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Neonectria bordenii sp. nov., a potential symbiote of the alder bark beetle, and its detection by quantitative PCR

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Abstract: A taxonomically comprehensive perspective on the fungal associates of bark beetles (*Coleoptera*: *Curculionidae*: *Scolytinae*), and powerful molecular tools for detection of these fungi, are imperative to understanding bark beetle impacts on forest ecosystems. The most common filamentous fungi living alongside bark beetles in infested trees are ophiostomatoids (*Ascomycota*: *Ophiostomatales* and *Microascales*), yet an undescribed species of *Neonectria* (*Neonectria sp. nov.*; *Ascomycota*: *Hypocreales*) was recently identified cohabitating with the alder bark beetle, *Alniphagus aspericollis*, in red alder, *Alnus rubra*. The hardwood-infesting alder bark beetle is found throughout the range of its red alder host in the Pacific Coast region of North America and is associated with *Neonectria sp. nov.* in southwestern British Columbia, Canada. The aim of this study was to describe and name *Neonectria sp. nov.* and to develop a quantitative PCR (qPCR) assay to enable rapid detection of *Neonectria sp. nov.* from individual adult alder bark beetles and to define the distribution of the fungus. *Neonectria sp. nov.* was phylogenetically and morphologically determined to represent a distinct species closely related to *N. ditissima* and is described herein as *Neonectria bordenii sp. nov. Neonectria bordenii* was reliably detected from individual whole-beetle DNA extractions using a probe-based qPCR assay targeting multi-copy internal transcribed spacers (ITS) of nuclear ribosomal DNA. The qPCR assay amplified the fungus from 87.8 % (36/41) of individual alder bark beetle samples and was highly sensitive to *N. bordenii*, with a lower limit of detection of 1×10^{-6} ng/µL of culture DNA (or ~262 genome copies). Application of the qPCR assay developed in this study will expedite future research evaluating *N. bordenii* as a potential symbiote of the alder bark beetle.

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INTRODUCTION

Bark beetles (*Coleoptera*: *Curculionidae*: *Scolytinae*) are recognised for their consistent associations with filamentous fungi, typically symbiotic ophiostomatoids (*Ascomycota*: *Ophiostomatales*) (Six 2012). Awareness of the diversity of bark beetle fungal symbiotes is increasing, supported by newly developed molecular detection methods and widespread availability of reference sequences (Miller *et al*. 2016, Hulcr *et al.* 2020, Rizzo *et al.* 2020, Araújo *et al*. 2021, Kolařík & Hulcr 2023, Pepori *et al*. 2023). Both conifer- and hardwood-infesting bark beetles are associated with a wide range of fungi, including *Ceratocystis* spp. (*Ascomycota*: *Microascales*) (Six 2012, Ploetz *et al*. 2013), *Geosmithia* spp. (*Ascomycota*: *Hypocreales*) (Kolařík *et al*. 2011, Kolařík & Hulcr 2023), and *Entomocorticium* spp. (*Basidiomycota*: *Russulales*) (Six 2020, Araújo *et al*. 2021) in addition to ophiostomatoids. For some beetle–fungus associations there is evidence of symbiosis between the organisms and for others the functionality of the relationship has yet to be tested (Ploetz *et al*. 2013, Six 2013, 2020, Six & Klepzig 2021). It is also likely that many fungal associates of understudied bark beetles are yet to be described (Hulcr *et al*. 2020, Araújo *et al*. 2021).

The alder bark beetle, *Alniphagus aspericollis*, attacks and colonises red alder, *Alnus rubra*, often killing apparently healthy trees as their broods feed and develop within the phloem layer (Chamberlin 1958, Borden 1969). Red alder is an ecologically important, nitrogen-fixing tree species that is distributed throughout the Pacific Coast of North America, from California to southern Alaska (Torrey 1978, Binkley 2003, Harrington 2006, Perakis *et al*. 2012, Perakis & Pett-Ridge 2019). As the primary host of the alder bark beetle, red alder is affected by the beetle across its range (Chamberlin 1958, Borden 1969, Wood 1982). A recent investigation of the fungal associates of the alder bark beetle found no evidence of a potential ophiostomatoid nutritional symbiote, but identified a consistent association with a hitherto unidentified species, *Neonectria sp. nov.* (Lee *et al.* 2023).

The genus *Neonectria* (*Ascomycota*: *Hypocreales*: *Nectriaceae*) includes several well-known phytopathogens, including apple canker and the causal agents of beech bark

disease (Castlebury *et al*. 2006, Chaverri *et al*. 2011), but no previously known associates of bark beetles. The objectives of this study were to (i) describe and name *Neonectria sp. nov.* upon isolation from alder bark beetles, and (ii) to design a quantitative PCR (qPCR) probe-based assay to facilitate reliable detection of *Neonectria sp. nov.* from individual alder bark beetle DNA extractions. A description of *Neonectria sp. nov.* will expand the known diversity of bark beetle-associated fungi to include a new hypocrealean species, and a direct detection method for *Neonectria sp. nov.* will enable follow-up research on the role of the fungus in the alder bark beetle system.

MATERIALS AND METHODS

Alder bark beetle collections

To obtain *Neonectria sp. nov.* cultures, adult alder bark beetles were collected using single fine-mesh emergence traps attached to one infested tree at each of two Lower Mainland sites, Pacific Spirit Regional Park (PSP) and Aldergrove Regional Park (ARP) and two southern Vancouver Island sites, Sooke Hills Wilderness Regional Park (VW, adjacent to Greater Victoria Water Supply Area) and Royal Roads University campus (RR), in British Columbia, Canada (location coordinates listed in the Taxonomy part part of the Results section). Beetles were collected from traps as they emerged from brood trees in summer 2019 (PSP, n = 4; ARP, n = 5; VW, n = 6; and RR, n = 6).

To acquire field materials for evaluation of the qPCR assay (pending development), individual adult alder bark beetles were hand-collected from surfaces where they had landed during spring 2019. Beetles were obtained from PSP, VW, and a third Lower Mainland site, Malcolm Knapp Research Forest (MK; location coordinates listed in the Taxonomy part of the Results section). Hand-collected beetles at PSP (n = 17) and MK (n = 16) were gathered from the bark surfaces of three trees per site and were not boring into the trees at the time of collection (ranging from 3–7 beetles per tree). At VW (n = 13), landed beetles were collected from the surface of a vehicle and swarming beetles gathered directly from the air using gloves that were bleach-cleaned between captures.For both hand and trap collections, individual beetles were transferred into sterile 1.5 mL microcentrifuge tube using 10 % bleach-cleaned forceps, transported to the laboratory, and either frozen at ~ -30 °C (for qPCR) or maintained in a refrigerator at ~4 °C (for culturing).

Alder bark beetle culturing and fungal isolate barcoding

Alder bark beetles that were collected via emergence trapping were used for culturing of *Neonectria sp. nov.* (n = 21). In addition, five hand-collected beetles from MK (obtained from the surface of the same tree; see 'Alder bark beetle collections' above) were also cultured such that each sampling site was represented in the collection of *Neonectria sp. nov.* isolates. All beetles were processed within 1 wk of collection. Beetles were transferred from their collection tubes into new, sterile 1.5 mL microcentrifuge tubes. Each tube received 50 µL of autoclaved distilled water and a drop of Tween® 20 (MP Biomedicals, Irvine, California, USA) detergent. Tubes were vortexed on high for 30 s to wash fungal inoculum from the beetles. The 50 µL beetle wash was then spread evenly across an antibiotic malt extract agar (MEA) plate and sealed with Parafilm. Antibiotic MEA plates were prepared using 200 µg/mL streptomycin (Streptomycin Sulphate, Fisher Scientific, Waltham, Massachusetts, USA) in 8.33 g MEA (MilliporeSigma, Burlington, Massachusetts, USA), 7.5 g agar (Fisher Scientific, Waltham, Massachusetts, USA), and 500 mL distilled water. Wash plates were monitored for fungal colonies and sub-cultured within three to ten days depending on rate of colony growth, aiming to isolate visually distinct colonies. Unique colonies were isolated onto antibiotic-free MEA plates and as needed, sub-cultured repeatedly onto MEA until only a single morphologically unique isolate was present on each plate.

