Metabolomic-guided discovery of cyclic nonribosomal peptides from *Xylaria ellisii* sp. nov., a leaf and stem endophyte of *Vaccinium angustifolium*

Ashraf Ibrahim\(^1,7\), Joey B. Tanney\(^2,3,4\), Fan Fei\(^1\), Keith A. Seifert\(^4\), G. Christopher Cutler\(^5\), Alfredo Capretta\(^1\), J. David Miller\(^2\) & Mark W. Sumarah\(^2,6*\)

Fungal endophytes are sources of novel bioactive compounds but relatively few agriculturally important fruiting plants harboring endophytes have been carefully studied. Previously, we identified a griseofulvin-producing *Xylaria* species isolated from *Vaccinium angustifolium*, *V. corymbosum*, and *Pinus strobus*. Morphological and genomic analysis determined that it was a new species, described here as *Xylaria ellisii*. Untargeted high-resolution LC-MS metabolomic analysis of the extracted filtrates and mycelium from 15 blueberry isolates of this endophyte revealed differences in their metabolite profiles. Toxicity screening of the extracts showed that bioactivity was not linked to production of griseofulvin, indicating this species was making additional bioactive compounds. Multivariate statistical analysis of LC-MS data was used to identify key outlier features in the spectra. This allowed potentially new compounds to be targeted for isolation and characterization. This approach resulted in the discovery of eight new proline-containing cyclic nonribosomal peptides, which we have given the trivial names ellisiiamides A-H. Three of these peptides were purified and their structures elucidated by one and two-dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR) and high-resolution tandem mass spectrometry (HRMS/MS) analysis. The remaining five new compounds were identified and annotated by high-resolution mass spectrometry. Ellisiamide A demonstrated Gram-negative activity against *Escherichia coli* BW25113, which is the first reported for this scaffold. Additionally, several known natural products including griseofulvin, dechlorogriseofulvin, epoxy/cytochalasin D, zygosporin E, hirsutatin A, cyclic pentapeptides #1–2 and xylariotide A were also characterized from this species.

*Vaccinium angustifolium* (wild lowbush blueberries or commonly wild blueberries) were consumed fresh and preserved for the winter by the Indigenous peoples of northeastern North America and rapidly incorporated into the diets of European settlers in Canada from the early 17th century\(^1,2\). Today, blueberries comprise more than half of all fruit production in Canada. Wild blueberries often grow in forests where *Pinus strobus* (eastern white pine) is the dominant tree species. Eastern white pine is an economically, ecologically, and culturally important keystone tree species in eastern N. American forests, especially for bird species\(^3,4\).

Endophytes are an ecological category of phylogenetically diverse fungi that can asymptptomatically colonize healthy plant tissues. Ascomycetous endophytes of various species of *Vaccinium* have been reported over the past three decades. This includes from surface-sterilized tissues of *Vaccinium vitis-idaea* (lingonberry, European

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\(^1\)Department of Chemistry and Chemical Biology, McMaster University, Hamilton, Ontario, L8S 4M1, Canada. \(^2\)Department of Chemistry, Carleton University, Ottawa, Ontario, K1S 5B6, Canada. \(^3\)Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, Victoria, British Columbia, V8Z 1M5, Canada. \(^4\)Ottawa Research and Development Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, K1A 0C6, Canada. \(^5\)Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada. \(^6\)London Research and Development Centre, Agriculture and Agri-Food Canada, London, Ontario, N5V 4T3, Canada. \(^7\)Present address: LifeMine Therapeutics, Cambridge, Massachusetts, 02140, USA. *email: mark.sumarah@canada.ca*
Endophytes belonging to the family Xylariaceae (Xylariales, Sordariomycetes) are ubiquitous and detected in varying abundance in most studies involving woody plants, regardless of geographic location or host, whether by isolation of cultures or by studies of DNA, often exhibiting little host preference and including known saprotrophs. Xylariaceae endophytes are common but difficult to identify to species because of a lack of reference sequences and the limited taxonomic resolution of the asexual states (usually the only morphological characters produced in vitro). However, careful field observations can provide connections between the often conspicuous Xylariaceae stromata found in nature and the corresponding endophytes isolated in culture or detected by DNA sequences from the same forests. Taxonomically, Xylariaceae comprises at least 37 genera with likely more than 1,000 species. Many endophyte studies based on morphological identification of cultures report geniculosporium-like morphs attributable to Anthostomella, Rosellinia and Xylaria species. The classical nature of most taxonomic studies of Xylariaceae is reflected by the need for the sexual state to confirm identification, with a relative paucity of species-specific DNA barcodes and phylogenetic markers compared to many other ascomycete groups. Thus, xylariaceous endophytes may include species and genera known to classical taxonomy but not included in sequence databases (i.e.: named-but-unsequenced species).

Species of Xylariaceae are a rich source of secondary metabolites, and chemotaxonomy is often part of taxonomic studies. Species in this family can produce diverse metabolites from multiple biosynthetic families including dihydroisocoumarins, punctaporinins, cytochalasins, butyrolactones, and succinic acid derivatives. Exploration of Xylaria metabolites using newer chemical methods led to discovery of a broad array of metabolites from both tissues of stromata and culture extracts.

Although there have been many studies of metabolites from fungal endophytes, there are few reports from endophytes of Vaccinium. We previously described production of the antifungal compounds griseofulvin and piliforinc acid from an unknown Xylaria species isolated as a foliar endophyte from wild blueberry in natural and commercial sites, and from white pine. After the Richardson et al. (2014) study, we continued to isolate the same unidentified species of Xylaria as an endophyte of white pine needles and as an endophyte of leaves and stems of both wild and highbush blueberry at three different locations in Nova Scotia, New Brunswick, and Ontario. We also conducted field sampling to discover the putative sexual state of this unknown Xylaria species. This would provide information about the morphological characters of sexual structures, permitting its identification. This previously unknown endophyte is described here as Xylaria ellisi based on morphological and genomic evidence. Representative sequences in NCBI GenBank from other studies indicate that X. ellisi has been isolated many times as an unidentified endophyte from a wide variety of plant hosts, allowing us to infer additional information about its distribution, biology, and chemistry.

In our effort to discover novel natural products, we applied a LC-MS metabolomic-guided discovery approach to these Xylaria strains from wild and highbush blueberry plants (Fig. 1). This approach allows for a global survey of small molecule metabolites from an extract and visual representation of metabolite variances between groupings or extracts. Thus, discriminating between like and different features allows extracts to be prioritized for further investigation. Fifteen strains were grown on two media and the resulting ethyl acetate extracted filtrates and mycelium were screened using standardized LC-UV/MS conditions. Multivariate statistical analysis was used to organize resulting analytical data to reveal extracts that appeared to have differences in their major secondary metabolites. This approach led to the discovery of a family of eight new proline-containing cyclic nonribosomal pentapeptides named ellissiamides A–H. Ellissiamide A is an alanine (Ala) substituted variant, a first report for this scaffold, and demonstrated modest activity against Escherichia coli.

