophane and rinsed with water to remove excess polysaccharides secreted by hyphae. DNA extraction was performed according to the CTAB method described by Möller et al. (1992), with the following modifications: mat tissue samples were flash-frozen in liquid nitrogen and put into 500 µl of TES buffer in tubes containing a garnet matrix and a single 1/4-in ceramic sphere (Lysing Matrix A, MP Biomedicals). Sample material was homogenized in a FastPrep-24 (MP Biomedicals) tissue and cell homogenizer for 45s with the speed set to 5.0. Lysates were treated with 4 µl of Proteinase K (~20 µg/µl) (Millipore-Sigma) and then clarified by centrifugation at 16,000 × *g* for 5 min at room temperature. Sample incubation time with chloroform/isoamyl alcohol (24:1) was increased to 4.5 h at room temperature, while subsequent centrifugation was performed at 18,000 × *g* for 5 min at room temperature. Chloroform extraction was performed twice on each sample due to excess cloudiness remaining in samples after the first extractions. Following chloroform extraction, samples were transferred to fresh 1.5-ml tubes and treated with 3 µl (10 µg/µl) of RNase-A (Millipore-Sigma) with a 30-min incubation at 37°C. Sample tubes were centrifuged at 18,000 × *g* for 1 min and the supernatant was transferred to a fresh 1.5-ml tube where treatment with 150 µl of ammonium acetate (3M) and 500 µl of ice-cold isopropanol for DNA precipitation were combined into a single step, with overnight incubation at -20°C. Sample tubes were centrifuged at 18,000 × *g* for 5 min at 4°C in a precooled centrifuge rotor and the supernatant was aspirated off the pellet, which was then washed with 300 µl of ice-cold 75% ethanol by dislodging the pellets from the sides of the tubes and vortexing the mixture for 1 min. Sample tubes were centrifuged at 14,000 × *g* for 15 min at 4°C and then allowed to air dry for 30 min at room temperature before being resuspended in 50 to 100 µl of TE buffer. DNA concentration was estimated by spectrophotometry using the NanoDrop ND-1000 (ThermoFisher Scientific) and the Qubit DNA BR assay kit (ThermoFisher Scientific) on a Qubit fluorometer (ThermoFisher Scientific).

For sequencing library preparation, DNA samples were sheared using the Covaris M220 ultrasonicator (Covaris, Inc.) to 400 bp. Sequencing libraries were prepared with the Ion Xpress gDNA Fragment Library Kit (ThermoFisher Scientific) on the Ion PGM System (ThermoFisher Scientific) as described by Tremblay et al. (2018). The libraries were size selected (300 to 450 bp) on the PippinPrep automated size selection system (Sage Science) and 2% DF Marker L cassette type (BDF2010). Libraries were prepared and quantified using the Ion Library TaqMan Quantitation Kit (ThermoFisher Scientific). Quality assessment of the unenriched template-positive Ion Spheres was performed on aliquots of bead-template preparations prior to the enrichment step using the Ion Sphere Quality Control Kit (ThermoFisher Scientific). Enriched template-positive sample libraries were diluted to 16 pM and loaded onto 318v2 chips for sequencing using the Ion Torren PGM sequencer (ThermoFisher Scientific).

Sequencing reads were checked for their quality and to provide graphical guidance for filtering, trimming and reformatting using the PREINSEQ-LITE v.0.20.4 and PRINSEQ-graphs v.0.6 software packages (Schmieder and Edwards 2011). Reads were trimmed using a sliding window approach (window size 7, shift 1 base). From the 3' end, read bases were kept until the average quality score Q of 7 bases was below Q = 10. Reads were additionally trimmed and genome assembly was performed using Newbler v.2.6 with default parameters (Margulies et al. 2005). De novo repetitive element libraries were created for each of the *Lachnellula* assemblies with RepeatModeler v.1.0.11 using the RMBlast-NCBI search engine (Smit and Hubley 2017). The derived repeat consensi for each species were combined and sequences classified as “unknown” repeats were queried for conserved domains against the NCBI nucleotide collection database (BLASTx) and RFAM (BLASTn) (Gardner et al. 2009; Madden 2013). Consensus sequences producing significant alignments (E-value $\leq 1e-5$) to predicted genes, gene families, or RNA features were removed. Three iterations of repeat masking were performed. In the first iteration, RepeatMasker (RepBase RepeatMasker Edition v.4.0.7) (Smit et al. 2016) was used to soft-mask the genomes using the RepBase and Dfam databases (releases 20170127) (Bao et al. 2015; Hubley et al. 2015). In the second iteration, the masked output fasta from the first iteration was soft-masked using the set of consensi sequences previously classified as “known” repeats. Finally, the masked fasta output from the second iteration were soft-masked using the set of repeat consensi previously classified as “unknown” repeats. Results from each round were analyzed together to produce a final repeat annotation and formatted to work with MAKER as described by Campbell et al. (2014).