Suspected *Neonectria sp. nov.* cultures (maximum of one per beetle), identified using the description by Lee *et al*. (2023), were plated onto MEA plates containing porous cellophane (MilliporeSigma, Burlington, Massachusetts, USA) for DNA extraction, and filter paper MEA plates (six autoclaved filter paper sections, per plate) for long-term storage. Once mycelial growth of sufficient volume for DNA extraction was observed for each isolate on its cellophane plate, mycelia were collected into sterile 1.5 mL Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes (Fisher Scientific, Waltham, Massachusetts, USA). Two sterile 3.2 mm stainless steel beating beads were added to each mycelia-containing tube and the samples frozen at ~ -30 °C until extraction. For long-term storage, cultures were allowed to grow until mycelia covered at least 80 % of the surface of the filter paper sections. Mycelia-covered filter paper sections were removed from each plate and placed into sterile Petri dishes for drying inside a sealed plastic container containing desiccant crystals. Once dry, each set of mycelia-covered filter paper sections was transferred to an autoclaved paper envelope, placed inside a clean zip-lock freezer bag, and stored at ~ -30 °C in the Genomics and Forest Health Laboratory at the University of British Columbia.

Suspected *Neonectria sp. nov.* cultures were processed for gDNA extraction, and subsequent PCR and sequencing of internal transcribed spacers (ITS) of nuclear ribosomal DNA for isolate identification. DNA from each tube containing frozen mycelia was extracted using the Qiagen DNeasy® Plant Mini Kit (Valencia, California, USA) according to manufacturer instructions. The ITS regions were amplified from each extracted sample using ITS1F (Gardes & Bruns 1993) and ITS4 primers (White *et al*. 1990) (Table S1). PCR was conducted using Invitrogen™ Platinum™ *Taq* DNA Polymerase (ThermoFisher Scientific, Waltham, Massachusetts, USA) in 26 µL reactions (including 3 µL of genomic DNA at a maximum concentration of 5 ng/µL) cycled at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 53.5 °C for 30 s, 72 °C for 1 min, and held for 10 min at 72 °C. Amplification products were visualised on a 1 % agarose gel using Safe-Green™ (Applied Biosystems, Waltham, Massachusetts, USA). Sanger sequencing was performed in both directions using PCR primers in the 3730xl DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) at the Centre de Recherche du CHUL (CHUQ), Université Laval (Quebec City, Quebec, Canada). Resultant forward and reverse sequences for each PCR product were pairwise aligned using MAFFT v. 7.490 alignment (Katoh *et al.* 2002, Katoh & Standley 2013), and the consensus sequence extracted and trimmed in Geneious Prime v. 2020.2.5 (https://www.geneious.com). Fungal isolates confirmed as *Neonectria sp. nov.* by homology of their ITS sequences (Lee *et al.* 2023) were set aside for subsequent species description and specimen examination.

Neonectria sp. nov. morphology and sequencing

Five isolates, one per site, were selected from a collection of 19 *Neonectria sp. nov.* cultures for species description. Cultures were maintained on Petri dishes containing 2 % malt extract agar (MEA; 20 g Bacto malt extract, Difco Laboratories, Sparks, Maryland, USA; 15 g agar, EMD Chemicals Inc., New Jersey, USA; 1 L distilled water) at 20 °C in the dark. To induce asexual and sexual reproductive structures, isolates were inoculated onto different media and substrates including: cornmeal agar (CMA; Difco Laboratories, Detroit, Michigan, USA), oatmeal agar (OA; extract of 30 g/L boiled oatmeal, 15 g agar, 1 L distilled water), potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan, USA), and water agar (WA; 15 g agar, 1 L tap water). Small (< 2 cm diam) branches and twigs of red alder were autoclaved (121 °C, 15 PSI for 30 min) in beakers containing distilled water and added to Petri dishes containing CMA or MEA, or placed in 500 mL flasks containing sterile perlite and 50 mL tap water and autoclaved for an additional 30 min; cultures and flasks with red alder branches and twigs were subsequently inoculated with mycelial plugs of individual isolates and incubated for 2 wk at 20 °C in the dark and then exposed to ambient light near a window for up to four months. Perithecia formed on inoculated branches and twigs but never fully matured to produce ascospores. To induce the development of fertile perithecia, mycelial blocks or conidia from different isolates were introduced into cultures and flasks containing red alder material, incubated at room temperature near a window, and checked weekly.

Micromorphological characters of colonies and asexual (sporodochia) and sexual (ascomata) morphs were visualised, described, and measured from living material mounted in tap water. Measurements are presented herein as ranges calculated from the mean (± SD) of each measured value with the minimum and maximum outliers in parentheses. Observations were made with a Leica DM4 B light microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and Leica M165 C stereomicroscope with a TL5000 Ergo light base (Leica Microsystems Ltd., Singapore). Images were captured from the light microscope using a Leica DFC450 C camera and from the stereomicroscope using a Leica DMC5400 camera, both using the Leica LAS X v. 3.7.1 software. The photographic plates were assembled using Adobe Photoshop CC 2019 v. 23.0.1 (Adobe Systems, San Jose, California, USA).

Isolates of *Neonectria sp. nov.* are maintained in J.B. Tanney's culture collection as mycelial plugs in cryovials containing sterile deionised water and stored at 4 °C and in cryovials containing 10 % glycerol v/v and sterile deionised water and stored at -80 °C. Select living cultures were deposited in the Canadian Collection of Fungal Cultures (Ottawa, Canada; DAOMC) and preserved specimens were deposited in the Department of Agriculture, Victoria, Forest Pathology (DAVFP) herbarium at the Pacific Forestry Centre (Canadian Forest Service, Natural Resources Canada, Victoria, British Columbia, Canada). Additional barcode sequences for the ex-type isolate (PSP-Trap1b-03-iso1) and a second isolate (MKHC19-T3-03-iso1) were obtained via PCR and Sanger sequencing of partial RNA polymerase II second largest subunit (*RPB2*), and partial translation elongation factor 1-alpha (*EF1-α*) gene regions (see Table S1 for primers). *RPB2* and *EF1-α* PCR reactions (16 µL, including 4 µL of 10 ng/µL DNA) were performed by Centre de Recherche du CHUL (CHUQ), Université Laval (Quebec City, Quebec, Canada) using Phusion™ Plus DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA) and cycled at 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 1 min (*RPB2*) or 15 s (*EF1-α*), followed by 5 min at 72°C. The *RPB2*, *EF1-α*, and ITS sequences for PSP-Trap1b-03-iso1 and MKHC19-T3-03-iso1 were deposited into the GenBank database.

Phylogenetic analysis

The three barcode sequences of the *Neonectria sp. nov.* isolates [PSP-Trap1b-03-iso1 (ex-type) and MKHC19-T3-03-iso1] were separately aligned with MAFFT v. 7.490 (Katoh *et al*. 2002, Katoh & Standley 2013) in Geneious Prime v. 2023.2.1 (https://www.geneious.com) to homologous GenBank sequences of *Neonectria* spp. (1–3 strains/isolates per species) that were selected to optimise taxon resolution (Lee *et al*. 2023), as well as two barcode sequences (ITS, *RPB2*) generated in this study for a strain of *N. major* [DAOMC 216524/ATCC 74260/PFC-082, see Table S1 for the corresponding primers applied to DNA from a culture provided to J.B. Tanney by the Canadian Collection of Fungal Cultures (DAOMC)]. *Corinectria fuckeliana* was included as the outgroup in the alignment (Salgado-Salazar *et al.* 2021). GenBank sequences for all three barcodes were obtained for each fungal strain/isolate used in the analysis, inclusive of sequences for available type and authentic strains (Table 1). Alignments were trimmed to consistent length and concatenated (ITS–*RPB2*–*EF1-α*) for phylogenetic analysis using Geneious Prime.

Maximum likelihood (ML) analysis was performed using IQ-TREE v. 2.2.2.6 (Minh *et al.* 2020) with the following parameter settings: edge-linked partition model (Chernomor et al. 2016); automated substitution model selection for each partition (evaluated by BIC in ModelFinder; Kalyaanamoorthy *et al.* 2017); FreeRate heterogeneity (Yang 1995, Soubrier *et al.* 2012) to account for variation in evolutionary rates for each region; 1 000 bootstrap alignments [ultrafast bootstrap (UFBoot); Minh *et al*. 2013, Hoang *et al.* 2018], Approximate Bayes test (aBayes; Anisimova *et al*. 2011), and 1 000 SH-like approximate likelihood ratio tests (SH-aLRT; Guindon *et al.* 2010) for branch support; IQ-TREE stopping rule = 500; and *C. fuckeliana* (CBS 239.29) selected as the outgroup. Bayesian inference (BI) analysis was also performed with the same partitioned dataset using MrBayes v. 3.2.7a (Ronquist *et al.* 2012) with the following parameter settings: two parallel runs of four chains each, run for 500 M generations but with the stop value set at 0.01, sample frequency every 100 generations, and *C. fuckeliana* (CBS 239.29) selected as the outgroup. The best-fit model for BI was selected based BIC score using jModelTest v. 2.1.7 (Darriba *et al.* 2012). The first 25 % of trees were discarded as burn-in and the remaining trees kept and combined into a 50 % majority rule consensus tree. The resulting trees were imported into FigTree v. 1.4.4 (Rambaut 2012) for visualisation and comparison and then formatted using Adobe Illustrator CC v. 23.0.1 (Adobe Inc., San Jose, California, USA). SH-aLRT (≥ 80 %), aBayes (≥ 0.95), and ultrafast bootstrap (≥ 95 %) support values were plotted at the nodes of the ML tree while Bayesian posterior probabilities (PP) were plotted at the nodes of the BI tree.

