The North American cranberry fruit rot fungal community: a systematic overview using morphological and phylogenetic affinities

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Cranberry fruit rot (CFR) is caused by many species of fungi, with the contribution of any given species to the disease complex varying among plantings of Vaccinium macrocarpon within a site, sites within regions, and among regions and years. This study assessed the morphological and molecular variability of five widespread CFR-causing fungi: Phyllosticta vaccinii, Coleophoma empetri, Colletotrichum acutatum, Colletotrichum gloeosporioides and Physalospora vaccinii. Although the majority of isolates had morphological characteristics consistent with published descriptions, some were atypical. For example, non-chromogenic isolates of C. acutatum were recovered from British Columbia and white isolates of Physalospora vaccinii were recovered in addition to the more common dark isolates. On the basis of sequence analysis of the ITS and large subunit rDNA (LSU), it appears that Phyllosticta vaccinii, Coleophoma empetri, C. gloeosporioides and C. acutatum are genetically uniform on cranberry in North America. This suggests the possibility that these fungal species were introduced to cultivated cranberries and concomitantly moved with planting material to new locations. In contrast, white isolates of Physalospora had ITS and LSU sequences distinct from those of their dark counterparts, with phylogenetic analyses suggesting that these isolates represent either different species or distinct members of highly divergent populations. Taxonomic placement of all species based on phylogenetic relationships was consistent with morphological placement, with the exception of Physalospora vaccinii. Unlike other Physalospora species, CFR isolates of Physalospora vaccinii were not allied with the Xylariomycetidae; instead, these fungi were members of the Sordariomycetidae. A deeper taxonomic analysis is needed to resolve this inconsistency in familial affiliation.

Keywords: Coleophoma empetri, Colletotrichum acutatum, Colletotrichum gloeosporioides, Phyllosticta vaccinii, Physalospora vaccinii, Vaccinium macrocarpon

Introduction

In North America, more than 10 fungal species are reported to parasitize the native American cranberry (Vaccinium macrocarpon) (Oudemans et al., 1998). Of these, Coleophoma empetri, Colletotrichum acutatum, Colletotrichum gloeosporioides, Phyllosticta vaccinii and Physalospora vaccinii are among the principal species responsible for cranberry fruit rot (CFR), either in the field or after harvest (storage rot) (Stiles & Oudemans, 1999; Blodgett et al., 2002; McManus et al., 2003; Olatinwo et al., 2003, 2004). Fruit rot is a significant threat to cranberry production in all North American growing regions and can be expressed either as a field rot, resulting in reduced berry yield, or as a storage rot that affects the quality of fresh fruit. In New Jersey and Massachusetts, the field-rot phase can destroy 50–100% of the fruit if the crop is not protected by fungicides (Oudemans et al., 1998). Even with the use of fungicides, fruit rot in New Jersey and Massachusetts ranged from less than 1% up to 15% (Oudemans et al., 1998). In Wisconsin, field rot is more sporadic, varying widely among years and locations (McManus et al., 2003). In the western cranberry-growing regions of Washington, Oregon and British Columbia, field rot is pervasive, although the losses sustained are generally lower than those experienced in the East (Oudemans et al., 1998). Even though the importance of different fungal species in the CFR disease complex varies

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among regions, recommendations for fungicide application are similar across North America (Oudemans & Vaiciunas, 2005; Caruso, 2006).

The taxonomy of the CFR fungi has never been examined at the molecular level, although it has long been assumed that each of the CFR pathogens conforms to individual species. Within-species population structure and genetic diversity are similarly obscure for these organisms. Given the disparity in the composition of the CFR disease complex across the major North American cranberry-growing regions, determining the identity and genetic variability of each of the major CFR pathogens may prove to be particularly important for the development of DNA markers for genotypic analyses of CFR. Such analyses would shed light on community structures, which could ultimately lead to the development of more targeted, precise and effective disease-management programmes.

This paper describes a combined molecular and morphological approach to address three broad objectives, providing an initial glimpse into the taxonomy and variation of five important CFR fungal pathogens. First, the morphological variability in culture within a species for isolates collected from the primary cranberry-growing regions of North America was investigated. Secondly, genetic variability within each of the five species was evaluated through analysis of the nuclear internal transcribed spacer (ITS) sequence. Thirdly, using both morphological and molecular sequence data from the ITS and nuclear large subunit (LSU) rDNA, the taxonomic identities and phylogenetic placement of the five CFR pathogens were examined.

**Materials and methods**

**Pathogen collection and isolation**

Rotted cranberry fruits were collected from commercial cranberry farms in New Jersey (NJ), Massachusetts (MA) and Wisconsin (WI) in the USA, and British Columbia (BC) in Canada, during the growing season as well as during harvest (September 2005 and September 2006). Berries were collected from 18–24 geographically separate locations from each region and fungal isolates of the same species were always selected from different fields in an attempt to maximize genetic diversity. The isolates were further selected from within each growing region to maximize morphological variability. For example, isolates displaying different growth rates or distinct colony morphology, e.g. colour and growth pattern, were chosen to represent the greatest possible diversity. Initially, a goal of five isolates of each species was targeted for each region. However, after completing the post-harvest sampling, certain species were rare or absent in one or more regions, whereas other species were common and more isolates were selected.