Results
Identification, biology and ecology of Xylaria sp. Approximately 30 strains of Xylaria sp. were isolated from surface-sterilized blueberry tissues collected from highbush and wild blueberry fields within a ~300 × 100 km triangular area. All fields were surrounded by forested lands. Preliminary phylogenetic analysis using the internal transcribed spacer (ITS) barcode combined with morphological features confirmed conspecificity of isolated endophytic Xylaria sp. strains. However, identification of the strains to species was not possible using molecular or in vitro morphological data. Based on a BLAST query of the Xylaria sp. ITS and RPB2 sequences with available GenBank sequences, the endophyte strains were closest related to sequences identified as Xylaria berteri, X. castorea, X. cubensis, X. laevis, and X. longipes, species that form conspicuous sexual reproductive structures (stromata) from decaying hardwood. Given the close phylogenetic relationship of the unknown Xylaria endophyte to these species and evidence of prevalent endophytic-saprotrophic life histories within Xylariaceae, we inferred that the unknown Xylaria endophyte likely produces stromata from decaying hardwood in mixed-wood stands in the Acadian forest. Thus, Xylaria stromata were selectively sampled during ongoing field surveys to collect the putative sexual state of the endophyte. This would provide material for identification and insight into its life history.
A Xylaria sp. producing stroma reminiscent of X. corniformis and X. curta was collected from decaying, often partially buried, Acer saccharum branches or logs in late summer and autumn. Sequences (ITS, SSU, LSU, BenA, EF1-α, RPB2) obtained from stromatal tissue and ascospore cultures were identical to those obtained from the Xylaria sp. endophyte cultures, indicating they are conspecific and evincing a saprotrophic-endophytic life history. Based on morphological study of the stromata, this species is equivalent to X. corniformis var. obovata Sacc., Xylaria corniformis sensu Laessøe44, and Xylaria curta sensu Rogers45. From the RPB2 phylogeny, X. corniformis var. obovata is weakly supported (posterior probability value (PP) = 0.56) sister to X. laevis and other species within the strongly-supported (PP = 1.0) X. cubensis aggregate clade. Xylaria is polyphyletic, including Amphirosellinia nigrospora, Stilbohypoxylon quisquiliarum, and Nemania serpens, and the type species (X. hypoxylon) occurs in a basal clade sister to X. bambusicola. Additional RPB2 sequences for related Xylaria species are needed to generate a more comprehensive phylogeny (Fig. 2).

Several DAOMC herbarium specimens identified as X. corniformis from Acer spp. wood in Ontario and Quebec were morphologically similar to X. laevis. The resulting ITS sequences from these specimens showed that they formed a clade sister to X. longipes and X. primorskensis and were distinct from the griseofulvin-producing X. corniformis var. obovata (Fig. 3). We support the distinction of X. corniformis var. obovata from X. corniformis, and thus describe a new species, Xylaria ellisii, to accommodate its novelty and fulfill the need to delineate boundaries in species complexes with robust species concepts connected to authenticated reference sequences and specimens.

**LC-MS analysis of culture extracts and multivariate data analysis.** Fifteen strains of X. ellisii were subject to further study: four from cultivated highbush blueberry plants and 11 from wild blueberry plants. Ethyl acetate extracts of the culture filtrate and associated mycelium were screened using standardized LC-UV/MS conditions.

In order to identify unique secondary metabolite differences between extracts of Xylaria isolates of highbush and wild blueberry plants we compared the extracted filtrates and mycelium with three different pair-wise comparisons. These comparisons included: ethyl acetate extracts of Xylaria strains grown on 2% malt extract broth (ML) versus those grown in potato dextrose broth (PDB) cultures; ML media cultures of highbush versus wild varieties; and, PDB medium cultures of highbush versus wild isolates (Fig. 1S). A supervised multivariant analysis
method, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), was used to identify outlier metabolites biosynthesized under the different culture conditions tested. OPLS-DA correlates differences in secondary metabolite feature abundances (X variables) to various treatment groups (Y variables) by identifying principle components that describe differences. R²X, R²Y, and Q² parameters are important validation parameters used for OPLS-DA, where R²X and R²Y describes the percentage of X and Y variables described by the model (Fig. 4 and Supplementary Fig. 2S). A valid model is defined as having a prediction statistic of Q² > 0.4, with values above 0.7 being highly significant. Metabolite features with a high Variable Importance in Projection (VIP) scores (>0.7) are responsible for driving the differences between treatment groups, and these values are considered significant. Their metabolic features can be viewed at both ends of the OPLS-DA S-plot.

Fractions with VIP scores above 0.7 were selected for further study and compounds were identified where possible. OPLS-DA validation parameters for each of the extracted filtrates and mycelium metabolite models tested are summarized in Table 1 and Supplementary Table 1S. In total, 3856 metabolite features were identified from
Figure 3. Bayesian 50% majority rule ITS consensus tree containing *Xylaria ellisi* and related species. All unlabeled branches have Bayesian posterior probability values of 1.0; values lower than 1.0 are presented at nodes. The tree was rooted to *Nemania serpens* and the scale bar indicates the expected number of changes per site. GenBank accession numbers and host information follow species names (when applicable). Type specimens are indicated in bold.
the extracted filtrates, with a Q² value of 0.615 for ML versus PDB, and Q² values of 0.778 for ML and PDB, as well as highbush versus wild varieties.

**Metabolomic-guided discovery and metabolite identification of knowns 1–11.** We first evaluated metabolites with the top VIP (30, 50, 100) scores for ethyl acetate extracts of the filtrates and methanol/acetone (1:) extracted mycelia from *Xylaria*. The initial focus was on metabolites that displayed UV absorption maxima at ~210, 254, 275 or 350 nm (Table 2 and Supplementary Tables S1–8). Compounds (100–2000 μg) were purified by reverse phase semi-preparative HPLC and characterized by NMR (Bruker Advance III 700 MHz NMR with cryoprobe) (Supplementary Fig. 1S). Metabolites were dereplicated against natural product databases including Antibase (https://www.wiley.com/en-us/AntiBase%3A+The+Natural+Compound+Identifier-p-9783527343591), Dictionary of Natural Products (http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml) and NORINE (https://bioinfo.lifl.fr/norine/) using molecular formulas dictated by HRMS data. In addition, a comparative analysis was conducted against known fungal metabolites34–37. Using this dereplication approach, the previously reported compounds 1–11 were identified; (1) griseofulvin, (2) dechlorogriseofulvin, (3)
cytochalasin D, (4) zygosporin E, (5) epoxycytochalasin D, (6) hirsutain A, (7) piliformic acid, (8) 2,3-dihydro-2,4-dimethylbenzofuran-7-carboxylic acid, (9) cyclic pentapeptide 1, (10) xylarotide A, and (11) cyclic pentapeptide 2 (Table 2). LC-HRMS, NMR, and spectroscopic data for compounds 1–11 confirm their structures can be found in the Supplementary Methods, Figs. 2 and 3S, 32–49S and Tables 1–9S).

Structure elucidation of ellisiiamides A–C (12–14).

Ellisiiamides A–C (12–14) were identified by metabolomic analysis of the extracted filtrates and mycelium with high VIP scores (2.6–11.59; Fig. 4, Supplementary Tables 1–8S). These new cyclic pentapeptides are structurally similar to cyclic pentapeptide 1 (9), with amino acid differences at positions 2 (Ala/IsoLeu vs. Val) and 3 (Val vs. IsoLeu) within the peptide scaffold (Fig. 5 and Supplementary Fig 5S, Tables 9–12S).

Ellisiiamide A (12) was isolated as a white powder and afforded a protonated molecular ion at m/z 556 (C30H46N5O5 with 11 double bond equivalents). Examination of the 1H and 13C NMR data revealed the

| Model | Variables* | R2X(cum) | R2Y(cum) | Q2(cum) | Conditions |
|-------|------------|----------|----------|---------|------------|
| 1a    | 3856       | 0.15     | 0.939    | 0.615   | ML, PDB    |
| 1b    | 100        | 0.313    | 0.955    | 0.737   | ML, PDB (including top 100 VIP) |
| 1c    | 3756       | 0.299    | 0.982    | 0.434   | ML, PDB (excluding top 100 VIP) |
| 1d    | 3556       | 0.207    | 0.954    | 0.394   | ML, PDB (excluding top 300 VIP) |
| 2a    | 3856       | 0.477    | 0.998    | 0.778   | ML-H, ML-L |
| 2b    | 30         | 0.861    | 0.984    | 0.864   | ML-H, ML-L (including top 30 VIP) |
| 2c    | 3826       | 0.544    | 1        | 0.718   | ML-H, ML-L (excluding top 30 VIP) |
| 2d    | 3156       | 0.121    | 0.73     | -0.187  | ML-H, ML-L (excluding top 700 VIP) |
| 3a    | 3856       | 0.648    | 1        | 0.778   | PDB-H, PDB-L |
| 3b    | 50         | 0.589    | 0.995    | 0.885   | PDB-H, PDB-L (including top 50 VIP) |
| 3c    | 3806       | 0.651    | 1        | 0.668   | PDB-H, PDB-L (excluding top 50 VIP) |
| 3d    | 2956       | 0.0851   | 0.894    | -0.175  | PDB-H, PDB-L (excluding top 900 VIP) |