MAKER v. 2.31.9 (Campbell et al. 2014) was used to annotate genes using protein-homology-based and *ab initio* predictions. A total of three iterations of MAKER were run. Initial predictions were generated from MAKER using, as input (i) *Botrytis cinerea* as the species model for Augustus v. 3.2.3 (Stanke and Waack 2003) gene prediction, (ii) the protein sets of the related species for protein-homology-based prediction (est2genome = 1, protein2genome = 1), and (iii) and the custom-created repeat library for repeat masking. The species and assemblies used for protein-homology-based prediction were: *Botrytis cinerea* (ASM83294v1), *Phialocephala scopiformis* (GCA_001500285.1), *Phialocephala subalpina* (GCA_900073065.1), *Rhynchosporium commune* (GCA_900074885.1), and *Sclerotinia borealis* (GCA_000503235)—retrieved from EnsembleFungi; *Marssonina brunnea* (GCA_000298775.1) and *Sclerotinia sclerotiorum* (GCA_000146945.2)—retrieved from NCBI; and *Glarea lozoyensis* (GCA_000409485.1)—retrieved from EMBL-EBI. Gene models from the first iteration of MAKER were used to train Augustus using the Benchmarking Universal Single-Copy Orthologs (BUSCO) v. 3 program (Simão et al. 2015) as well as to train the Semi-HMM-based Nucleic Acid Parser (SNAP) program (version 2006-07-28) (Korf 2004). For SNAP, confident gene models generated in the first iteration of MAKER (annotation edit distance [AED] = 0.25, length = 50 amino acids) were used to create a new parameter file to be used by SNAP with the fathom parameters, categorize and export, set to 1,000 and default settings for the forge and the hmm-assembler elements of SNAP.

Regions that contained mRNA annotations plus 1000 bp of flanking sequence were excised from the gene models generated from the first iteration of MAKER using BEDTools v. 2.27.1 (Quinlan and Hall 2010) and used as input for optimization of Augustus gene model parameters using the BUSCO v.3 pipeline (Waterhouse et al. 2017). BUSCO was used to optimize the HMM search model to retrain Augustus and produce a trained, species-specific HMM model for subsequent use with MAKER using the genome mode, long option, pezizomycotina_odb9 BUSCO dataset as lineage option and pretrained Augustus gene prediction parameters for *Botrytis cinerea*. In parallel, GeneMark-ES v. 3.54 (Ter-Hovhannisyan et al. 2008) was trained on the unmasked genome assemblies to produce *ab initio* gene models, using fungal settings, which were provided to MAKER for the second and third iterations.

Repeat annotations generated from the first MAKER iteration, as well as the optimized Augustus gene models, SNAP HMM file, and GeneMark-ES predictions were incorporated into a second iteration of MAKER, with the parameters est2genome and protein2genome set to 0 and tRNA predictions enabled (tRNAscan-SE v. 1.3.1) (Lowe and Eddy 1997). The results from the second MAKER iteration were then used to train Augustus and SNAP again, before running the final round of MAKER. The model_org, max_DNA_length, and min_contig options were adjusted from simple, 100,000 and 1,000 to default, 300,000 and 1, respectively, for the second and third MAKER iterations.