To isolate the fruit-rotting organisms, whole berries were surface-sterilized for 5–20 min in 10% household bleach (final concentration 0.525% sodium hypochlorite), rinsed in sterile water, cut in half and placed cut-side-down on Petri plates containing solid medium [V8 juice agar (Miller, 1955), potato dextrose agar (PDA; Difco, Becton, Dickinson and Company), or corn meal agar (CMA; BBL, Becton, Dickinson and Company)]. If CMA was used, the medium was supplemented with either lactic acid or streptomycin to inhibit bacteria. The plates were incubated at room temperature for 5–10 days and fungi growing from the rotted fruits were subcultured on fresh plates. Attempts were made to obtain isolates of five important CFR-causing species, namely *Coleophoma empetri*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Phyllosticta vaccinii* and *Physalospora vaccinii* from each of the four growing regions (Table 1). Some species and/or morphological variants were not recovered from some growing regions, as detailed in Table 1.

**Morphological assessment**

All pure cultures were grown in the laboratory on V8 juice agar at 25°C for 7–21 days under ambient light. Species identifications were performed using key morphological characters including colony morphology and colour, spore size and shape, as well as the morphological characteristics of teleomorphic stages, if present (Barr, 1970; Weidemann, 1980; Brown, 1982; Sutton, 1992).

**Genomic DNA isolation**

For DNA extraction, fungal cultures were grown on V8 juice agar; after 7–10 days, aerial mycelium was harvested using a sterile spatula. Approximately 20 mg mycelium was suspended in 500 μl cetyl trimethylammonium bromide (CTAB) buffer (Stewart & Via, 1993) and frozen at −20°C for at least 30 min, followed by heating at 65°C for 30 min. The suspension was extracted twice with an equal volume of chloroform. After centrifugation at 12 000 g for 5 min, the aqueous supernatant was transferred to a new tube and nucleic acid was precipitated by adding 0.7 volumes of isopropanol to each tube, followed by centrifugation at 12 000 g for 10 min. Pellets were resuspended overnight at 4°C in 1 ml Tris-EDTA (ethylenediaminetetraacetic acid) (TE) buffer. Nucleic acids were incubated with 0.50 μg RNase A at 37°C for 30 min, followed by chloroform extraction. After a final ethanol precipitation, DNA was resuspended in 25 μl TE (pH 7.6).

**PCR amplification and sequencing**

The nuclear internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA gene and ITS 2 region (hereafter this entire region, including internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, is referred to as the ‘ITS region’) was amplified by PCR using primers ITS1 and ITS4 (White et al., 1990). A portion of the nuclear large subunit ribosomal RNA (28S ribosomal RNA gene, partial sequence, hereafter referred to as ‘LSU’) sequence was PCR-amplified using the primers LROR and LR5 (Vilgalys & Hester, 1990). Amplification reactions contained 1X PCR buffer A (Fisher Scientific),...
were assembled and edited using various modules of the automated sequencer (Beckman Coulter). Sequence data were obtained using the QuickStart kit (Beckman Coulter) and run on a CEQ 8000 instrument. Reactions were performed in both directions from PCR amplicon or plasmid DNA templates using the DCT S reaction mix. Sequencing was performed using the ExoSAP-IT (USB Corp.) according to the manufacturer’s protocol. In the few instances where PCR products were not of sufficient quantity and/or quality for direct sequencing, the amplicon was ligated into the pGemT vector (Promega Corp.) according to the manufacturer’s protocol. Sequence analyses were performed using the program T-COFFEE (Poirot et al., 2003) of the National Center for Biotechnology Information (NCBI) GenBank database. Multiple sequence alignments were conducted with T-COFFEE (Poirot et al., 2003) according to the manufacturer’s protocol and transformed into E. coli strain DH5α. Plasmids with inserts of the correct size were purified using the UltraClean plasmid miniprep kit (MoBio Labs). Sequencing reactions were performed in both directions from PCR amplicon or plasmid DNA templates using the DCT S Quick Start kit (Beckman Coulter) and run on a CEQ 8000 automated sequencer (Beckman Coulter). Sequence data were assembled and edited using various modules of the LASERGENE software package (DNAStar Inc.).