Table 1. A summary of validation parameters (R2X, R2Y, Q2) of all calculated OPLS-DA models for extracted filtrates of X. ellisii endophytes isolates from wild and highbush blueberries cultured in ML and PDB media. ML-H, endophyte isolates from highbush blueberries cultured in ML medium; ML-L, endophyte isolated from wild blueberries cultured in ML medium; PDB-H, endophyte isolates from highbush blueberries cultured in PDB medium; PDB-L, endophyte isolates from wild blueberries cultured in PDB medium. *Number of metabolomic features included in the OPLS-DA analysis.

| #   | Compound            | Class   | Rt     | Molecular Formula | Measure and Calculated [M+H]+ ppm error |
|-----|---------------------|---------|--------|-------------------|----------------------------------------|
| 1   | Griseofulvin        | PKS     | 11.42  | C17H18ClO6      | 353.0793 353.0786 -1.98                |
| 2   | Dechlorogriseofulvin| PKS     | 10.01  | C17H19O6        | 319.1173 319.1176 0.94                  |
| 3   | Cytochalasin D      | PKS-NRPS| 11.81  | C30H38NO6       | 508.2687 508.2694 1.38                  |
| 4   | Zygosporin E        | PKS-NRPS| 13.94  | C30H38NO4       | 492.2742 492.2744 0.41                  |
| 5   | Epoxycytochalasin D | PKS-NRPS| 10.87  | C30H38NO4      | 524.2651 524.2661 1.91                  |
| 6   | Hirsutain A         | NRPS    | 15.85  | C30H52N5O10  | 677.3741 677.3756 2.21                  |
| 7   | Piliformic acid     | PKS     | 10.84  | C11H18O4Na     | 237.1094 237.1097 1.27                  |
| 8   | 2,3-dihydro-2,4- dimethylbenzofuran-7-carboxylic acid| PKS | 11.15 | C11H13O3 | 193.0857 193.0859 1.04 |
| 9   | Cyclic pentapeptide 1* | NRPS | 16.20 | C30H38N5O6 | 584.3816 584.3806 -1.71                |
| 10  | Xylarotide A        | NRPS    | 15.97  | C30H52N5O5  | 550.3973 550.3963 -1.82                 |
| 11  | Cyclic pentapeptide 2| NRPS   | 14.28  | C30H50N5O5  | 536.3819 536.3806 -2.42                 |

Table 2. Identification of known and new secondary metabolites from X. ellisii via LC-UV/HRMS and LC-HRMS/MS analysis. Select metabolites have been further isolated and characterized by 1D and 2D NMR. *Structures elucidated by 1D and 2D NMR, HRMS and MS/MS analysis.
presence of five α protons (δ 5.08/55.8, 4.49/46.0, 4.17/55.7, 4.74/46.5, 5.10/58.9 ppm), and three key amide N-H protons for Ala (δ 8.52), Isoleucine (δ 6.94) and Leu (δ 8.49), and the N-Methyl group at (δ 3.04/30.2 ppm). Examination of the multiplicity edited 1H-13C HSQC, 1H-13C HMBC, and 1H-1H COSY NMR data revealed the individual amino acid spin systems within the peptide scaffold based on α proton correlations to the individual carbonyl carbon, including amide protons to neighboring amino acid carbonyls, and α, β and γ proton correlations (Fig. 5, Supplementary Figs. 4–13S and Tables 9 and 10S). These correlations supported the amino acid sequence of cyclo-(NMePhe-Ala-IsoLeu-Leu-Pro). NOESY through-space correlations of αΗ (N–MePhe)/NH (Ala), NH(Ala)/βΗ (Ala), NH(Isoleu)/αΗ (Ala), NH(Leu)/αΗ (Isoleu) and H3-NMe (N-MePhe)/βΗ (Pro) further supported the amino acid sequence and relative stereochemistry. Analysis of the LC-MS/MS spectra of ellisiiamide A revealed key diagnostic b-ion fragments of m/z 459.3 (-Pro), 346.2 (-Leu), 233.1 (-Isoleu), 162.1 (-Ala) and the presence of two fragmentation pathways as seen in cyclic pentapeptide 1 with ring-opening cleavage events at the N-MePhe-Pro and Pro-Leu sites52.

Ellisiiamide B (13) was isolated as a white powder with a protonated molecular ion at m/z 570 affording a molecular formula of C31 H47N5O5 with 11 double bond equivalents. Examination of 1H and 13C NMR data revealed presence of five α protons (δ 5.10/56.0, 3.95/57.6, 4.10/56.8, 4.72/46.6, 5.08/58.7 ppm), key amide N-H protons for Val (δ 8.18), Val2 (δ 6.98), and Leu (δ 8.43), and the N-Methyl group at (δ 3.03/30.2 ppm). Ellisiiamide B (13) differs from (9) with Val substituted for Isoleucine at position # 3 (Fig. 4, Supplementary Figs. 4S and 14–21S, Table 9 and 11S). Examination of the MS/MS spectra revealed a similar fragmentation pattern as in (9) and (12), with key diagnostic b-ion fragment ions at m/z 471.3 (-Pro), 360.2 (-Leu), 261.2 (-Val), and 162.1 (-Val). The cyclo-(NMePhe-Val1-Val2-Leu-Pro) amino acid sequence was confirmed with key HMBC correlations of αH(N–MePhe)/CO (N-MePhe), H3-NMe (N-MePhe)/CO (Pro), αH(Val1)/CO(Val1), αH(Val2)/CO(Val2), αH (Leu)/CO (Leu), and αH (Pro)/CO (Pro). Key NOESY correlations of αH(N–MePhe)/NH(Val1), NH(Val1)/αΗ (Val1), NH(Val2)/αΗ (Val2), NH(Val2)/βΗ (Val2), NH(Leu)/NH(Val2), and H3-NMe (N-MePhe)/βΗ (Pro) further supported the assignments.

Ellisiiamide C (14) was isolated as a white powder with a protonated molecular ion at m/z 598 affording a molecular formula of C33H51N5O5 with 11 double bond equivalents. Examination of 1H and 13C NMR data revealed the presence of five α protons (δ 5.10/56.0, 4.05/55.8, 4.25/55.1, 4.72/46.5, 5.09/58.7 ppm), key amide

Figure 5. Ellisiiamides A–H (12–19), new cyclic nonribosomal peptides from Xylaria ellisii. (a) ellisiiamides A–C (12–14) isolated and characterized by 1D and 2D NMR, LC-HRMS and LC-HRMS/MS analysis with new amino acid substituent highlighted. Corresponding COSY/TOCSY (1H -1H), HMBC (1H-13C) and long-range through-space NOESY/ROESY correlations are shown. (b) Structures of ellisiiamides D–H (15–19) based on LC-MS/MS, comparative LC-MS/MS analysis of cyclic pentapeptides 9, 11 and 12–14 (c) Amino acid scaffold of the cyclic pentapeptide family of compounds. Cyclic pentapeptide 1 (9) shown with established amino acid substituents.
N-H protons for IsoLeu1 (δ 8.12), IsoLeu2 (δ 6.94) and Leu (δ 8.45), and the N-Methyl group at (δ 3.04/30.2 ppm). Ellisiiamide C (14) differs from (9) with IsoLeu substituted for Val at position #2 (Fig. 4, Supplementary Fig. 4S and 22–31S, and Tables 9 and 11S). Examination of the 1H−1H COSY, multiplicity edited 1H−13C HSQC and HMBC NMR data revealed the individual spin system for the new IsoLeuC group with correlations of β−3H(IsoLeu)/αH (IsoLeu) and δH(IsoLeu)/βH (IsoLeu). Correlations of the remaining α protons to the individual carbonyl carbons, amide protons to neighboring amino acid carbonyl, and HMBC α-, β- and γ-proton correlations for Leu, Pro and N-MePhe is consistent with the cyclic peptide scaffold (Supplementary Table 9S.). NOESY through-space correlations of δH(N−MePhe)/NH (IsoLeu1), NH (IsoLeu1)/NH (IsoLeu2), NH (Leu)/αH (IsoLeu2) further supported the amino acid sequence. Analysis of the MS/MS spectra of (14) revealed key diagnostic b-ion fragments of m/z 501.3 (-Pro), 388.3 (-Leu), 275.2 (-IsoLeu2), and 162.1 (- IsoLeu1) further confirming the amino acid sequence of cyclo-(N-MePhe-IsoLeu1-IsoLeu2-Pro).