qPCR assay design

For development of a qPCR detection method for *Neonectria sp. nov.*, a qPCR assay set consisting of forward and reverse primers and a probe were designed using IDT PrimerQuest™

(Integrated DNA Technologies, Coralville, Iowa, USA) to target internal transcribed spacers (ITS) of nuclear ribosomal DNA, based upon known *Neonectria sp. nov.* sequences (Lee *et al*. 2023). The ITS regions were selected for assay design because they are multi-copy in fungal genomes and therefore ideal for optimising qPCR detection from environmental samples of low template quantity. Primers and probes were designed using the following optimal targets: primer Tm = 60 °C, GC = 50 %, and length = 20 nucleotides; probe Tm = 65 °C, GC = 50 %, and length = 20–27 nucleotides; amplicon length = 100 base pairs (80–150 bp). All primer and probe assay sets were screened *in silico* using Primer-BLAST (Ye *et al.* 2012) and BLASTn (Altschul *et al.* 1990) against non-target fungal genera present in the authors' samples and those found by Lee *et al*. (2023) and verified using IDT OligoAnalyzer™. The most *Neonectria*-specific assay was selected (Table 2; amplicon length = 124 bp) and primer oligos and probes (PrimeTime Eco Probe, 5' 6-FAM/ZEN/3' IBFQ) ordered from IDT (Coralville, Iowa, USA) and used according to manufacturer guidelines.

Assay specificity screening

The ITS assay was first screened for specificity to *Neonectria sp. nov.* using culture DNA from *Neonectria sp. nov.* (five isolates), *Ophiostoma* (two isolates), *Beauveria* (two isolates), *Acremonium* (one isolate), and *Fusarium* (one isolate), obtained from the alder bark beetle system (D.L.W. unpubl. data), as well as DNA from two additional *Fusarium* isolates from a separate study not associated with the alder bark beetle. These reactions were run on a calibrated StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA) using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts, USA) selected to manage known PCR inhibitors present in bark beetle DNA samples. TaqMan Environmental Master Mix 2.0 reactions were run using ROX as the passive reference dye according to manufacturer instructions: 10 min hold at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 20 µL reaction contained 2 µL of 5 ng/µL culture DNA and primers and probes at final concentrations of 500 nM and 250 nM, respectively. Duplicate reactions for each DNA sample and two negative (water) controls were run on each plate (MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL with MicroAmp Optical Adhesive Film covers, both from Applied Biosystems, Waltham, Massachusetts, USA).

Subsequent specificity screening of the ITS assay included *Neonectria sp. nov.* and randomly-chosen representatives (1–3) of other species or genera of all available non-target culture DNA (2 µL at 1 ng/µL per 20 µL reaction) from the authors' alder bark beetle-associated fungus collection (D.L.W. unpublished data and Lee *et al.* 2023) to evaluate assay specificity to *Neonectria sp. nov. Neonectria major* DNA extracted from a culture (DAOMC 216524/ATCC 74260/PFC-082) obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA; culture formerly available from Cedarlane, Burlington, Ontario, Canada), deposited by Dorworth (1995), was screened as an indicator of genus-level specificity. Undiluted DNA extracts from six spruce beetles, *Dendroctonus rufipennis* (collected from Prince George, British Columbia, Canada), were included as negative controls in the specificity assays. Reactions were run under the same conditions described above.

Twelve non-target culture DNA samples showed duplicate amplification before Ct 40, so these amplicons (1/2 per sample) were sequenced in both directions using the forward and reverse ITS qPCR primers by the Centre de Recherche du CHUL (CHUQ), Université Laval (Quebec City, Quebec, Canada), and MAFFT-aligned (Katoh *et al.* 2002, Katoh & Standley 2013) in Geneious Prime v. 2022.1.1. It was determined that amplification of non-target DNA was due to trace amounts of *Neonectria sp. nov.* contamination in the corresponding source cultures and in a single case due to mis-priming (of *Beauveria* culture DNA) irrelevant at environmental sample concentrations, confirming that the ITS qPCR probe-based assay was specific to *Neonectria sp. nov.* in context of the alder bark beetle system.

Assay efficiency and detection limits

ITS assay efficiency (standard curve) and limit of detection of *Neonectria sp. nov.* were determined by serial dilution qPCR of stock *Neonectria sp. nov.* culture DNA. *Neonectria sp. nov.* stock (5 ng/µL) was produced by combining four culture DNA samples at equal concentrations, diluted in Qiagen DNeasy Plant Mini Kit elution buffer (Valencia, California, USA), into a single volume that was aliquoted into separate tubes and stored at ~ -30 °C for dilution applications.

A standard curve was produced using duplicate reactions of 10-fold serial dilutions from the 5 ng/µL *Neonectria sp. nov.* culture DNA stock, starting at 1 ng/µL and ending at 1×10^{-10}. All duplicate reactions were within 0.5 Ct of one another (1 to 1×10^{-6} ng/µL), and loss of amplification was observed at 1×10^{-7} ng/µL. Based upon the observed point of amplification loss from the standard curve, a limit of detection test was conducted using 10-fold serial dilutions from 1 to 1×10^{-4} ng/µL in duplicate and ten 5-fold serial dilution replicates each at 1×10^{-5}, 2×10^{-6}, 1×10^{-6}, 2×10^{-7}, 1×10^{-7} ng/µL. Thresholds for all qPCR reactions were set such that the Ct for the 1 ng/µL dilution replicates averaged 16.00. ITS assay efficiency was determined from the standard curve to be 83.653 % ($R^2 = 0.999$; Fig. S1). 10/10 replicates at 1×10^{-6} ng/µL (mean ± SE = Ct 38.64 ± 0.22) and above amplified, while only 1/10 replicates at each 2×10^{-7} and 1×10^{-7} ng/µL amplified, indicating a clear detection limit of 1×10^{-6} ng/µL. The limit of quantification was also determined to be 1×10^{-6} ng/µL for this assay (3/10 replicates within 0.3 Ct of one another).

Detection of *Neonectria sp. nov.* from alder bark beetles by qPCR

To generate genomic DNA from field-collected samples for evaluation of the qPCR assay, DNA was extracted from individual alder bark beetles (see 'Alder bark beetle collections' in the Materials and Methods section) using the KingFisher™ Duo Prime and KingFisher Plastics for 96 deep-well format (both from Thermo Scientific, Waltham, Massachusetts, USA) with the MagMAX™ Microbiome Nucleic Acid Isolation Kit and MagMAX_Microbiome_Soil_Duo program (Applied Biosystems, Waltham, Massachusetts, USA). Extractions were performed according to manufacturer instructions, with the following exceptions: beetles were ground in their tubes inside a bead beater for 5 min at 30 Hz; 400 µL of lysis buffer was added to each sample instead of the 800 µL suggested in the MagMAX Microbiome protocol, and all the resultant supernatant was transferred to the sample plate. DNA was frozen at ~ -30 °C for qPCR application.