Sequence analyses

Similarities of the sequence data (ITS region and LSU) from this study to published sequences were determined by performing BLAST searches (Altschul et al., 1990) of the National Center for Biotechnology Information (NCBI) GenBank database. Multiple sequence alignments were prepared using the program T-COFFEE (Poirot et al., 2003), followed by manual refinements in the program BIOEDIT 7.0.9 (distributed by the author at http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Gaps were re-coded as multistate characters, with any questionably aligned sites excluded from the dataset. Phylogenetic trees were generated from maximum likelihood (ML) searches executed in PAUP* v.4.0b10 (Sinauer Associates) using heuristic search strategies with 10 random addition sequence replicates and tree-bisection reconnection branch swapping. Bootstrap analyses were performed using 100 replicates. Bayesian posterior probabilities supporting the ML tree topologies were generated using MRBAYES v.3.1 (Huelsenbeck & Ronquist, 2003) by performing two simultaneous runs of one cold and four incrementally heated Metropolis-coupled Monte Carlo Markov chains (MCMC) for 5 000 000 generations and sampling trees every 500 generations. Trees drawn from the posterior probability distribution were identified using TRACER v.1.3 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer), then imported into PAUP* v.4.0b10 and used to construct a 50% majority-rule consensus tree from which support values were derived. The best fit model of DNA sequence evolution, as identified using the Akaike information criterion (AIC) in MODELTEST v.1.7 (Posada & Crandall, 1998) was incorporated into all likelihood and Bayesian analyses (Table 2).

Results

Physalospora vaccinii

Physalospora vaccinii was isolated, with varying frequency, from all cranberry-growing regions except BC; 18 isolates were evaluated morphologically and through DNA sequence analysis (Table 1). Morphological features were consistent with published descriptions for the species (Barr, 1970). Colonies grown on V8 juice agar were generally petaloid with mycelium closely appressed to the agar surface. Perithecia developed immersed in the growth medium. Ascospores were ejected from perithecia within 14–21 days and adhered to the surface of the Petri dish lid, as previously described for this species (Brown, 1982). Although most isolates were morphologically similar, there was considerable variation in pigmentation. Isolates ranged from varying shades of grey to pure white and sectoring was very common (Fig. 1A). The pure white isolates (Fig. 1B) were less common, with only a single isolate collected from NJ and three from WI. The darker isolates sporulated profusely, while the white isolates sporulated sparsely. Those isolates that were varying shades of grey all had a similar ascospore size and shape (obovoid with blunt ends and 26–32 × 12–17 μm in size) (Fig. 1J). In contrast, pure white isolates possessed

### Table 1 Summary of cranberry fruit fungal isolates used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates</th>
<th>BC</th>
<th>WI</th>
<th>NJ</th>
<th>MA</th>
<th>GenBank accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleophoma empetri</td>
<td></td>
<td>8</td>
<td>13</td>
<td>11</td>
<td>10</td>
<td>FJ480104-FJ480145</td>
</tr>
<tr>
<td>Physalospora vaccinii</td>
<td></td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td>FJ603575-FJ603591</td>
</tr>
<tr>
<td>Physalospora vaccinii (Dark)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>FJ603593, FJ603595-FJ603596, FJ603600-FJ603610</td>
</tr>
<tr>
<td>Physalospora vaccinii (White)</td>
<td></td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>FJ603594, FJ603597-FJ603599</td>
</tr>
<tr>
<td>Colletotrichum acutatum (C)</td>
<td></td>
<td>10</td>
<td>11</td>
<td>9</td>
<td>3</td>
<td>FJ478047-FJ478075, FJ478077, FJ478080-FJ478082</td>
</tr>
<tr>
<td>Colletotrichum acutatum (NC)</td>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>FJ478076, FJ478078, FJ478079, FJ478083-FJ478085</td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td></td>
<td>2</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>FJ539181-FJ539191</td>
</tr>
</tbody>
</table>

*Two types of colony phenotype were found for Physalospora vaccinii: varying degrees of dark and ‘pure’ white. 

Colletotrichum acutatum was chromogenic (C; red diffusible pigment) or nonchromogenic (NC).

BC, British Columbia; WI, Wisconsin; NJ, New Jersey; MA, Massachusetts.

0.2 mM dNTPs, 2.5 U Taq polymerase (Fisher Scientific), 0.2 μM primers and approximately 5 ng template DNA in a final volume of 20 μL. All PCR consisted of one cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min; and a final cycle of 72°C for 5 min. After amplification, PCR reactions were treated with ExoSAP-IT (USB Corp.) according to the manufacturer’s protocol. In the few instances where PCR products were not of sufficient quantity and/or quality for direct sequencing, the amplicon was ligated into the pGemT vector (Promega Corp.) according to the manufacturer’s protocol and transformed into E. coli strain DH5α. Plasmids with inserts of the correct size were purified using the UltraClean plasmid miniprep kit (MoBio Labs). Sequencing reactions were performed in both directions from PCR amplicon or plasmid DNA templates using the DCT S Quick Start kit (Beckman Coulter) and run on a CEQ 8000 automated sequencer (Beckman Coulter). Sequence data were assembled and edited using various modules of the LASERGENE software package (DNAStar Inc.).

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spores distinct from the pigmented types (Fig. 1K), with greater length (44–50 \( \mu \)m) and width (16–19 \( \mu \)m).