The optical rotation for Ellisiiamides A–C were measured at [α]21 =−86.1 (0.06, MeOH), [α]21 =−43.1 (0.04, MeOH), and [α]20 =−47.8 (0.06, MeOH) respectively, and were consistent with 9 at [α]21 =−63.4 (0.18, MeOH) (Supplementary Table 9S).

**LC-MS/MS analysis and putative identification of new cyclic pentapeptides.** Ellisiiamide D–H (15–19) was identified by metabolomic analysis of the extracted filtrates and mycelium models as unique outliers with high VIP scores (1.92–6.48). Evaluation of the HRESIMS derived molecular formulas and MS/MS fragmentation patterns of (15–19) indicated that the fragmentation sequence and ring-opening events were consistent with ellisiiamides A–C and cyclic pentapeptide 1. We have therefor assigned putative identification and annotated structures for ellisiiamides D–H. LC-HRMS/MS characterization data can be found in the Supporting Methods and Figs. 4S and Table 1S and 9S.

**Bioactivity activity screening.** Compounds 9 and 12–14 were screened for biological activity against three species of microorganisms in accordance with the Clinical Laboratory Standards Institute (CLSI) protocols (National Committee for Clinical Laboratory Standards, 2000, 1997). The microorganism included E. coli ATCC# 90028. Ellisiiamide (A) (12) showed modest activity against E. coli with a minimum inhibitory concentration (MIC) of 100μg/mL. Such activity against E. coli is a first report for the cyclic pentapeptide scaffold. Compound 9 showed no antifungal activity against S. cerevisiae or C. albicans at 100μg/mL, which is consistent with reported data52. Similarly, compounds 13–14 showed no activity against any test microorganisms at concentrations between 50–200μg/mL.

**Taxonomy of Xylaria ellisii.** Xylaria ellisii. J.B. Tanney, Seifert & Y.M. Ju, sp. nov. MycoBank MB832257 (Fig. 6).

=Xylaria corniformis (Fr.: Fr.) Fr. var. obovata M.C. Cooke & J.B. Ellis, Grevillea 6: 92. 1878.

**Etymology.** Named for the prolific mycologist Job Bicknell Ellis who, with Mordecai Cubitt Cooke, described Xylaria corniformis var. obovata Sacc., a synonym of X. ellisii.

**Typus.** Canada: New Brunswick, Alma, Fundy National Park, East Branch Trail, 45.6433 –65.1156, stromata on branches of Acer saccharum at 50–80 cm above ground, 28 Sep 2014, J.B. Tanney NB-623 (holotype DAOM 628556). Ex-type culture DAOMC 252031.

Colonies 32–38 mm diam after 14 d in the dark at 20 °C. Colonies 32–38 mm diam after 14 d in the dark at 20 °C on MEA; white, velvety, appressed, sometimes sectored; margin diffuse, hyaline; surface and reverse white. Exudates and soluble pigments absent. Mycelium consisting of hyaline, smooth, septate, branched, hyphae 1.5–3 μm diam.

Conidiophores on MEA macronematous, arising vertically from mycelium, hyaline to pale brown, smooth, cylindrical, thin-walled, dichotomously branched several times, septate, 30–60 × 3–4 μm, or occurring in synnemata, grey to olive brown (4D2–4E3). Synnemata cylindrical to clavate, occurring singly, gregariously, or in clusters joined at base, up to 10 mm high by 1–3 mm diam, surface appearing powdery due to conidia. Conidiogenous cells intercalary and terminal, cylindrical, straight or undulating to geniculate, 7–16(−20) × 3–4 μm, hyaline to pale brown, producing one or more conidia holoblastically from lateral or apical regions, crater-shaped protruding scission scars 1–1.5 × 1–1.5 μm. Conidia pyriform to obovoid, subhyaline to pale brown, (5–)5.5–7(−7.5) × (2.5–)3(−3.5) μm, flattened basal scar indicating former site of attachment to conidiogenous cell.

Sporodochia, solitary, unbranched or occasionally branched once, cylindrical to spathulate or clavate, apices broadly rounded, divided into fertile head and sterile stipe, (2–)2.5–4(−5) × 0.8–1.2 cm including stipes (0.4–1.5 cm high); surface even to irregularly flattened or wrinkled, frequently cracked into a network of light brown to brownish orange (6D4–6D5) angular plates above black basal layer; stromatal interior white; stipes brownish orange to light brown (6D4–6D5) frequently with black longitudinal cracks extending from fertile head; arising from brown (7D7) to black pannose bases, black mycelia often appearing iridescent. Perithecium immersed, subglobose to globose, 0.3–1 mm diam, lining the perimeter of the stromata. Ostiolae conspicuous, papillate, 100–300 μm diam. Ascii 95–130 × 6–7 μm, partis sporiferae 50–80 μm, eight-spored, cylindrical, with ascospores arranged uniseriately; apical apparatus inverted hat-shaped, amloid, 1.5–2 μm long. Ascospores (8–)9–9.5(−10) × (4.5–)5–5.5(−6) μm, dark brown, smooth, unicellular, ellipsoid-inequilateral, narrowly or broadly rounded ends, 1–2 guttules frequently observed, inconspicuous long, straight germ slits which are more or less the spore length, occurring on convex side; small ephemeral cellular appendage 1.5–2 × 1.5 μm, visible on less pigmented immature ascospores and disappearing as spores reach maturity.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.
Host range: Stromata on decaying hardwood including *Acer, Betula, Fagus,* and other hardwood trees. Foliar endophyte of *Abies balsamea, Picea glauca, P. mariana, P. rubens,* and *Pinus strobus.* Foliar and stem endophyte of *Vaccinium angustifolium* and *V. corymbosum.* Closely related ITS sequences in GenBank suggest a broad endophytic and endolichenic host range.

**Figure 6.** *Xyaria ellisi* morphology. (A, B) Stromata on partially buried, decaying *Acer saccharum* branches, arrow pointing to longitudinal section of stroma with perithecia lining outer surface. (C) Base of stroma showing ostioles and reticulations. (D) Ostioles on stroma surface. (E) Eight-week-old colony on oatmeal agar. (F) Longitudinal section of perithecium. (G) Asci and paraphyses. (H) Conidiogenous cells. (I) Conidia. (J, K) Asci with amyloid, inverted hat-shaped apical apparatuses. (L) Ascospores, arrow denoting germ slit. Scale bars: 
(F, G) = 100 µm, (H, J–L) = 10 µm, I = 5 µm.
Distribution: Eastern Canada and U.S.A.