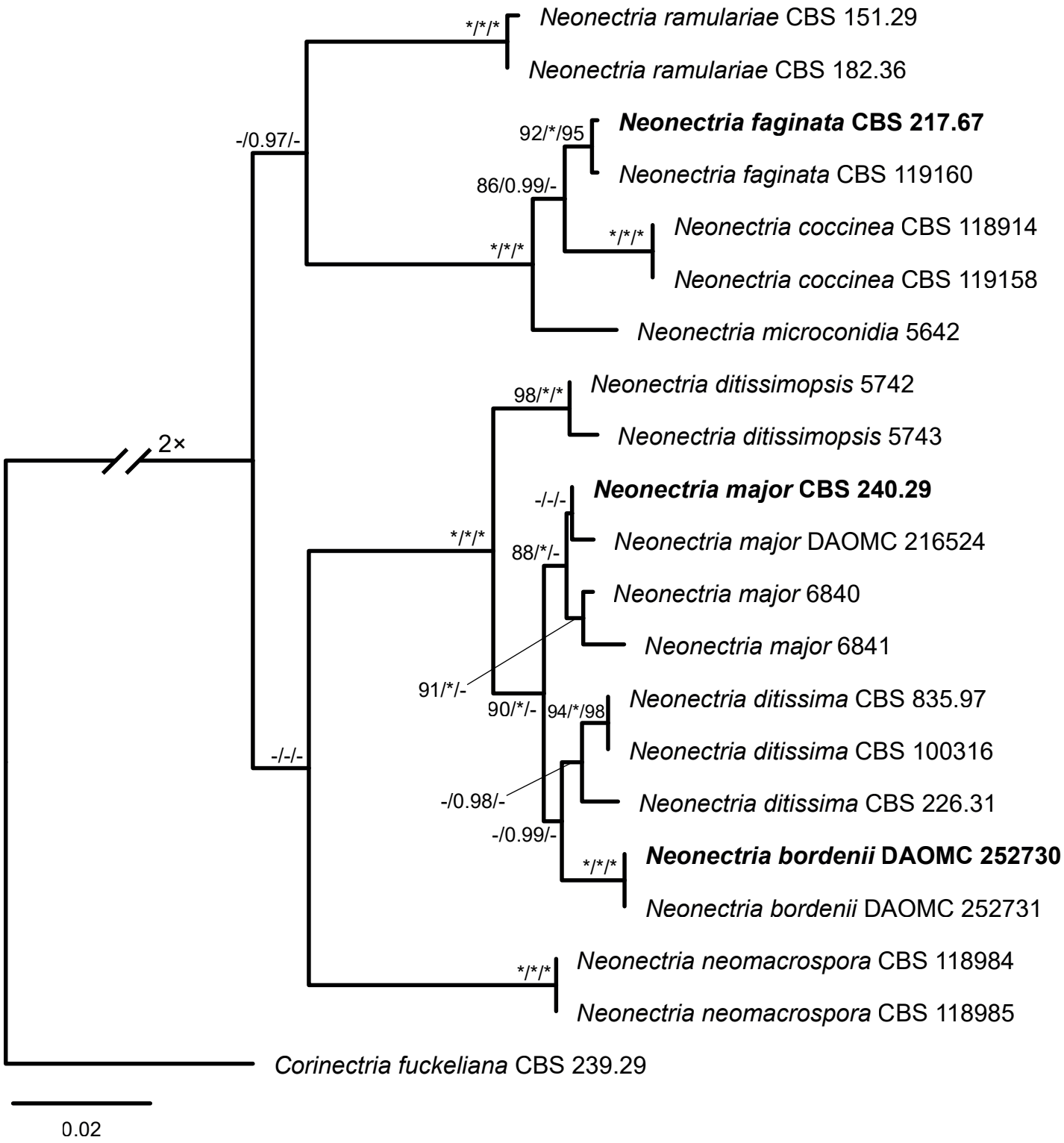

Fig. 1. Maximum likelihood phylogenetic tree of the concatenated partial ITS, *RPB2*, and *EF1-α* sequences of *Neonectria* (*Ascomycota*: *Hypocreales*: *Nectriaceae*) showing the relative position of *Neonectria bordenii sp. nov.* Strain/isolate identifiers are shown, with ex-type strains indicated in bold. Node labels indicate SH-aLRT support (≥ 80 %)/aBayes support (≥ 0.95)/ultrafast bootstrap support (≥ 95 %) with hyphen (-) indicating lack of statistical support and asterisk (*) indicating full support (100 % or 1.0). The tree is rooted to the outgroup *C. fuckeliana* (CBS 239.29; *Hypocreales*: *Nectriaceae*) and the scale bar shows the number of substitutions per site.

The *Neonectria sp. nov.* qPCR probe-based assay was applied to each alder bark beetle DNA extraction. Duplicate reactions for each sample and negative (water) controls were run on 96-well plates under the same conditions outlined above on undiluted DNA. A standard curve consisting of duplicate reactions ranging from 1 to 1 × 10^{-6} ng/µL was run alongside samples on each experimental plate. Data were threshold-standardised so that the Ct values of the 1 ng/µL standard curve culture DNA averaged 16.00. Samples were considered positive for *Neonectria sp. nov.* when Ct values of duplicate reactions were within 0.5 of one another and below 40. Samples with replicates below Ct 40 and ≥ 0.5 Ct apart, or with amplification of 1/2 replicates below Ct 40, were repeated a maximum of two additional times. If samples did not meet criteria for positive *Neonectria sp. nov.* assignment after three trials, they were considered negative for *Neonectria sp. nov.*

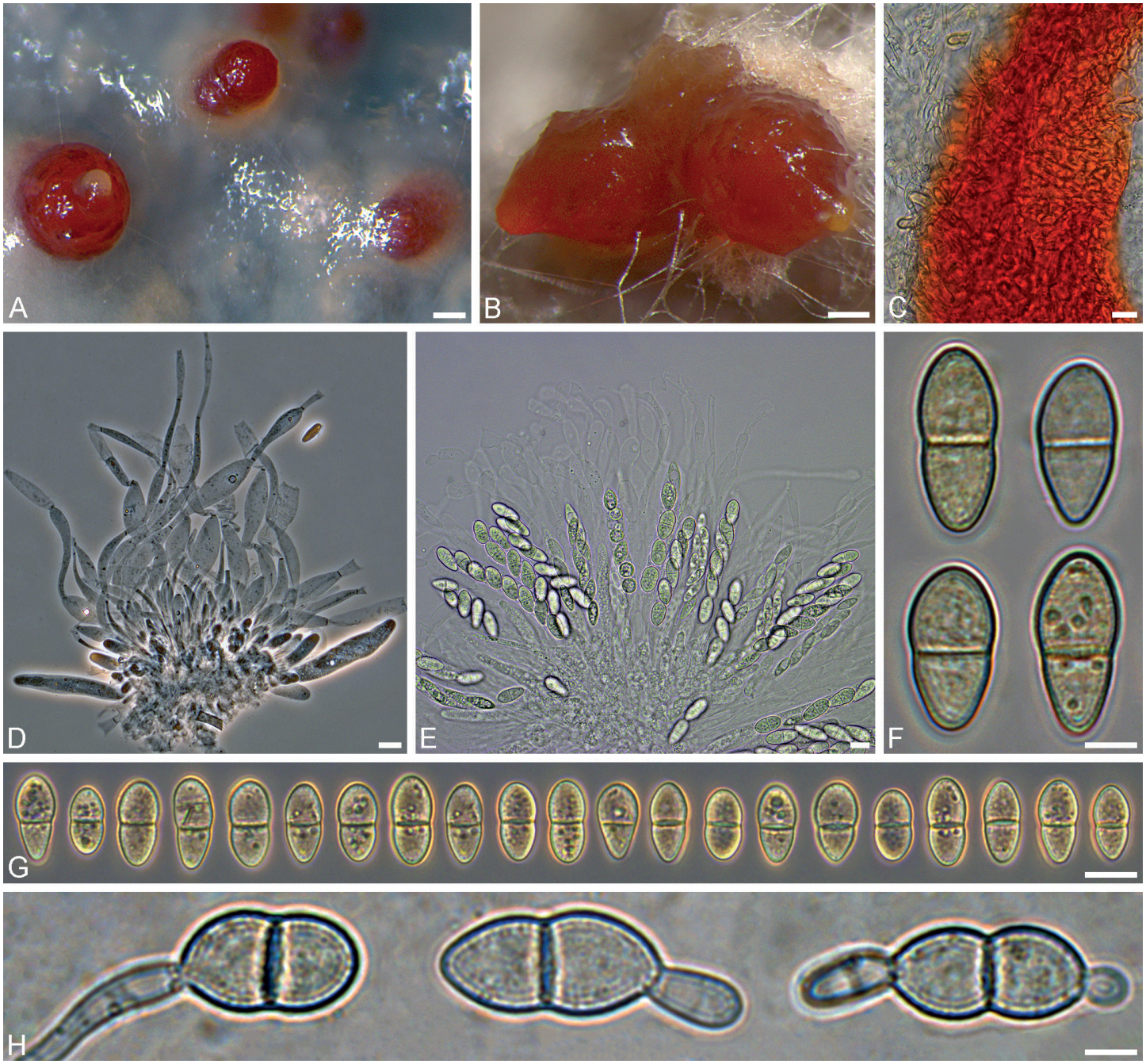

Fig. 2. Morphological characters from *Neonectria bordenii* perithecia formed on autoclaved red alder, *Alnus rubra*, twigs on cornmeal agar (CMA) resulting from addition of DAOMC 252730 conidia to a colony of DAOMC 252731. **A, B.** Immature and mature perithecia with ascospores oozing from ostioles. **C.** Cross section of perithecial wall. **D.** Developing asci and paraphyses. **E.** Mature asci and paraphyses. **F, G.** Ascospores. **H.** Germinating ascospores. Scale bars: A, B = 100 µm; C–E, G = 10 µm; F, H = 5 µm.