Alignment of the ITS dataset was unambiguous, and the introduction of only three gaps was adequate to accommodate indels. Sequence variation in the 484-bp ITS region of this sample was minimal, with 95% of the characters constant and pairwise distances between ingroup taxa ranging from 0.00 to 0.03. Two major groups dividing the white \((n=4)\) and dark isolates \((n=14)\) were recovered through phylogenetic analysis of the dataset (Fig. 2); thus differences in pigmentation and spore morphology corresponded with molecular characters. From a taxonomic standpoint, species of *Physalospora* are reported to be members of the Sordariomycete subclass Xylariomycetidae (Eriksson & Winka, 1998), and although morphologically the isolates evaluated in this study are well within the circumscription of *Physalospora vaccinii*, molecular identification was problematic. BLAST searches of the NCBI GenBank database (performed 9 December 2007) identified the ITS sequences from two unpublished *Physalospora vaccinii* isolates from cranberry (DAOM 229646 and DAOM 229650; accessions AY075113 and AY075112, respectively) as sharing significant sequence similarity to the ITS sequences generated in the present study (98% maximum identity, e-values = 0). However, outside of these two sequences, BLAST searches showed the ITS region sequence data from this study to be most similar to members of the Sordariomycete subclass Sordariomycetidae, including *Podospora anserina*, *Chaetomium globosum*, *Thielavia hyalocarpa* and *Chaetosphaeria fusiformis* (86–88% maximum identity; e-values 1e-127 to 2e-118). Because the only isolates of *Physalospora* spp. in the GenBank database associated with published systematic analyses consisted of LSU or SSU ribosomal sequence data, not the ITS region, the LSU from four CFR isolates [two white (*Physa57, Physa62*) and two dark (*Physa53 and Physa74*)] was sequenced and these data used to perform phylogenetic analyses that included the vouchered isolates of *Physalospora* posted on GenBank and representative members of the Xylariomycetidae and the Sordariomycetidae (Fig. 3). The *Physalospora vaccinii* isolates from cranberry noted above (accession numbers AY075113 and AY075112) were not used in these

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Length</th>
<th>Model</th>
<th>Base frequencies</th>
<th>Proportion invariable sites</th>
<th>Gamma shape (alpha)</th>
<th>Rate matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coleophoma empetri</em></td>
<td>ITS</td>
<td>477 bp</td>
<td>GTR+G+I</td>
<td>0.21/0.28/0.25/0.26</td>
<td>0.53</td>
<td>2.82</td>
<td>A → C=2.07</td>
</tr>
<tr>
<td></td>
<td>LSU</td>
<td>883 bp</td>
<td>TrN+I+G</td>
<td>0.27/0.19/0.30/0.24</td>
<td>0.60</td>
<td>0.56</td>
<td>A → C=1.00</td>
</tr>
<tr>
<td><em>Colletotrichum acutatum</em></td>
<td>ITS</td>
<td>517 bp</td>
<td>JC+G</td>
<td>Equal</td>
<td>–</td>
<td>1.73</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LSU</td>
<td>886 bp</td>
<td>TrN</td>
<td>0.25/0.23/0.30/0.21</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Colletotrichum gloeosporioides</em></td>
<td>ITS</td>
<td>464 bp</td>
<td>TrN+I</td>
<td>0.22/0.29/0.26/0.23</td>
<td>0.9174</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Phyllosticta vaccinii</em></td>
<td>ITS</td>
<td>592 bp</td>
<td>TrNef</td>
<td>Equal</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Physalospora vaccinii</em></td>
<td>ITS</td>
<td>484 bp</td>
<td>TrN+I</td>
<td>0.24/0.29/0.27/0.20</td>
<td>0.77</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LSU</td>
<td>961 bp</td>
<td>TrN+I+G</td>
<td>0.25/0.22/0.32/0.21</td>
<td>0.07</td>
<td>0.56</td>
<td>A → C=1.00</td>
</tr>
</tbody>
</table>

Table 2 Characteristics of the ITS and LSU regions analysed for the five cranberry fruit rot fungal species, as determined using the Akaike information criterion (AIC) in MODELTEST.
analyses since the LSU sequence data available for these isolates were much shorter than those used for all other isolates. Similar to the results obtained using the ITS data, a BLAST search (performed 15 May 2008) using the LSU from this study data identified only members of the Sordariomycetidae as similar to the CFR Physalospora vaccinii isolates here. None of the GenBank accessions of Physalospora were recovered through the search.

**Colletotrichum acutatum**

*Colletotrichum acutatum* was isolated from all four cranberry-growing regions. All 28 isolates from NJ, MA and WI and five isolates (of 11) from BC exhibited typical morphological features when grown on V8 juice agar (Table 1, Fig. 1D). These morphologically typical isolates all possessed grey mycelium and produced red pigmentation, most visible on the reverse of the culture plates (Fig. 1E). All *C. acutatum* isolates produced abundant acervuli on both the original isolation plates with rot- ted fruit and on V8 juice agar with characteristic masses of orange spores. Conidia were fusiform, with sizes consistent with the current circumscription of *C. acutatum* (10–12 × 3–4 μm) (Fig. 1L) (Sutton, 1980, 1992).