Additional specimens and cultures examined: DAOM 696463, DAOM 696464, DAOM 696466, DAOM 696480, DAOM 696488, DAOM 696489, DAOM 696492, DAOM 69649, DAOM 696503, NB-699, NB-701, NB-702, NB-703, NB-708, NB-721, NB-722, NB-723, NB-727, NB-746, CH-12, CH-15, CH-16, CH-37, CH-38, CH-4, CH-5, DT-181, DT-6, NB-236-1F, NB-236-2F, NB-236-2L, NB-285-10A, NB-285-10D, NB-285-1A, NB-285-3A, NB-285-6B, NB-285-7A, NB-285-7B, NB-285-7C, NB-285-7D, NB-365-4E, NB-365-71G, NB-365-8A, NB-366-1F, NB-366-2E, NB-366-3L, NB-366-4C, NB-382-1C, NB-382-3A, NB-382-3C, NB-382-3D, NB-382-4B, NB-391-1E, NB-391-2C, NB-391-4C, NB-406-2A, NB-406-2B, NB-406-2F, NB-406-5A, NB-421-1B, NB-437-5E, NB-464-10A, NB-487-5B, NB-487-5C, NB-487-6A, NB-487-6H, NB-488-6L, NB-505-4D, NB-746, RS-910E, RS-912C, T1-3B-2, T1-4B-1, T2-4A-2, T3-2A-2, T3-2B-1, T3-3A-3, T4-3A-1, T5-1A-1, T5-3B-1-1, T6-4B-1, T6-5A-1-2.

Notes: Xylaria ellisii is equivalent to X. corniformis var. obovata, e.g.: Ju et al. (2016) recorded blackish-brown ascospores, 8–10.5 × 4.5–5.5 (–6) μm from the X. corniformis var. obovata holotype45. The X. corniformis species concept is unresolved and consequently the name has been misapplied to various species within the X. corniformis and X. polymorpha aggregates46. The Xylaria corniformis aggregate is a polyphyletic morphotaxonomic concept comprising species characterized by stromata with a wrinkled surface and a thin outer layer that gradually cracks into fine scales with maturation, including X. bipindensis, X. cuneata, X. curta, X. divisa, X. fieveensis, X. humosa, X. luteostromata, X. maumeetii, X. montagnei, X. plebeja, and X. rhytidophloea45,53–55. Rogers (1983) noted the taxonomic confusion surrounding X. corniformis and its misapplication to X. buliosa, X. castorea, X. curta, and other morphologically similar species, and recommended that Xylaria taxonomy would be best served if the name X. corniformis were no longer used45. Xylaria corniformis s.s. is possibly a rare species known only from Swedish and Polish collections and is characterized by delicate, horn-like stromata with attenuated or sterile apices versus the robust stromata of X. ellisii, which also have darker coloured ascospores45,53–55. Ju et al. (2009) concluded that X. corniformis var. obovata was an equivalent of X. corniformis sensu Læssøe (1987)45,56. Læssøe (1987) noted that X. corniformis var. obovata was probably the most frequently encountered member of the X. corniformis complex in northern temperate regions45. Ju et al. (2009) considered X. corniformis and X. corniformis var. obovata as distinct species but refrained from making a formal taxonomic decision pending additional evidence45. Xylaria ellisii is common on decaying fallen Acer saccharum branches in New Brunswick during late summer and autumn and is a frequently isolated endophyte of Picea, Pinus strobus, and Vaccinium angustifolium in Eastern Canada48. Conspecific ITS sequences in GenBank suggest that X. ellisii is capable of endophytically infecting a wide range of hosts.

Discussion

Xylaria ellisii was the most commonly isolated Xylariaceae endophyte from Picea and Pinus in Eastern Canada57. Stromata of X. ellisii were commonly found on decaying Acer saccharum branches or stems in the same forest stands where it was isolated as a Picea endophyte. Endophyte ITS sequences in GenBank corresponding to X. ellisii originate from an exceptional diversity of hosts, including Tsuga canadensis, bryophytes (e.g.: Hypnum sp.), liverworts (e.g.: Metzgeria furcata, Trichocolea tomentella), and lichens (e.g.: Flavoparmelia caperata, Sticta beauvoisii, Xanthoparmelia conspersa) (Fig. 3). In New Brunswick, corresponding X. ellisii stromata were commonly found in late summer and early fall only on decaying Acer saccharum wood; however, the stromatal host range is likely broad. For example, Læssøe (1987) examined European specimens of Xylaria corniformis (probably X. ellisii) from Carpinus and Fagus44 and Rogers (1983) examined North American collections from Betula, Fagus, Malus, and Tsuga45.

Xylaria ellisii is a common Picea and Pinus endophyte even in conifer-dominated stands lacking Acer saccharum or any other hardwood hosts possibly suitable for the production of stromata. This indicates that the fungus is capable of persisting in the environment in the prolonged absence of a suitable primary host. The method of transmission between foliage is currently unknown. It is conceivable that the dry, powdery masses of conidia produced from conidiomata in vitro are also produced on dead foliage and capable of infecting new foliage by means of air currents or insect vectors48,59. Abscised foliage infected with X. ellisii is probably capable of saprotopically colonizing hosts by means of direct contact (viaphytism), as demonstrated in other Xylaria species25. The known range of hosts that X. ellisii can endophytically infect includes lichens and various understory and overstory plant species with different successional statuses, allowing for its persistence across forest succession pathways and disturbances (e.g.: as an endophyte of the fire-adapted aseral species Vaccinium angustifolium). A proposed endophytic-saprotrophic life history is described and illustrated for Xylaria ellisii (as Xylaria sp.) by Tanney et al.60.

The production of the potently antifungal compound griseofulvin by X. ellisii, an apparently ubiquitous endophyte with a broad host range, is significant. Griseofulvin is toxic to a wide variety of plant pathogens44–46 and is systemically translocated within plants46, suggesting that X. ellisii endophyte infections could increase host resistance to plant pathogens. For example, Park et al. (2005) described griseofulvin production in an unidentified Xylaria endophyte of Abies holophylla and showed its ability to control the development of plant diseases such as barley powdery mildew (Blumeria graminis f. sp. hordei), rice sheath blight (Corticium sasakii), wheat leaf rust (Puccinia recondita), and rice blast (Magnaporthe grisea)44. Griseofulvin and related compounds are reported from Xylaria endophytes of Asimina triloba, Chrysobalanus icaco, and Garcinia hombriana66–68. Richardson et al. (2014) reported the production of the antifungal compound griseofulvin by Xylaria ellisii (as Xylaria sp.) isolated as a foliar endophyte of Pinus strobus and Vaccinium angustifolium18. These isolates produced griseofulvin and its de-halogenated analogue (Fig. 1), along with piliformic acid18. Subsequent investigations of white pine seedlings infected with this Xylaria species found griseofulvin at biologically effective concentrations in the needles69.

SCIENTIFIC REPORTS | (2020) 10:4599 | https://doi.org/10.1038/s41598-020-61088-x
Nonribosomal peptides (NRPSs) are of great interest as they represent a unique class of natural products with diverse therapeutic applications such as antimicrobial agents (caspofungin, penicillin, vancomycin), anticancer compounds (bleomycin, daptomycin), immunosuppressants (cyclosporine, rapamycin) and as insect toxins (beauvericin, enniatin)⁹⁰⁻⁹⁴. This complex structural diversity of linear, cyclic, and cyclic branched architectures is synthesized through a modular enzymatic assembly line process⁹⁵⁻⁹⁷. In principle, this enzyme complex is capable of incorporating >500 proteinogenic and nonproteinogenic building blocks, including polyketide and terpene hybrid moieties.

In this study, we have applied a LC-MS metabolomic guided discovery approach to profile the chemical space of a novel endophytic species described here as Xylaria ellisii. Our collections of isolates have identical ITS DNA sequences yet differ in their LC-MS metabolite profiles and bioactivity. OPLS-DA and S-plot analysis identified features separated by a statistical toll, Variable Importance in Projection (VIP) scores. VIP scores from the extracted filtrates and mycelium extracts were calculated and extracts differentiated by this method were targeted for compound isolation and structural characterization. This approach resulted in the discovery of three new cyclic pentapeptides given the trivial names ellisiamides A–C (12–14) and the putative identification and annotation of ellisiamides D–H by LC-HRMS and LC-HRMS/MS analysis. Additionally, 11 known compounds are reported to be produced by these strains. Ellisiamide A (12) was active against Gram-negative bacteria and is a first report for this scaffold. These findings are of interest as the isolates were also reported from eastern white pine needles in a pine-blueberry forest ectotype. Endophytes from wild Vaccinium species may be an interesting source of novel bioactive compounds. This information provides a better understanding of the chemical ecology of plant-fungi microbiomes. In the long term, opportunities may present to employ this information for integrated pest management crop protection strategies.