RESULTS

The combined alignment consisted of 10 *Nectriaceae* species (represented by 21 strains/isolates; see Table 1) having 1 268 characters including gaps (ITS: 446; *RPB2*: 547; *EF1-α*: 275 bp), of which 996 are constant sites, 227 are distinct patterns, and 184 are parsimony-informative. For the ML analysis, ModelFinder selected the following models for each data partition: Tne+I for ITS and *RPB2*, and TPM2+I for *EF1-α*; and for the BI analysis, jModelTest selected GTR+G+I. The ML and BI analyses reconstructed phylogenetic trees with virtually identical topologies (Figs 1, S2). *Neonectria* was resolved into two weakly-supported major clades consisting of (1) *N. coccinea*, *N. faginata*, *N. microconidia*, and *N. ramulariae*, and (2) *N. ditissima*, *N. ditissimopsis*, *N. major*, *N. neomacrospora*, and *Neonectria sp. nov.* (= *N. bordenii*, see below). The second clade consisted of a fully-supported [100 %/1/100 % (SH-aLRT/aBayes/ultrafast bootstrap)] subclade with *N. neomacrospora* placed basally with weak support (72 %/0.83/81 %). *Neonectria sp. nov.* was placed in a distinct branch sister to *N. ditissima* with varying support (78 %/0.99/66 %) (Fig. 1). Morphologically, *Neonectria sp. nov.* is comparable to *N. ditissima* by its yellow to pale orange colonies on MEA and PDA and asci and ascospores with similar dimensions. *Neonectria sp. nov.* differs from *N. ditissima* by its macroconidia, which are sometimes curved or weakly sigmoid and smaller, *e.g.*, 5-septate conidia: (44–)52–65(–75) × 5–6(–7) µm ($\bar{x}$ = 58.4 × 5.7 µm) *vs* (48.8–)58.7–74.9(–86.3) × (4.9–)6–7.8(–9.3) µm ($\bar{x}$ = 66.8 × 6.9 µm), 6-septate conidia:

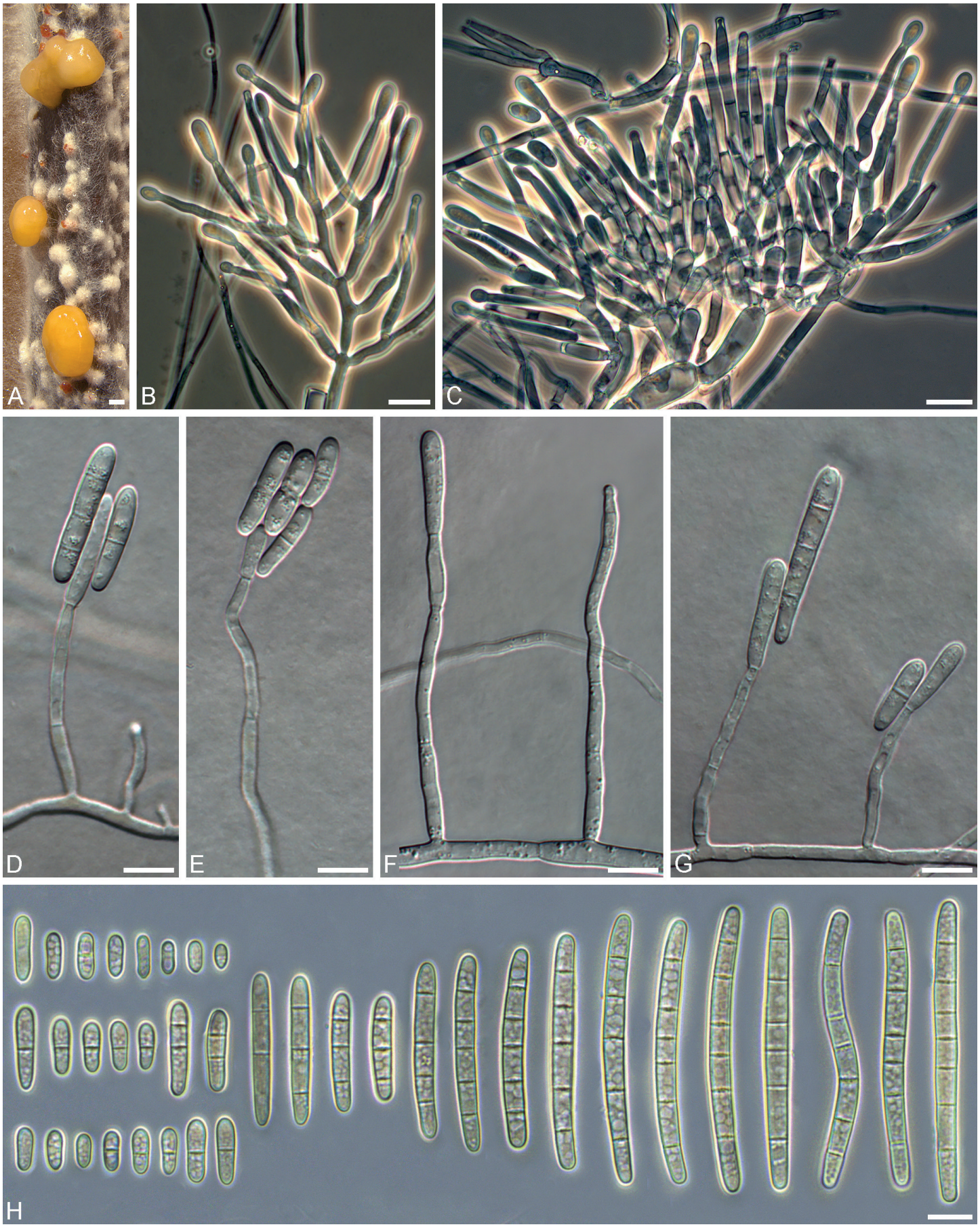

Fig. 3. Asexual morphology of *Neonectria bordenii* (DAOMC 252730). **A.** Sporodochia with yellow-orange slimy mass of conidia on an autoclaved red alder, *Alnus rubra*, twig placed on cornmeal agar (CMA). **B, C.** Penicillately branched conidiophores with cylindrical phialides and conidia. **D–G.** Micronematous conidiophores from surface of colony growing on CMA. **H.** Micro- and macroconidia. Scale bars: A = 500 µm; B–H = 10 µm.