In addition to normally pigmented isolates of *C. acutatum* (Fig. 1D, E and C-left side), atypical non-chromogenic isolates were also recovered from BC, accounting for six of the 11 isolates collected from this region (Table 1, Fig. 1C-right side). Apart from their colour, the atypical, non-chromogenic isolates were morphologically similar to the standard, chromogenic isolates. Comparison of the ITS region and LSU sequences generated in the present study against sequences curated in GenBank (from BLAST search performed 9 December 2007) supported the morphological identification of these isolates as *C. acutatum*.

Multiple sequence alignment of the ITS region for the 34 *C. acutatum* isolates indicated that 56% of the 475 nucleotides were constant among the ingroup taxa, with pairwise distances from 0.00 to 0.42. Few gaps were introduced into the alignment, except to accommodate 46 indels present in isolate Cacu214 but not the other sequences. From the phylogenetic analysis, two sub- groups were generated, but these were not well supported...
Figure 2 Midpoint rooted maximum likelihood ITS gene tree illustrating the evolutionary relationships of the putative Physalospora vaccinii isolates from cranberry [shown as Physa(isolate number)] obtained from North America (Table 1) alongside the Sordariomycete fungi which were identified as sharing the greatest sequence similarity through BLAST searches of NCBI GenBank. Bayesian posterior probabilities and bootstrap values supporting the topology are shown below the branches (posterior probability/bootstrap); support values below 50 are not labelled. ITS region sequences can be accessed through GenBank under accession numbers FJ603593–FJ603610.

Figure 3 LSU maximum likelihood gene tree illustrating the evolutionary relationships of the Physalospora vaccinii isolates from cranberry [shown as Physa(isolate number)] obtained from North America (Table 1) alongside sequences curated by NCBI GenBank. Bayesian posterior probabilities and bootstrap values supporting the topology are shown below the branches (posterior probability/bootstrap); support values below 50 are not labelled. The tree is rooted using members of the Diaporthales as outgroup taxa. LSU sequences can be accessed through GenBank under accession numbers FJ588246–FJ588249.
by the Bayesian posterior probabilities (not shown); nor did bootstrap resampling of the dataset using maximum parsimony optimality criteria support the subgroups. The 878-bp LSU sequences generated from five C. acutatum isolates (Cacu154, Cacu156, Cacu167, Cacu176 and Cacu202) were invariable except for two nucleotides (not shown). When the C. acutatum LSU data were used to construct a phylogeny using C. acutatum, C. phormii, C. nymphaeae and C. luteostramine C. luteostramine sequences curated by GenBank, 99% of the nucleotide positions were constant. Based upon these low levels of variability, four of the isolates grouped with C. acutatum, while isolate Cacu202 was more closely associated with C. phormii, C. luteostramine and C. nymphaeae (not shown), but support for the topology was too low to be definitive.

**Colletotrichum gloeosporioides**

*Colletotrichum gloeosporioides* was found only in samples collected from BC and NJ (Table 1). All C. gloeosporioides isolates possessed similar morphologies in culture (Fig. 1F, G). Isolates of C. gloeosporioides were easily distinguished from *C. acutatum* through differences in colony colour and spore morphology consistent with those described in the literature (Sutton, 1980, 1992), and the intermittent presence of the teleomorph (*Glomerella cingulata*). Colonies were uniformly grey and non-chromogenic, with no diffusible pigment visible in the growth media. Pale yellow masses of conidia were produced in acervuli on both the rotted cultured fruits and on V8 juice agar. Conidia were rounded at the ends and variable in size (10–25 × 4–5 µm) (Fig. 1M). Perithecia sometimes formed on the rotted fruits and in pure culture. Ascospores, when present, were allantoid to oblong elliptical and measured 12–20 × 3.5 µm (Fig. 1N).

Nucleotide similarity of the ITS sequences between the 17 isolates of *C. gloeosporioides* ranged from 96 to 100% identity in the 464-nt dataset, with 98% of the characters constant; pairwise distances ranged from 0.00 to 0.005. Phylogenetic comparison of ITS sequences with published sequences (BLAST searches performed 9 December 2007) confirmed that the cranberry isolates sampled in the present study were members of the genus *Phyllosticta*. The *Phyllosticta* sequences in this study grouped in a sister clade relative to isolates identified as *Phyllosticta pyralae*, including a sample isolated from Ericaceous plants in Japan (Fig. 5). Similarity was also high to an unpublished GenBank sequence of *Phyllosticta vaccinii* (EF672227) from cranberry and several sequences from *Guignardia* spp., the teleomorph of *Phyllosticta*. Based upon the ITS tree topology, the inclusion of the GenBank sequence identified as *Phyllosticta vaccinii* rendered the species paraphyletic, as an isolate of *Phyllosticta elongata* was positioned within the group. This result suggests that the taxonomic structure of the *Phyllosticta* species needs to be further investigated. Although Weideman et al. (1982) demonstrated convincingly that *Phyllosticta vaccinii* and *Phyllosticta elongata* are morphologically distinct species, a more in-depth study of this genus should be conducted.