**Methods**

**Sampling, isolation, and culturing.** Plant material, including leaves and stems from highbush and wild blueberries, were collected from three different locations within the Acadian forest region of Nova Scotia, Canada. Highbush blueberry endophyte isolates were obtained from a commercial field in Rawdon, Nova Scotia and wild blueberry endophyte isolates were collected from commercial fields in Mount Thom, Debert, and Portapique, Nova Scotia. Specimens were collected in labelled bags and stored at -20 °C for fungal isolation. Plant tissues were first washed with sterile deionized water to remove any loose debris and surface contaminants, followed by a chemical surface-sterilization process using sodium hypochlorite bleach (6%) and ethanol (70%). Small segments were then cut and/or incised and placed in Petri plates containing 2% malt extract agar (MEA; 20 g Bacto malt extract, Difco Laboratories, Sparks, USA; 15 g agar, EMD Chemicals Inc., Gibbstown, USA; 1 L deionized H₂O). Inoculated plates were incubated at 25 °C for 4–8 weeks, depending on the presence of filamentous hyphae. Endophytic fungi that grew from cut ends were then transferred to potato dextrose agar (PDA, Sigma-Alrich, Canada) plates and incubated at 25 °C.

Field specimens of stromata were collected and stored in paper bags. Single-ascospore isolates were made by affixing with petroleum jelly a small (ca. 5 mm²) piece of stroma containing mature perithecia to the lid of a Petri dish containing water agar (WA; 15 g agar, EMD Chemicals Inc., Gibbstown, USA; 1 L deionized H₂O). Germination of ejected ascospores on the agar surface was confirmed by stereo microscope (Olympus SZX12, Olympus, Tokyo, Japan) and germinating ascospores were transferred to individual Petri plates containing 2% MEA and incubated at 20 °C. Dried specimens were accessioned in the Canadian National Mycological Herbarium (Ottawa, Ont.; DAOM). Living cultures were deposited in the Canadian Collection of Fungal Cultures (Ottawa, Ont.; DAOMC). Additional specimens used for morphological comparison and phylogenetic analyses were also obtained from DAOM, DAOMC, and the personal culture collection of J.B. Tanney. Xylaria strains from highbush blueberry and wild blueberry were cultured in PDB (24 g/L potato dextrose broth) and ML (30 g/L malt) fermentation media. Each strain was grown in 1 L Roux bottles containing 200 mL of media and grown statically for 4–6 weeks at 25 °C. The culture broth was then separated from the mycelium by vacuum filtration using a Whatman #4 filter paper. The filtrate was extracted with equal volumes of ethyl acetate, while the mycelium was first lyophilized for 24 h and then extracted with equivalent volumes of methanol and acetone (1:1). Organic fractions were then dried under reduced pressure by rotary vacuum. Extracts were then centrifuged at 13,000 rpm for 15 min and Acro-disk (13 mm, 0.45 µm GHP) filtered prior to LC-MS analysis.

**Morphological study.** Sections of stromata were cut by hand using a safety razor blade or with a freezing microtome (ca. 15–30 µm thick) and mounted in either water, 5% KOH, 85% lactic acid, or Lugol’s solution with or without 5% KOH pretreatment to test amyloid reactions⁹⁵. Stromata and colony colours were described using alphanumeric codes⁹⁶. Observations of the asexual morph were made from living cultures grown on oatmeal agar (OA)⁹⁷. Microscopic measurements were taken from living material mounted in deionized water and are presented as ranges calculated from the mean ± standard deviation of each measured value with outliers in brackets. Observations were made using an Olympus BX50F4 light microscope and an Olympus SZX12 stereo microscope (Olympus, Tokyo, Japan). Images were captured with an InfinityX-32 camera (Lumenera Corp., Ottawa, Canada) using Infinity Analyze v. 6.5.2 (Lumenera Corp.) software. Photographic plates were assembled using Adobe Photoshop CC 2017.1.1 (Adobe Systems, San Jose, California, USA). Cardinal temperatures were assessed for the type strain (DAOMC 252031) by incubating single-point inoculated Petri dishes containing MEA at 5 °C intervals from 5–40 °C. Each treatment was conducted in triplicate and colony diameters were measured two weeks after inoculation.
DNA extraction, sequencing, and phylogenetic analyses. DNA was extracted from cultures and stromata using the Ultracean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, CA) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). Stromatal tissue from fresh collections and herbarium specimens underwent an initial grinding stage in liquid nitrogen using an Oxygen polypropylene pestle (PES-15-B-SI, Union City, CA, USA).

Loci chosen for sequencing included the internal transcribed spacer rDNA region (ITS), β-tubulin (BenA), translation elongation factor 1-alpha (EF1-α), the second largest subunit of RNA polymerase II (RPB2), 18 s rDNA (SSU), and 28 s ruc DNA (L5). Primer pairs used for PCR amplification and sequencing included: ITS1 and ITS472 or ITS4A and ITS578 for ITS, Bt2a and Bt2b for BenA79; RPB2-5f2 and RPB2-7CR80 for RPB2; and EF1-728F and EF1-986R41 for EF1-α. LSU was amplified using LR0R and LR5 and sequenced using the primers LR0R, LR3, LR3R, and LR546. SSU was amplified using the primers NS1 and NS4, and sequenced using the primers NS1, NS2, NS3, and NS477. PCR and sequencing were performed as described by Tanney and Seifert (2017)79. To improve ITS amplification in herbarium specimens, 0.5 μm of 20 mg/ml bovine serum albumin (BSA) was added per reaction.

For all analyses, sequences were aligned using MAFFT v773 and visually inspected and manually aligned when necessary in Geneious R8 v8.1.5 (Biomatters, Auckland, New Zealand). The most suitable sequence evolution model was determined based on the optimal Akaike information criterion scores in MrModeltest v2.2.646. Consensus trees were visualized in FigTree 1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/) and exported as SVG vector graphics for assembly in Adobe Illustrator v10 (Adobe Systems, San Jose, CA, USA).

Three separate phylogenetic analyses were performed. The first phylogeny included ITS sequences of diverse representative endophytes isolated from highbush and wild blueberry leaves and stem. The ex-type of *Mucor ellipsoides* (ATCC: MYA-4767; NR_111685) was selected as outgroup because of its basal position (Mucoromycotina). Maximum likelihood (ML) analysis was performed using RAxML v8.2.4 in PAUP v4.0b10 starting from a random starting tree with 1000 bootstrap replicates85,86.

The second phylogenetic analysis included RPB2 sequences from related Xylaria species. The resulting alignment was 1058 bp long and consisted of 47 taxa, including the outgroup *Barrmaelia rhamicola* (CBS 142772). Bayesian analysis was performed using MrBayes v3.2.687. Three independent Markov Chain Monte Carlo (MCMC) samplings were performed with 12 chains (11 heated and one cold) with sampling every 500 generations until the standard deviation of split frequencies was <0.01. The first 25% of trees were discarded as burn-in and the remaining trees were kept and combined into one consensus tree with 50% majority rule consensus. Convergence was assessed from the three independent runs using Tracer v1.686. The third phylogenetic analysis included ITS sequences from related endophytic *Xylaria* isolates. The alignment was 593 bp long and included sequences from 107 isolates or samples. The resulting phylogenetic analysis was performed in the same manner as described above, with *Nemania serpens* (GU292820) as the outgroup.

All novel sequences used in this study were accessioned in GenBank (Supplementary Table 13S) and taxonomic novelties and associated metadata were deposited in MycoBank (www.MycoBank.org).