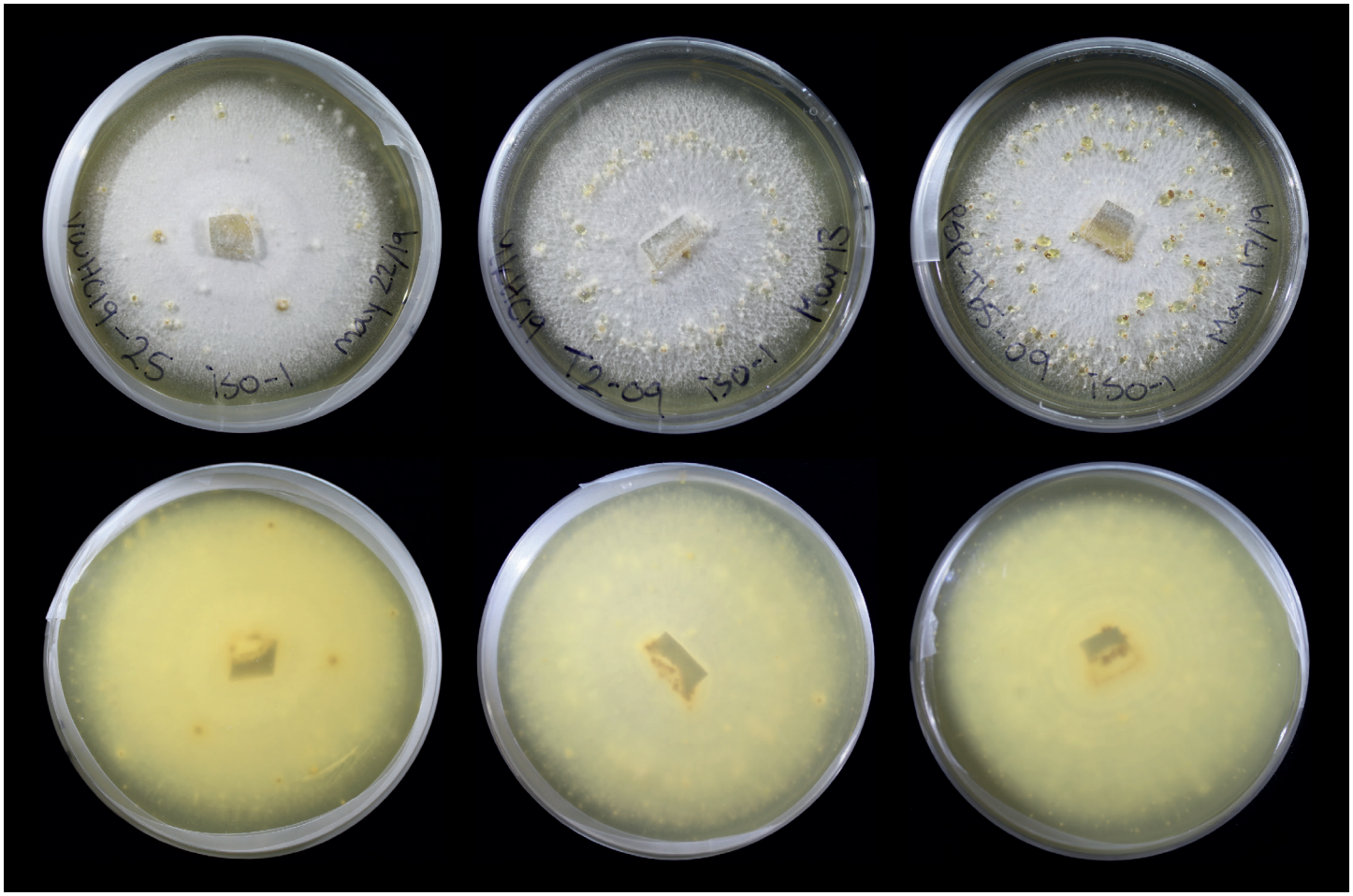

Fig. 4. Photographs depicting *Neonectria bordenii* cultures (on malt extract agar, MEA) obtained during this study; top row shows the colony surface, and the bottom row shows the corresponding colony reverse. *Neonectria bordenii* cultures were isolated from individual alder bark beetles, *Alniphagus aspericollis* at (left to right) the Sooke Hills Wilderness Regional Park (adjacent to Greater Victoria Water Supply Area) on southern Vancouver Island, and Malcolm Knapp Research Forest and Pacific Spirit Regional Park in the Lower Mainland of British Columbia, Canada. Each Petri dish is 10 cm in diameter.

(60.5–)64–73(–78.6) × (4.5–)5–6(–6.5) µm ($\bar{x}$ = 68.4 × 5.7 µm) *vs* (58.9–)67.7–84.1(–93.5) × (4.9–)6.2–8(–9.3) µm ($\bar{x}$ = 75.9 × 7.1 µm) (Castlebury *et al*. 2006). Based upon its phylogenetic position and morphological differences, *Neonectria sp. nov.* was determined to represent a separate species, as previously suggested by Lee *et al.* (2023), and is described below as *Neonectria bordenii sp. nov.*

Taxonomy

Neonectria bordenii J.B. Tanney, ***sp. nov.*** MycoBank MB 850108. Figs 2–4.

Etymology: Named in honour of Dr. John H. Borden for his exceptional contributions to forest entomology in British Columbia and foundational work on the alder bark beetle, *Alniphagus aspericollis* (Borden 1969, Wertman *et al*. 2022).

Diagnosis: *Neonectria bordenii* is most closely related to *N. ditissima*, *N. ditissimopsis*, and *N. major* (Figs 1, S2). *Neonectria bordenii* differs from *N. ditissima* by its shorter 5- and 6-septate conidia and larger perithecia, from *N. ditissimopsis* by its slightly smaller ascospores that are often constricted at the septum and not fusoid-ellipsoid, and from *N. major* by its larger perithecia and ascospores that are often constricted at the septum.

Typus: **Canada**, British Columbia, Vancouver, Pacific Spirit Regional Park (49.272117, –123.239275), 96 m a.s.l., isolated from the exoskeleton of a live adult alder bark beetle, *A. aspericollis*, collected from an emergence trap affixed to a host tree, *Alnus rubra*, 13 Jul. 2019, *A. Gonczar*, PSP-Trap1b-03-iso1 (**holotype** DAVFP 29789, culture ex-holotype DAOMC 252730). GenBank: OR435282 (ITS); OR441310 (*RPB2*); OR441309 (*EF1-α*).

Mycelium composed of branched and anastomosing, smooth-walled, septate, hyaline hyphae, 2–8 µm diam, cylindrical, some hyphae distally inflated and appearing racquet-like. *Ascomata* perithecial, solitary or in groups of up to six, developing apparently heterothallically (see Notes below) on sterile pieces of *A. rubra* wood, ovoid to obpyriform, slightly papillate ostiolar region, orange to red when fresh, darker red when dry, becoming reddish-purple in 3 % KOH, smooth to somewhat warted, 400–650 × 300–400 µm. *Ascomatal wall* 35–75 µm thick, comprised of two layers: outer layer 10–48 µm thick, cells 4–12 × 3–7 µm, *textura globulosa* to *angularis*, cell wall 1–2 µm thick, occasional clavate to obovoid cell protruding from surface, 6.5–16 × 4.5–6 µm; inner layer 8–26 µm thick, *textura prismatica*, 7–22 × 2.5–6 µm, cell wall 1–1.5 µm thick. *Paraphyses* extending beyond asci, hyaline, smooth, consisting of inflated cells (18–)22–43(–53) µm long, 2–4(–5.5) µm wide at narrowest point (at septum with neighboring cell), (3.5–)6.5–11(–13) µm at widest point, apical

Table 1. All strains/isolates and corresponding sequences used for phylogenetic analysis in this study, including metadata and GenBank accession numbers.

Species	Strain/isolate[1]	Host	Source	Location	GenBank accession number (ITS, *RPB2*, *EF1-α*)	References
Corinectria fuckeliana	CBS 239.29[2]	*Picea sitchensis*	Bark	Scotland	HQ840386, JF268707, DQ789728	Zhao *et al*. (2011b), Castlebury *et al*. (2006)
Neonectria bordenii	DAOMC 252730 (PSP-Trap1b-03-iso1)[3]	*Alnus rubra*	Emergent *Alniphagus aspericollis*	British Columbia, Canada	OR435282, OR441310, OR441309	This study
	DAOMC 252731 (MKHC19-T3-03-iso1)	*Alnus rubra*	Emergent *Alniphagus aspericollis*	British Columbia, Canada	OR435283, OR441312, OR441311	This study
Neonectria coccinea	CBS 118914	*Fagus sylvatica*	Unknown	France	KC660500, DQ789760, DQ789688	Hirooka *et al*. (2013), Castlebury *et al*. (2006)
	CBS 119158	*Fagus sylvatica*	Unknown	Germany	JF268759, DQ789818, JF268734	Zhao *et al*. (2011b), Castlebury *et al*. (2006)
Neonectria ditissima	CBS 226.31[4]	*Fagus sylvatica*	Unknown	Germany	JF735309, DQ789798, DQ789726	Cabral *et al*. (2012), Castlebury *et al*. (2006)
	CBS 835.97	*Salix cinerea*	Branch	Belgium	JF735310, DQ789808, DQ789737	Cabral *et al*. (2012), Castlebury *et al*. (2006)
	CBS 100316[4]	*Malus domestica* cv. Bramley	Unknown	Ireland	KM515890, DQ789787, DQ789715	Lombard *et al.* (2014), Castlebury *et al*. (2006)
Neonectria ditissimopsis	5742 (HMAS 98328)	Dicotyledon tree	Twigs	China	JF268762, JF268703, JF268743	Zhao *et al*. (2011b)
	5743 (HMAS 99206)	Unknown	Unknown	China	JF268763, JF268704, JF268744	Zhao *et al.* (2011b)
Neonectria faginata	CBS 217.67[3]	*Fagus grandifolia*	*Cryptococcus fagi* nymph	Canada	HQ840385, DQ789797, JF268746	Zhao *et al.* (2011b), Castlebury *et al*. (2006)
	CBS 119160	*Fagus grandifolia*	Unknown	USA	HQ840384, DQ789811, DQ789740	Zhao *et al*. (2011b), Castlebury *et al.* (2006)
Neonectria major	CBS 240.29[3]	*Alnus incana*	Canker	Norway	JF735308, DQ789800, DQ789729	Cabral *et al.* (2012), Castlebury *et al.* (2006)
	6840 (HMAS 183183)	Unknown	Unknown	China	JF268766, JF268712, JF268752	Zhao *et al.* (2011b)
	6841 (HMAS 183184)	Unknown	Unknown	China	JF268767, JF268713, JF268753	Zhao *et al.* (2011b)
	DAOMC 216524 (ATCC 74260/ PFC-082)	*Alnus rubra*	*Alnus rubra* tissue sections, or spores	British Columbia, Canada	OR673730, —, OR712762	This study[5]
Neonectria microconidia	5642 (HMAS 98295)	Unknown	Twigs	China	JF268761, JF268698, JF268737	Zhao *et al.* (2011b)

Table 1. (Continued).