**Phyllosticta vaccinii**

*Phyllosticta vaccinii* was isolated from all growing regions except BC. Colonies were slow growing, irregular in shape, and dark grey to dark green (Fig. 1H). Pycnidia were densely packed on the surface of the V8 juice agar and on fruits placed on culture medium. Conidia were obovate, 6.9 × 10 µm with a short gelatinous appendage (Fig. 1O). Thus, morphological features were consistent with published descriptions of the fungus (Weidemann et al., 1982).

The sequences of the ITS region from *Phyllosticta vaccinii* exhibited little polymorphism. Of 592 nucleotides, 99% were constant among the ingroup taxa. The LSU from three representative isolates was sequenced (Phyllo101, Phyllo106 and Phyllo122); this region (LSU) exhibited variation at only one of the 939 bases (not shown). Phylogenetic comparisons of both the ITS and LSU with sequences curated on GenBank (BLAST searches performed 9 December 2007) confirmed that the cranberry isolates sampled in the present study were members of the genus *Phyllosticta*. The *Phyllosticta* sequences in this study grouped in a sister clad relative to isolates identified as *Phyllosticta pyralae*, including a sample isolated from Ericaceous plants in Japan (Fig. 5). Similarity was also high to an unpublished GenBank sequence of *Phyllosticta vaccinii* (EF672227) from cranberry and several sequences from *Guignardia* spp., the teleomorph of *Phyllosticta*. Based upon the ITS tree topology, the inclusion of the GenBank sequence identified as *Phyllosticta vaccinii* rendered the species paraphyletic, as an isolate of *Phyllosticta elongata* was positioned within the group. This result suggests that the taxonomic structure of the *Phyllosticta* species needs to be further investigated. Although Weideman et al. (1982) demonstrated convincingly that *Phyllosticta vaccinii* and *Phyllosticta elongata* are morphologically distinct species, a more in-depth study of this genus should be conducted.

**Coleophoma empetri**

*Coleophoma empetri* was isolated from all growing regions sampled. Cultures were dark grey to black on V8 juice agar (Fig. 11). Cultures often developed white crystals in the medium as they aged. Margins were smooth and often lobed. Pycnidia (65–100 µm in diameter) were common on the surface of fruits and on the edges where fruits contacted the growth medium. Pycnidia were absent in pure culture. Mature pycnidia had an obvious circular ostiole and oozed conidia. Conidia (12–18 × 2–4 µm) were cylindrical, guttulate and hyaline (Fig. 1P). Paraphyses were present among the conidiophores. The observed morphological characteristics were within the species circumscription (Duan et al., 2007).

This species exhibited little ITS sequence variation among the isolates sampled in the present study, with 92% of the 477 nucleotides invariable and pairwise distances ranging from 0.00 to 0.22 between the ingroup taxa. Although variability was identified at the nucleotide...
level, it did not translate into phylogenetic distinctness, as the 42 C. empetri isolates grouped into 15 haplotypes, all of which formed a polytomy within the tree topology (Fig. 6). BLAST searches (performed 9 December 2007) showed the 42 cranberry isolates of this study to be similar to an isolate from cranberry posted as Gloeosporium sp. (EF672242), several endophytes and members of the Leotiomycetes/Helotiales group, consistent with the broader taxonomic placement of Coleophoma spp. There was one ITS sequence of Coleophoma sp. (EF672243) from cranberry curated by the database, but none of the isolates of this study were similar to that sequence. Given that the Coleophoma sequence (EF672243) in GenBank did not possess significant ITS sequence similarity to any member of the Leotiomycetes/Helotiales group, it is likely that the sequences of this study were legitimate C. empetri isolates, while the GenBank sequence was incorrectly classified.

To more robustly test the ITS-based placement of the C. empetri samples within the Helotiales clade, the LSU
of four representative isolates was sequenced (Coleo1, Coleo8, Coleo14 and Coleo27). The LSU sequences from the four *C. empetri* isolates were very similar to each other, with 99% of the 883 nucleotide bases invariant. **BLAST** searches of GenBank showed the *C. empetri* LSU sequences as most similar to fungi in the order Helotiales, including *Cryptosporiopsis actinidae* Epacris pulchella and *Neofabraea alba*. Phylogenetic analysis using the LSU sequence data from the four *C. empetri* isolates and well-characterized species of the Helotiales confirmed the placement of the *C. empetri* isolates from the present study as a member of this order, within the family Dermateaceae (not shown).

**Discussion**

The five CFR fungal pathogens evaluated in this research were described on cultivated cranberry plants early in the 20th century (Shear et al., 1931). Despite the tremendous impact of these pathogens on the cranberry industry, molecular methods have not previously been used to evaluate the taxonomic identity of these organisms, and, until this work, identification of CFR fungi was based solely upon morphological characters. A recent publication described the development of DNA array hybridization for the detection of CFR fungi from rotted fruit, and although the system was tested for cross reactivity to related fungi, that paper did not specifically evaluate the taxonomic identity of the CFR pathogens (Robideau et al., 2008). Using ITS and LSU sequence data, all of the CFR pathogen species examined in this study were shown to represent distinct taxonomic units with minimal intra-specific sequence variation. From a practical standpoint, the results confirm that the ITS sequence could be developed into an effective molecular-marker-based identification system to determine the presence or absence of these fungal species in the CFR disease complex.