LC-UV/HRMS and LC-UV/HRMS/MS screening. Extracts of endophytic cultures were screened using a Dionex Ultimate 3000 HPLC-UV system coupled to a Bruker maXis 4 G ultra-high-resolution-qTOF mass spectrometer operated in positive electrospray ionization (ESI+) mode using a scan range of 150–1100 m/z, with the nebulizer gas (nitrogen) at 3 bar, dry gas flow at 8 L/min, dry gas temperature at 240 °C, and capillary voltage at 4000 V. Chromatographic separations were performed using a standardized HPLC-UV method with a Supelco Ascentis Express C18 reverse-phase core-shell column (150 × 4.6 mm, 2.7 μm, Sigma Aldrich, USA) operating at 250 μL/min and at 40 °C. UV/vis data were acquired from 190–600 nm and monitored at four wavelengths (210, 254, 275 and 350 nm). Mobile phase composition was linear with a gradient of 5% organic from 0 to 1 min, 5–95% from 1 to 24 min, 95–100% from 25 to 31 min, and 100% from 25 to 31 min. Solvent A was H2O + 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid (v/v). HR-MS/MS analysis was performed on a Thermo Q-Exactive Orbitrap mass spectrometer operated in positive electrospray ionization (ESI+) and coupled to an Agilent 1290 HPLC system.

Data processing and multivariate statistical analysis. Data processing and analyses were modified from a previously published protocol (Fei et al., 2014). Post-acquisition internal calibration using sodium formate clusters in both ESI+ and ESI- were performed with Bruker's Data Analysis 4.0 SP4. LC-MS data files were converted to mzXML format using Bruker Compass.Xport. Metabolic features were extracted and aligned using open source XCMS with centWave algorithm88; adducts, isotopic ions, and in-source fragments were identified using CAMERA89,90. To acquire the final metabolite feature list, isotopic ions and features with integrated peak area under 10,000 were removed. For mycelium metabolome, metabolite features that eluted after 25 min were eliminated.

Both extracted filtrates and mycelium were analyzed using supervised multivariate OPLS-DA after pareto scaling by SIMCA-P v 12.0.1 (Umetrics, Kinnelon, NJ). The statistical parameters R2(X), R2(Y), and Q2 were calculated using open source XCMS with centWave algorithm. R2 and Q2 followed an upward trend from 0 to 1. For an over-fit model, R2 approached 1, and Q2 fell toward 0. Significant features between classes were identified based on OPLS-DA S-plot and their Variable Importance in Projection (VIP) score. To ensure the identified metabolites are the sole important markers, the two OPLS-DA analyses were conducted in parallel by only including the significant features or by removing the significant features from the raw data. A useful metabolite subset was produced if the first model was successful and the later model failed.
Metabolite Isolation and characterization. NMR experiments for 1D and 2D measurements were performed on a Bruker Advance III 700 MHz NMR spectrometer equipped with a 5 mm QNP cryoprobe, operating at 700.17 MHz for 1H NMR and 176.08 MHz for 13C NMR or a Bruker Advance III HD 850 MHz NMR spectrometer equipped with a 5 mm TXI probe operating at 850.21 MHz for 1H NMR and 213.81 MHz for 13C NMR, with chemical shifts referenced to the residual solvent signal. Nitrogen dried compounds were re-suspended in 200 μL of deuterated solvent (CD6D, CD3OD, or DMSO-d6) and transferred to 3-mm NMR tubes (Wilmad 335–pp–7) for NMR measurements. NMR data processing was done using MNOVA NMR software ver. 10.0.1 by Mestrelab Research. Optical rotation measurements were done using an Autopol IV Polarimeter (Rudolph Research Analytical).

Purification of metabolomic targeted metabolites was performed on a semi-preparative HPLC system consisting of an Agilent 1100 series HPLC with a G1311A Quaternary Pump, a G1379A Degasser, a G1367A Wellplate Autosampler, a G1316A Column Thermostat, a G1315B Diode Array Detector (DAD), and a G1364C Automatic Fraction Collector controlled by Agilent ChemStation software (Rev. B.03.02-SR2). Metabolites were isolated using a Phenomenex Synergi-Max reverse-phase C-12 column (250 × 10 mm, 4 μm) (Torrence, CA, USA) operating at 5 mL/min and 40°C. Mobile phase composition was a linear gradient of 5% organic from 0 to 3 min, 5–30% from 3 to 16 min, 30% from 16 to 20 min, and 30–85% from 20–37 min with fractions collected every 20 s.

Known isolated compounds (mg/L): dechlorogriseofulvin (3.8), as yet unidentified, 29.1 min (2.8 mg); cytochalasin D (1.1), 32.5 min (2.0 mg); ellisiiamide A (12) eluted at 32.8 min (2.0 mg); ellisiiamide B (13) eluted at 33.4 min (1.3 mg); and ellisiiamide C (14) eluted at 35.6 min (2.3 mg). Compound fractions, from multiple HPLC runs, were pooled together and dried under N2 gas in pre-weighed vials prior to NMR and optical rotation measurements (Supplementary Figs. 5–31S, Tables 10–12S).

Ellisiiamide A (12) C30H46N5O5; white powder; [α]21 +86.1 (0.18, MeOH); For 1H and 13C NMR (DMSO-d6) spectroscopic data see Supporting Table 9S: HRESIMS (m/z) 556.3501 [M+H]+ (calcd for C30H46N5O5, 556.3493).

Ellisiiamide B (13) C31H47N5O5; white powder; [α]21 +43.1 (0.04, MeOH); For 1H and 13C NMR (DMSO-d6) spectroscopic data see Supporting Table 10S: HRESIMS (m/z) 570.3650 [M+H]+ (calcd for C31H47N5O5, 570.3650).

Ellisiiamide C (14) C32H51N5O5; white powder; [α]21 +74.8 (0.06, MeOH); For 1H and 13C NMR (DMSO-d6) spectroscopic data see Supporting Table 11S: HRESIMS (m/z) 598.3968 [M+H]+ (calcd for C32H51N5O5, 598.3963).

Biological activity screening. Compounds were tested for their minimum inhibitory concentration (MIC) according to the Clinical Laboratory Standards Institute (CLSI) protocols M7-A5 and M27-A (National Committee for Clinical Laboratory Standards, 2000, 1997). Stock working solutions were made to 5, 10, and 20 mg/mL and tested at a maximum concentration of 200 μg/mL in 96-well liquid culture (National Committee for Clinical Laboratory Standards, 1997, 2003) as previously described. Preliminary evaluation of biological activity was against E. coli BW25113 ΔbamBΔtolC, a membrane and efflux pump compromised strain, Staphylococcus aureus ΔBW25113, E. coli ΔbamB, Micrococcus luteus, Saccharomyces cerevisiae B4741, and Candida albicans ATCC# 90028. A cut-off of < 25% growth was used for inhibition, with the trend across dilutions also considered.

Received: 2 October 2019; Accepted: 28 January 2020;
Published online: 12 March 2020