Species	Strain/isolate[1]	Host	Source	Location	GenBank accession number (ITS, *RPB2*, *EF1-α*)	References
Neonectria neomacrospora	CBS 118984	*Arceuthobium tsugense*	Unknown	Canada	HQ840388, DQ789810, JF268754	Zhao *et al.* (2011b), Castlebury *et al.* (2006)
	CBS 118985	*Tsuga heterophylla*	Unknown	Canada	HQ840389, DQ789816, JF268755	Zhao *et al.* (2011b), Castlebury *et al.* (2006)
Neonectria ramulariae	CBS 151.29[4]	*Malus sylvestris*	Fruit	England	AY677291, DQ789792, HM054091	Halleen *et al.* (2004), Castlebury *et al.* (2006), Zhao *et al.* (2011a)
	CBS 182.36	*Malus sylvestris*	Fruit	Unknown	HM054157, DQ789793, HM054092	Zhao *et al.* (2011a), Castlebury *et al.* (2006)

[1] Where there is more than one identifier per strain/isolate, the identifier included in the phylogenetic trees (Figs 1, S2) is shown outside parentheses.

[2] Outgroup, authentic strain.

[3] Ex-type strain.

[4] Authentic strain.

[5] DAOMC 216524/ATCC 74260/PFC-082 from Dorworth (1995), Dorworth *et al.* (1996).

cells sometimes cylindrical. *Asci* narrowly clavate to cylindrical, stipitate, apex subtruncate, eight-spored, uniseriate at base to obliquely biseriate towards apex, without an apical ring, (81–)96–125(–130) × (9–)10–12(–13) µm (n = 17, x̄ = 110.6 × 11 µm, SD = 14.1 × 1 µm). *Ascospores* hyaline, pale brown with age, smooth, 1-septate, broadly ellipsoidal to obovoid, tapering slightly towards one or both ends, constricted at septum, (14–)16–19(–22) × (5.5–)6.5–8(–8.5) µm (n = 85, x̄ = 17.2 × 7.1 µm, SD = 1.5 × 0.6 µm), germ tube forming from one or both cells. *Conidiophores* micronematous to macronematous, simple or complex, branching verticillately or penicillately, 1–3 times branched, aggregated into sporodochia, producing 0–7-septate conidia, microconidia presumably produced from simple, unbranched conidiophores but these were not observed; *Conidiogenous cells* phialidic, hyaline, smooth, cylindrical, slightly tapering towards apices, straight or occasionally bent to faintly sinuous, more or less conspicuous periclinal thickening with minute collarette, (13.5–)16–23(–30.5) × (2–)2.5–3(–3.5) µm (n = 78, x̄ = 19.7 × 2.7 µm, SD = 3.3 × 0.3 µm). *Macroconidia* formed in yellow to orange slimy masses, hyaline, smooth, 0–7-septate, predominately 5–6-septate, ellipsoidal to subcylindrical to cylindrical, straight or occasionally curved or weakly sigmoid, lacking prominent scar or hilum; 0-septate conidia: ellipsoidal to obovoid-cylindrical to subcylindrical, slightly tapering towards base, ends rounded, (4.5–)7–10(–12.5) × (3–)3.5–4(–4.5) µm (n = 235, x̄ = 8.4 × 3.7 µm, SD = 1.3 × 0.2 µm); 1-septate conidia: ellipsoidal to obovoid-cylindrical to subcylindrical, slightly tapering towards base, ends rounded, (9–)11.5–18.5(–25) × (3–)4–4.5(–5) µm (n = 168, x̄ = 14.8 × 4.1 µm, SD = 3.6 × 0.3 µm); 2-septate conidia: subcylindrical, ends rounded, (19–)20.5–31(–38.5) × (4–)4.5–5 µm (n = 18, x̄ = 25.9 × 4.6 µm, SD = 5.3 × 0.3 µm); 3-septate conidia: subcylindrical, ends rounded, (23–)28–39(–45) × (4–)4.5–5 µm (n = 59, x̄ = 33.4 × 4.8 µm, SD = 5.5 × 0.3 µm); 4-septate conidia: subcylindrical, occasionally bent, ends rounded, (35.5–)39.5–50(–54) × (4–)4.5–5.5(–6) µm (n = 36, x̄ = 44.7 × 4.8 µm, SD = 5.3 × 0.4 µm); 5-septate conidia: subcylindrical, occasionally bent to weakly sigmoid, ends rounded, (44–)52–65(–75) × 5–6(–7) µm (n = 198, x̄ = 58.4 × 5.7 µm, SD = 6.6 × 0.5 µm); 6-septate conidia: subcylindrical, occasionally bent to weakly sigmoid, ends rounded, (60.5–)64–73(–78.6) × (4.5–)5–6(–6.5) µm (n = 71, x̄ = 68.4 × 5.7 µm, SD = 4.7 × 0.5 µm); 7-septate conidia: subcylindrical, occasionally bent to weakly sigmoid, ends rounded, (67.5–)71–80.5(–82) × 5–6(–6.5) µm (n = 15, x̄ = 75.8 × 5.5 µm, SD = 4.7 × 0.4). *Microconidia* hyaline, smooth, 0–1-septate, ellipsoid to obovoid, oblong, and subcylindrical, with or without a conspicuous hilum, 3–4.5(–5.5) × 2–3(–3.5) (n = 120, x̄ = 3.9 × 2.3 µm, SD = 0.7 × 0.4 µm). *Chlamydospores* not observed.

Culture characteristics: Colonies on MEA at 20 °C in the dark reach 30–35 mm diam after 7 d, 66–71 mm diam after 14 d. Colony surface white to honey, luteus, or saffron, with hyaline to yellow exudate droplets, reverse same as surface but more intense honey or saffron, margin even, aerial mycelium sparse to moderate, irregularly tufted, no soluble pigments.

Ecology/Substrate/Host: *Neonectria bordenii* is found living in association with the alder bark beetle, *A. aspericollis,* in the phloem of red alder, *A. rubra*, host trees (Lee *et al.* 2023). *Alniphagus aspericollis* may vector *N. bordenii* to red alders.

Table 2. qPCR assays designed to target *Neonectria sp. nov.* internal transcribed spacers (ITS) of nuclear ribosomal DNA. Primer and probe sequences were designed from reference sequences using IDT PrimerQuest™ and verified using IDT OligoAnalyzer™ (Integrated DNA Technologies, Coralville, Iowa, USA). Primer-BLAST (Ye *et al.* 2012) and BLASTn (Altschul *et al*. 1990) were used to preliminarily assess primer and probe specificity compared to available non-target sequences in GenBank. Forward and reverse primer oligos and the probe (PrimeTime Eco Probe, 5' 6-FAM/ZEN/3' IBFQ) were obtained from IDT (Integrated DNA Technologies, Coralville, Iowa, USA).

Target	Assay component	Length (bp)	Tm (°C)	Sequence (5' to 3')
ITS	Forward primer	20	52.6	CCGAGTTTACAACTCCCAAA
ITS	Probe	23	58.3	56-FAM/CTGTGAACA/ZEN/TACCCATCGTTGCC/3IABkFQ
ITS	Reverse primer	21	52.3	ATCGTGTTACTCAGAAGATGC

Distribution: *Neonectria bordenii* is known to occur in coastal British Columbia (Lower Mainland and southern Vancouver Island) within the Pacific Maritime Ecozone (Lee *et al.* 2023).

Additional specimens examined: **Canada**, British Columbia, Maple Ridge, Malcolm Knapp Research Forest (49.274747, –122.582756), 156 m a.s.l., isolated from the exoskeleton of a live adult alder bark beetle, *A. aspericollis*, collected from the bark surface of a host tree, *A. rubra*, 1 May 2019, *C.M.R. Taylor* (MKHC19-T3-03-iso1, DAVFP 29790, DAOMC 252731), GenBank: OR435283 (ITS); OR441312 (*RPB2*); OR441311 (*EF1-α*); Aldergrove, Aldergrove Regional Park (49.009931, –122.465486), 56 m a.s.l., isolated from the exoskeleton of a live adult alder bark beetle, *A. aspericollis*, collected from an emergence trap affixed to a host tree, *A. rubra*, 15 Jul. 2019, *A. Gonczar* & *E.M.C. Stewart* (ARP-Trap3c-01-iso1, DAVFP 29791, DAOMC 252732); Juan de Fuca Electoral Area, Sooke Hills Wilderness Regional Park (48.435844, –123.601267), 232 m a.s.l., isolated from the exoskeleton of a live adult alder bark beetle, *A. aspericollis*, collected from an emergence trap affixed to a host tree, *A. rubra*, 20 Jul. 2019, *D.L. Wertman* & *C.M.R. Taylor* (VW-Trap10a-05-iso1); Colwood, Royal Roads University Campus (48.427347, –123.478125), 13 m a.s.l., isolated from the exoskeleton of a live adult alder bark beetle, *A. aspericollis,* collected from an emergence trap affixed to a host tree, *A. rubra*, 15 Jul. 2019, *D.L. Wertman* (RR-Trap8a-01-iso1, DAVFP 29792, DAOMC 252729; Victoria, Pacific Forest Centre (48.459827, –123.397277), 30 m a.s.l., autoclaved *A. rubra* branches inoculated with MKHC-T3-03-iso1 (DAOMC 252731) and 6 wk later inoculated with conidia from PSP-Trap1b-3-iso1 (DAOMC 252730) to induce the development of perithecia, 19 Aug. 2023, *J.B. Tanney* (DAVFP 29793).