Several questions became apparent when the sequence data were used as queries to search the GenBank sequence database, particularly because most of the sequence data available were not associated with well-described specimens. Very few sequences are curated by GenBank for *Physalospora vaccinii*, *Phyllosticta vaccinii* or *C. empetri*, and in some cases the sequences present in the database complicated rather than simplified the analyses. In three instances, uncorroborated, directly submitted sequences were inconsistent with the data and analyses of this study.

In the first occurrence, ITS sequences from the isolates of *Physalospora vaccinii* here showed significant similarity with two isolates accessioned as *Acanthorhynchus vaccinii* from cranberry (AY075112, AY075113; Fig. 2) which were not yet associated with any publication or voucher specimen (deposited in GenBank 24 January 2002). It is unclear why the isolates in GenBank were identified as *Acanthorhynchus* instead of *Physalospora*, since *Acanthorhynchus* is the former name for this genus and data supporting a taxonomic change have not been published. Barr (1970) supported inclusion of *Physalospora vaccinii* in the genus *Physalospora*, but noted that it should be classed in the subgenus *Acanthorhynchus*. All of the *Physalospora* species found on Ericaceae belong to this same subgenus.

A second inconsistency emanating from GenBank sequence identification occurred when the single GenBank-curated *Coleophoma* sp. from cranberry (EF672243) did not align with this study’s sequences for this species. Given that the *C. empetri* isolates evaluated in the present study were both morphologically consistent
with published descriptions and phylogenetically positioned correctly within the Leotiomycetes/ Helotiales group using both the ITS and LSU (Fig. 6; LSU data not shown), it was concluded that the C. empetri isolates here were authentic for the species. In contrast, it appeared that EF672243, not predicted as a member of the Helotiaceae, had been incorrectly identified and accessioned as Coleophoma sp. Based entirely upon BLAST search similarity (9 December 2007), sequence EF672243 may have been generated from an isolate of Phoma sp. from cranberry.

A third irregularity arose during the phylogenetic analysis of Phyllosticta. All 17 of the Phyllosticta vaccinii isolates obtained from cranberry in this study formed a single, well-supported monophyletic group, distinct from the isolate of Phyllosticta elongata isolated from cranberry in this study (Fig. 5). However, the single ITS sequence from cranberry curated as Phyllosticta vaccinii on GenBank (EF672227) was quite different at the molecular level from this study’s isolates of both Phyllosticta vaccinii and Phyllosticta elongata, and introduction of the sequence rendered Phyllosticta vaccinii paraphyletic. Morphology confirmed the isolates of Phyllosticta vaccinii and Phyllosticta elongata from this study as authentic for their respective species, suggesting two possibilities. First, the separation of these two species using morphological characteristics (Weidemann et al., 1982) may not reflect species boundaries as defined through phylogenetic methodology, which would mean that P. vaccinii is far more variable than suggested by the geographically diverse sample of 17 isolates in this study. Alternatively, another species of Phyllosticta (in addition to Phyllosticta vaccinii and Phyllosticta elongata) might be associated with the CFR disease complex.

The results from the phylogenetic analyses suggest the novel theory that the fungal isolates from cranberry currently described as Physalospora vaccinii are not members of the genus Physalospora in the Sordariomycete subclass Xylariomycetidae, but instead fall within the limits of the Sordariomycete subclass Sordariomycetidae. The ITS and LSU sequence data were extremely useful in the present study to identify the taxonomic inconsistencies surrounding the current circumscription of Physalospora vaccinii. However, multi-locus molecular systematics and more expansive morphological analyses are necessary to properly describe and name the fungus, and to place it within a family. LSU data showed the CFR isolates as most closely affiliated with the Sordariomycete incertae sedis group, but were inadequate for the purpose of determining whether these fungi are part of the order, part of another known order, or comprise an entirely new order within the Sordariomycetes. Importantly, the specific identity of the CFR isolates needs to be directly addressed through the comparison with authentic voucher isolates of Physalospora and other members of the Xylariomycetidae and Sordariomycetidae. Additional molecular screening of CFR fungi morphologically consistent with Physalospora vaccinii should also be undertaken to determine if true members of the genus Physalospora actually contribute to the disease complex.

On a finer scale, colony pigmentation, spore morphology and analysis of both the ITS and LSU sequence data clearly established that the white isolates of Physalospora vaccinii were distinct from the morphologically more typical dark isolates of the fungus. These data suggest that the Physalospora vaccinii dark and white isolates may be distinct species; however, based on the present analyses, the possibility of gene flow connecting the two groups cannot be ruled out. A larger sample size, multilocus evaluations and higher levels of polymorphic data are required to confidently resolve the relationships between these distinct groups.