The annotation edit distance (AED) distribution of MAKER-generated gene models was used to assess the quality of the gene predictions. Genome annotation files were processed by

Table 1. *Lachnellula* species draft genome assembly and completeness summary statistics

Statistics	Genome assembly metrics						
	<i>L. arida</i>	<i>L. cervina</i>	<i>L. hyalina</i>	<i>L. occidentalis</i>	<i>L. subtilissima</i>	<i>L. suecica</i>	<i>L. willkommii</i>
Genome size (Mb) ^a	42.8	51.6	33.8	48.4	35.2	43.5	51.6
GC (%) ^a	46.2	42.9	46.5	42.1	46.6	48.0	40.4
Coverage ^a	27.2	20.1	32.7	27.3	24.0	22.0	11.4
Contigs ^b	3,229	8,607	585	3,682	2,040	4,381	13,593
N50 contig length (kb) ^b	38.1	23.2	161.8	31.3	34.1	19.2	8.7
Longest contig length (kb) ^b	187.6	135.2	531.4	252.5	189.3	115.9	80.4
Genome completeness metrics							
BUSCO (%)	92.7	90.8	94.5	89.8	85.0	82.1	80.0
Predicted gene models ^c	9,660	9,494	9,248	8,984	9,027	10,393	9,047
Mean gene length (bp) ^d	1,672	1,646	1,665	1,650	1,620	1,605	1,614
% Genome covered by genes	37.8	30.4	45.5	30.7	41.5	38.4	28.6
Predicted complete CDS ^e	8,702	8,367	8,530	7,983	7,834	8,458	6,973

^a Coverage statistic completed with read duplicates removed using Qualimap v.2.2.2-dev (Okonechnikov et al. 2015).

^b Assembly metric based on Newbler large contig consensus results.

^c Predicted gene models value includes predicted tRNA.

^d Mean gene length was calculated from CDS, excluding tRNA.

^e Predicted number of complete CDS, excluding predicted tRNA.

the Genome Annotation Generator (GAG) v. 2.0.1 (Geib et al. 2018) to remove short introns (<10 bp) and add missing start and stop features, while invalid gene features such as proteins with internal stops flagged by GAG were manually corrected. Error detection and correction of annotation files using GAG was performed iteratively until error-free. The GAG-generated protein files were used in subsequent functional annotation. Additional *ab initio* gene predictions overlapping MAKER annotations were scanned for protein domains using InterProScan v. 5.24-63.0 (Jones et al. 2014) with PFAM Models v. 31.0 and Lookup Service v. 5.24-63.0 while putative functions were assigned using BLASTP (E-value $\leq 1e-6$) (Altschul et al. 1990) to identify the best homologs from the UniProt/Swiss-Prot protein database v. 2/21/2018 (UniProt Consortium 2018). The program Annie v. 1.0 (Tate et al. 2014) was used to convert InterProScan and BLAST annotations into three column tables so that GAG could be used to update genome annotation files. Renaming and integration of annotation features in GFF, protein, BLAST and InterProScan output files was performed using scripts included in the MAKER package. GAG was used along with NCBI's command line tool tbl2asn (NCBI 2011) to create the GenBank submission files.

Completeness of the genome assemblies were assessed using BUSCO in combination with the pezizomycotina_odb9 dataset (created 2016-02-13) (Simão et al. 2015). Summaries of the assembly and annotation metrics for the species described in this announcement are provided in Table 1. An average of 9,407 gene models per species assembly were predicted. While the genome assembly metrics varied for each of the *Lachnellula* species sequenced, all genomes had BUSCO completeness predictions over 80%. The GC content of the genomes sequenced ranged from 41.4 to 48.6%, while the estimated genome sizes ranged from 33.8 to 51.4 Mb. The genome assemblies produced in this study expand the genomic data available for creating enhanced molecular diagnostic tools. This resource can facilitate future comparative genomics studies, identify species-specific gene targets for marker development and identification, and coupled with monitoring data can help provide a better understanding of the pathway(s) of introduction and spread of this pathogen in Canada.

Accession numbers. The Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession numbers QGMF000000000 (*L. arida*), QGMG000000000 (*L. cervina*), QGMH000000000 (*L. hyalina*), QGMI000000000 (*L. occidentalis*), QGMJ000000000 (*L. subtilissima*), QGMK000000000 (*L. suecica*), and QGML000000000 (*L. willkommii*).

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