Notes: The description of *N. bordenii* is warranted primarily by its phylogenetic distinction from other known species (Figs 1, S2), as initially suggested by Lee *et al.* (2023). Phylogenetic analyses using a concatenated alignment of ITS, *RPB2*, and *EF1-α* sequences placed *N. bordenii* (= *Neonectria sp. nov.* in Lee *et al.* 2023) within a clade consisting of *N. ditissima*, *N. ditissimopsis*, *N. major*, and *N. neomacrospora*. Several of these species are known pathogens of trees, for example *N. ditissima* on *Malus* and *Fagus*, *N. major* on *Alnus*, and *N. neomacrospora* on *Abies* (Castlebury *et al*. 2006). While *N. major* is presumably sympatric with *N. bordenii* on *A. rubra* in the Pacific Coast region of North America, it was never isolated from alder bark beetles in this study, in follow-up work (D.L.W. unpubl. data), nor from beetles and beetle-infested phloem in a study by Lee *et al*. (2023). A strain of *N. major* (DAOMC 216524/ATCC 74260/PFC-082) was patented (as *Nectria ditissima*) as a mycoherbicide for the biological control of *A. rubra* (Dorworth 1995, Dorworth *et al.* 1996), GenBank: OR673730 (ITS); OR712762 (*EF1-α*); OR712763 (beta-tubulin, *TUB2*). Given the close relatedness of *N. major* and *N. bordenii*, the pathogenicity of *N. bordenii* in *A. rubra* should be investigated according to Koch's postulates (Koch 1891). Some *N. bordenii* isolates inoculated onto red alder branches and twigs formed perithecia that did not mature until conidia from a different isolate were introduced, suggesting that *N. bordenii* is heterothallic.

qPCR assay specificity and sensitivity

The qPCR assay enabled reliable detection of *N. bordenii* from culture and alder bark beetle DNA. The assay was specific to *N. bordenii* in context of the alder bark beetle system [as *N. bordenii* is the only *Neonectria* species that has been isolated from beetles and beetle-infested phloem (Lee *et al*. 2023, D.L.W. unpubl. data)], yet it is likely to detect closely related *Neonectria* species such as *N. major* (DAOMC 216524/ATCC 74260/PFC-082), for which amplification was observed in this study.. The lower limit of detection for *N. bordenii* using the qPCR assay was 1×10^{-6} ng/µL of culture DNA (mean ± SE = Ct 38.64 ± 0.71), which equates to ~262 genome copies per sample as calculated using the total number of ITS target copies (~1.49×10^4) (equation from University of Rhode Island Genomics & Sequencing Center 2004, https://cels.uri.edu/gsc/cndna.html) extrapolated to the mean number of nuclear ribosomal DNA copies found per nucleus in the *Ascomycota* (57, Lofgren *et al.* 2019). The *N. bordenii* qPCR assay amplified the fungus from 87.8 % (36/41) of hand-collected alder bark beetle samples: PSP: 100 % (17/17); MK: 90.9 % (10/11); and VW: 69.2 % (9/13). Mean Ct values from duplicate qPCR reactions of *N. bordenii*-positive alder bark beetle samples ranged from 29.58 to 38.12 (mean ± SE = 33.02 Ct ± 0.38). Raw qPCR data are provided for all evaluated beetles in Table S2. No amplification was observed in the *D. rufipennis* negative control samples.

DISCUSSION

Neonectria bordenii sp. nov. is described herein as a new species found living in association with alder bark beetles throughout southwestern British Columbia, Canada. Sexual and asexual morphologies of *N. bordenii* are consistent with the phylogenetic placement of *N. bordenii* (= *Neonectria sp. nov.*) relative to other *Neonectria* spp. in this study and as suggested by Lee *et al*. (2023), indicating that *N. bordenii* is found within the *N. ditissima* complex. *Neonectria bordenii* marks the second identified *Alnus*-specific *Neonectria* species after *N. major*, which was formerly thought to be the only *Neonectria* species in *Alnus* (Castlebury *et al.* 2006). That *N. major* was never isolated from the alder bark beetle system (Lee *et al.* 2023, D.L.W. unpubl. data), despite its known presence on *A. rubra*, should be subject to future investigation.

A custom-designed qPCR assay reliably detected *N. bordenii* in DNA extracted from individual alder bark beetles and excluded all other fungi known to be present in the alder bark beetle system. qPCR assays amplifying *N. neomacrospora* (Nielsen *et al.* 2019) and *N. ditissima* (Ghasemkhani *et al.* 2016) from infected wood exist, but have not been applied in context of association with an insect. Direct molecular detection methods including qPCR for bark beetle-associated fungi enable efficient screening of beetles and beetle-infested tree tissues, functioning as useful methodological counterparts to culturing of viable fungi from beetle exoskeletons, mycetangia, or infested wood (*e.g*., Schweigkofler *et al*. 2005, Khadempour *et al*. 2012, Lamarche *et al.* 2014, Miller *et al*. 2016, Rizzo *et al*. 2020, Pepori *et al*. 2023). Application of the *N. bordenii* assay to DNA extracted from whole alder bark beetles may allow access to structures that may harbour inoculum but not be accessible to culturing methods (Six 2003, Miller *et al*. 2016, Cruz *et al*. 2021). Future use of the assay for detection of *N. bordenii* throughout the range of the alder bark beetle should be complimented by culturing methods to account for the possible presence of *N. major* in the system, as this species is also amplified by the assay. The *N. bordenii* assay will permit forthcoming assessments of the newly described fungus as a potential symbiote of the alder bark beetle. Best practice next steps for determining whether the alder bark beetle–*N. bordenii* association is symbiotic should involve critical assessment of the system according to Leach's postulates for herbivorous insect vectors of plant pathogens (Leach 1940) — specifically, whether the alder bark beetle vectors *N. bordenii* to healthy red alder hosts.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Fig. S1. Standard curve for a qPCR probe-based assay targeting internal transcribed spacers (ITS) of nuclear ribosomal DNA of *Neonectria sp. nov.* (efficiency = 83.653 %, R^2 = 0.999). The curve was generated using 10-fold serial dilutions from 5 ng/µL *Neonectria sp. nov.* culture DNA stock, starting at 1 ng/µL and ending at 1×10^{-10}, in duplicate reactions [60 °C annealing temperature, using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, Massachusetts, USA)].

Fig. S2. Bayesian inference phylogenetic tree of the concatenated partial ITS, *RPB2*, and *EF1-α* sequences of *Neonectria* (*Ascomycota*: *Hypocreales*: *Nectriaceae*) showing the relative position of *Neonectria bordenii sp. nov.* Strain/isolate identifiers are included, with ex-type strains shown in bold. Node labels indicate Bayesian posterior probabilities. The tree is rooted to the outgroup *C. fuckeliana* (CBS 239.29; *Hypocreales*: *Nectriaceae*) and the scale bar indicates the number of substitutions per site.

Table S1. Summary of forward (Fwd) and reverse (Rev) PCR primers used to amplify internal transcribed spacers (ITS) of nuclear ribosomal DNA, the partial RNA polymerase II second largest subunit (*RPB2*) gene region, and partial translation elongation factor 1-alpha (*EF1-α*) gene region from *Neonectria sp. nov.* [the same primers used by Lee *et al.* (2023) for *Neonectria sp. nov.*] and ITS, *EF1-α*, and beta-tubulin (*TUB2*) from *Neonectria major* (DAOMC 216524/ ATCC 74260/PFC-082; Dorworth 1995, Dorworth *et al.* 1996).

Table S2. Raw qPCR data for individual hand-collected alder bark beetles, *Alniphagus aspericollis*, evaluated for the presence/absence of *Neonectria bordenii*.