Two distinct species of Colletotrichum contribute to the CFR disease complex – C. acutatum and C. gloeosporioides (Stretch, 1995; Stiles & Oudemans, 1999). In this study, the ITS gene tree topology and associated Bayesian posterior probability and bootstrap values support the conclusion that the C. gloeosporioides sensu lato isolates from cranberry are more closely allied with C. kahawae, a pathogen of coffee (Fig. 4; Buddie et al., 1999) than with other isolates of C. gloeosporioides. Despite the well-supported nesting of cranberry isolates within the C. kahawae group, given the uncertainty currently surrounding Colletotrichum systematics – particularly within the C. gloeosporioides species group (Cannon et al., 2000) – these results should be interpreted cautiously. Although the majority of molecular systematic studies performed on the genus Colletotrichum to date have been limited to the generation of ITS gene trees, the results from several studies have demonstrated that this region alone is inadequate for taxonomic purposes and routinely generates poorly supported relationships (e.g. Moriwaki et al., 2002; Crouch et al., 2005). Therefore, for the genus Colletotrichum, ITS gene trees are best used to generate hypotheses regarding more detailed evolutionary studies. The development and use of variable protein-coding genes and intronic regions have become increasingly common in recent years for Colletotrichum, and useful marker regions include sequences from the beta-tubulin, Sod2, HMG-box, glutamine synthase and the glyceraldehyde-3-phosphate dehydrogenase genes (Du et al., 2005; Crouch et al., 2006; Farr et al., 2006; Liu et al., 2007). More recently, the DNA lyase and mating type genes have been used as effective phylogenetic and population markers for the C. graminicola group (Crouch et al., 2009) and the DNA lyase, mating type and translation elongation factor genes are being developed for use in systematic studies of C. gloeosporioides (P. R. Johnston, S. Rehner, personal communication). The continued usage of the name C. gloeosporioides sensu lato, is therefore recommended until larger-scale resolution of this group affords a more precise and stable taxonomic assignment for the cranberry-associated members of this taxon.

The morphological and sequence data in this study demonstrated that both the chromogenic and non-chromogenic isolates of C. acutatum from cranberry
were members of the *Colletotrichum* taxon. At the molecular level, the cranberry isolates of *C. acutatum* were almost identical to isolates of the fungus described from grape, apple, blueberry and heavenly bamboo (*Nandina domestica*). As with *C. gloeosporioides*, *C. acutatum* is an acknowledged group species (Sutton, 1980, 1992), and as the present data attest, ITS and LSU sequences alone are not capable of adequately resolving questions regarding intraspecific relationships for this species. Whether this close molecular identity reflects a genuinely non-specialized, genetically invariant species or has resulted from sampling performed in a non-representative, invariant genomic region is unknown, but the identification of the cranberry-infecting isolates of this study as *C. acutatum* was unambiguous. Studies performed in the *C. acutatum* system using additional genomic regions, including the HMG-box, glutamine synthase, glycoldehyde-3-phosphate dehydrogenase and beta tubulin, show that invariability within the ITS region, rather than an invariant species, is more likely to be the case, as well-supported specific groups have been identified in many *C. acutatum* study systems (Talhinhas et al., 2002; Guerber et al., 2003; Du et al., 2005; Sreenivasaprasad & Talhinhas, 2005).

With the exception of pigmentation, the non-chromogenic isolates from BC were morphologically consistent with descriptions published for *C. acutatum*, and were likewise morphologically identical to the standard, chromogenic cranberry isolates. Since these isolates were recovered only from BC, it is possible that the observed variation in coloration represented a local mutation. ITS sequence data separated the chromogenic and non-chromogenic isolates into separate haplotypes based on few nucleotide differences, but from a phylogenetic perspective the separation into two groups was not statistically supported.

For purposes of propagation, cranberry vines have been moved freely between growing regions for decades, supporting the hypothesis that associated fungal pathogens have also inadvertently been transported across North America. In support of this hypothesis, the present data show that, with the exception of the sexually reproducing *Physalospora vaccinii*, the overall trend for these species of CFR-associated fungi is one of very low genetic diversity. However, the possibility that this pattern is the result of a lack of resolving power from the ITS and LSU sequence data cannot be entirely discounted. More detailed population-level investigations of *C. acutatum*, *C. gloeosporioides*, *C. empetri*, *Physalospora vaccinii* and *Phyllosticta vaccinii* genetic diversity will require the implementation of highly polymorphic markers derived from multiple loci, such as AFLPs (amplified fragment length polymorphisms) (Bonin et al., 2007) or microsatellites. However, the results of the present study offer some insight into the diversification of CFR pathogen populations across North America and further provide a foundation for additional research aimed at resolving both taxonomic positioning and population-level questions proposed by this research.

**References**


Garos FL 2006. Evaluation of different fungicide sequences for control of field and storage rot in cranberries, 2005. *Fungicide and Nematicide Tests (online)* 61, SMF002.