References
1. Cutler, M. An Account of Some of the Vegetable Productions, Naturally Growing in this Part of America: Botanically Arranged. (Lloyd Library, 1903).
2. Turner, N. J. & Aderkas, P. v. J. A. S. B. P. Sustained by First Nations: European newcomers use of Indigenous plant foods in temperate North America. 81 (2012).
3. Miller, J. D. In Endophytes of Forest Trees: Biology and Applications (eds. Anna Maria Pirttilä & A. Carolin Frank) 237–249 (Springer Netherlands, 2011).
4. Kirk, D. et al. Avian assemblages differ between old-growth and mature white pine forests of Ontario, Canada: a role for supercanopy trees. 7 (2012).
5. Petrin, O., Sieber, T. N., Toti, L. & Viret, O. Ecology, metabolite production, and substrate utilization in endophytic fungi. 1, 185–196, https://doi.org/10.1002/mnfr.2016010306 (1993).
6. Li, Z.-J., Shen, X.-Y. & Hou, C.-L. Fungal endophytes of South China blueberry (Vaccinium dunalianum var. urophyllum). 3, 482–487, https://doi.org/10.1111/j.1267.1267 (2016).
7. Carris, L. M. Chalara Vaccinii Sp. Nov., A Vaccinium Endophyte. 8, 875–879, https://doi.org/10.1080/00275514.1988.10227574 (1988).
8. Carris, L. M. A New Species of Dwayalomella from Vaccinium Corymbosum. Mycologia 81, 638–642, https://doi.org/10.1080/00275514.1988.12025797 (1989).
9. Martínez-Álvarez, P., Fernández-González, R. A., Sanz-Ros, A. V., Pando, V. & Diez, J. J. Two fungal endophytes reduce the severity of pitch canker disease in Pinus radiata seedlings. Biological Control 94, 1–10, https://doi.org/10.1016/j.biocontrol.2015.11.011 (2016).
10. Prihatini, L., Glen, M., Wardlaw, T. J. & Mohammed, C. L. Diversity and identification of fungi associated with needles of Pinus radiata in Tasmania. Southern Forests: a Journal of Forest Science 78, 19–34, https://doi.org/10.2989/20702620.2015.1092345 (2016).
11. Rai, T. N. C. Jo. B. Genera coelomycetum. XXI. Strasseria and two new anamorph-genera. Apostrasseria and Nothostrasseria. 61, 1–30 (1983).
12. Carris, L. M. Cranberry black rot fungi: Allantophomopsis cytopsore and Allantophomopsis lycopodina. Canadian Journal of Botany 68, 2283–2291, https://doi.org/10.1139/b90-291 (1990).
55. Ju, Y.-M., Hsieh, H.-M., Vasileva, L. & Akoulov, A. Three new Xylaria species from Russian Far East. Mycologia 101, 548–553 (2009).
56. Eries, E. Elences fungorum, sestens commentarium in Systema mycologicum. Vol. 2 (Symbitus Ernesti Mautitii, 1828).
57. Tanney, J. B. & Seifert, K. A. Lophodermium resinosum sp. nov. from red pine (Pinus resinosa) in Eastern Canada. Botany 95, 773–784, https://doi.org/10.1139/cjb-2017-0012 (2017).
58. Pažoutová, S. et al. A new endophytic insect-associated Daldinia species, recognised from a comparison of secondary metabolite profiles and molecular phylogeny. 60, 107–123, https://doi.org/10.1016/j.scbi.2013.01.039 (2013).
59. Pažoutová, S., Šrůtková, P., Holubík, J., Chudíčková, M. & Kolářík, M. Diversity of xylariaceous symbionts in Xylaria woodwasps: role of vector and a host tree. Fungal. Ecology 3, 392–401 (2010).
60. Tanney, J. B., McMullin, D. R. & Miller, J. D. In Endophytes of Forest Trees: Biology and Applications (eds. Anna Maria Porttila & A. Carolan Frank) 343–381 (Springer International Publishing, 2018).
61. Brian, P. Griseofulvin. Transactions of the British Mycological Society 43, 1–13 (1960).
62. Decker, J. & Tullenaers, I. J. M. G. The antibiotic griseofulvin, some aspects of its mode of action. 28, 574–579 (1963).
63. Napier, E. J., Turner, D. I. & Rhodes, A. The Translocation of Antibiotics in Higher Plants: II. The movement of griseofulvin in broad bean and tomato. Journal of Experimental Botany 7, 42–64 (1956).
64. Park, J.-H.
65. Casella, T. M.
66. Napier, E. J., Turner, D. I. & Rhodes, A. The Translocation of Antibiotics in Higher Plants: II. The movement of griseofulvin in broad bean and tomato. Journal of Experimental Botany 7, 42–64 (1956).
67. Casella, T. M. et al. Antimicrobial and cytotoxic secondary metabolites from tropical leaf endophytes: Isolation of antibacterial agent pyrocidine C from Lewia infectoria SNP-GTC2402. Phytochemistry 96, 370–377, https://doi.org/10.1016/j.phytochem.2013.10.004 (2013).
68. Sica, V. P.
69. Pažoutová, S.
70. Brian, P.
71. Gallo, A., Ferrara, M. & Perrone, G. Phylogenetic Study of Polyketide Synthases and Nonribosomal Peptide Synthetases Involved in the Biosynthesis of Mycotoxins. 5, 717–742 (2013).
72. Schwarzer, D., Finkinger, R. & Marahiel, M. A. Nonribosomal peptides: from genes to products. Natural Product Reports 20, 275–287, https://doi.org/10.1039/B111145K (2003).
73. Walsh, C. T. A chemocentric view of the natural product inventory. Nature Chemical Biology 11, 620, https://doi.org/10.1038/nchembio.1894 (2015).
74. Baral, H. O. Lugolf’s solution/IKI versus Melzer’s reagent: hemiamylolysis, a universal feature of the ascus wall. Mycota 29, 399–450 (1987).
75. Kornerup, A. & Wanscher, J. H. J. M. H. o. C. Methuen handbook of colour (1963).
76. Visagie, C. M.
77. Bruns, T. M., Gaunt, R. A. & Weber, D. J. Estimating bladder pressure from sacral dorsal root ganglia recordings. Conference proceedings:... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference 2011, 4239–4242 (2011).
78. Larena, I., Salazar, O., González, V., Julián, M. A. & Rubio, V. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. Journal of Biotechnology 75, 187–194, https://doi.org/10.1016/S0168-1656(99)00154-6 (1999).
79. Glass, N. L. & Donaldson, G. C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 91, 1323 (1995).
80. Liu, Y. J., Whelen, S. & Hall, B. D. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16, 1799–1808, https://doi.org/10.1093/oxfordjournals.molbev.a026992 ([2005?).
81. Carbone, I. & Kohn, L. M. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553–556 (1999).
82. Vilgalys, R. & Hester, M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. Journal of Bacteriology 172, 4238–4246, https://doi.org/10.1128/jb.172.8.4238-4246.1990 (1990).
83. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution 30, 772–780, https://doi.org/10.1093/molbev/msu010 (2013).
84. Nylander, J. A. A. MrModeltest v2. Program distributed by the author ([2001?].
85. Bruns, T. M., Gaunt, R. A. & Weber, D. J. Estimating bladder pressure from sacral dorsal root ganglia recordings. Conference proceedings:... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference 2011, 4239–4242 (2011).
86. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313 (2014).
87. Ronquist, F. et al. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61, 539–542, https://doi.org/10.1093/sysbio/syq029 (2012).
88. Rambaut, A., Suchard, M., Xie, D. & Drummond, A. (Retrieved from http://beast.bio.ed.ac.uk/Tracer, 2014).
89. Smith, C. A., Want, E. J., O’Maille, G., Abagyan, R. & Suzdak, G. XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. Analytical Chemistry 78, 779–787, https://doi.org/10.1021/ac051437y (2006).
90. Kuhl, C., Tautenhahn, R. & Neumann, S. J. o. n. c. NMR chemical shifts of common laboratory solvents as trace impurities. 62, 7512–7515 (1997).

Acknowledgements

The authors would like to thank M. Kelman (AAFC), T. McDowell (AAFC) and D. Sorensen (McMaster University) for technical assistance. We would like to thank Linda Ejim (McMaster University) for antimicrobial testing of purified compounds. We thank The Center for Microbial Chemical Biology (CMCB), and the Biointerfaces Institutes (BI) at McMaster University for access to state-of-the-art instrumentation. J.B. Tanney thanks Jacques Fournier, Ju Yu-Ming, and Marc Stadler for insightful discussion on Xylaria taxonomy. A.I was
funded through an Ontario Graduate Scholarship (OGS) Doctoral Research Award. This project was funded by an AAFC grant to MWS and KAS. Additional support was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC SYN 479724-15) to J.D. Miller and by J.D. Irving Ltd.

**Author contributions**
Ashraf Ibrahim, Joey Tanney, and Mark Sumarah conducted the primary research. Fan Fei performed the statistical analysis. Ashraf Ibrahim, Mark Sumarah, J. David Miller and Keith Seifert conceived the experiments. Alfredo Capretta discussed research and structural characterization. Chris Cutler provided blueberry samples for endophyte isolation. All authors contributed to the manuscript writing and review.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-61088-x.

Correspondence and requests for materials should be addressed to M.W.S.

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