DNA Barcoding Reveals Diversity of *Cytospora* Species Associated with Branch Dieback and Canker Diseases of Woody Plants in Canada

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Article

DNA Barcoding Reveals Diversity of *Cytospora* Species Associated with Branch Dieback and Canker Diseases of Woody Plants in Canada

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Abstract: Branch dieback and canker diseases caused by *Cytospora* species negatively affect health of woody plants worldwide. In this study, 59 *Cytospora* isolates were obtained from symptomatic trees and shrubs growing in southwest Ontario and Saskatchewan, Canada. DNA barcoding approach combined with morphological (culture) characterization identified 15 known species of *Cytospora* associated with the diseases: *C. chrysosperma*, *C. curvata*, *C. euonymina*, *C. hoffmannii*, *C. kantschavelii*, *C. leucosperma*, *C. leucostoma*, *C. nitschkeana*, *C. piceae*, *C. populina*, *C. pruinopsis*, *C. pruinosa*, *C. ribis*, *C. schulzeri*, and *C. sorbina*. The obtained results contribute to the study of diversity, host affiliation, geographical distribution, and pathogenicity of *Cytospora* species occurring on woody plants in both natural habitats and agricultural systems. The findings also support the effectiveness of using sequence barcodes (ITS, *act1*) in fungal taxonomy and plant pathology studies.

Keywords: branch dieback; canker disease; Cytospora; DNA barcoding; pathogen

1. Introduction

DNA barcoding is a standardized approach that can be applied for correct identification and recognition of fungi while overcoming the issues with traditional criteria used for description of fungal species [1]. DNA barcode is a relatively short gene region of the highly variable parts of a genome, unique for the species identification. The main advantage of this approach is that a single specimen can provide information about the species, regardless of its morphology or lifestage characteristics [2]. Currently, the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene cluster is employed as the main fungal barcode [3].

Cytospora species are well known as causal agents of branch dieback and canker diseases on different woody plants [4–6]. Considered as weak pathogens, the species of *Cytospora* can reduce longevity and productivity of related hosts in the long term [7]. Disease symptoms can initially be observed on twigs and branches in a form of sunken areas with dark discoloration. As disease progresses, cankers form in other tree parts allowing for pathogen overwintering. *Cytospora* fruiting structures (pycnidia) appear on infected wood tissues during spring and fall seasons mainly. Under favorable conditions (e.g. high moisture), conidia or spores eject from pycnidia and disseminate on nearby hosts with rain splash or wind infecting plant tissue through bark wounds [8].

Traditional approach for *Cytospora* identification was primarily based on species morphological and ecological characteristics [9,10]. These criteria seem to be insufficient for *Cytospora* species delimitation because of significant overlap in morphological traits and lack of host specificity within the genus. Besides, different species of *Cytospora* can co-occur on the same host [7]. However, occurrence of some species can be restricted to a single host genus or family [11,12]. Currently, identification of *Cytospora* species includes both morphological and molecular (sequence) data analyses. ITS region was initially employed to resolve the phylogeny of *Cytospora* [7]. The partial

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protein-coding genes (PCG), such as actin (*act1*), RNA polymerase II subunit (*rpb2*), translation elongation factor 1-alpha (*tef1-α*), and beta-tubulin (*tub2*) were further applied to phylogenetic analyses of the genus to address the issues with species recognition. Combined multi-gene (ITS+PCGs) sequence data have been used to correctly identify or describe *Cytospora* species in the most recent studies [13–15]. Therefore, a DNA-based approach including multi-locus phylogenetic analysis is critical to uncovering *Cytospora* species diversity associated with plant diseases.

Trees and shrubs with symptoms of branch dieback and canker diseases (Figure 1) have been observed during the surveys in southwest Ontario and Saskatchewan (Canada) in 2020-23. Since *Cytospora* are among the main causal agents of these diseases, exhaustive knowledge on the identity of the fungi isolated from symptomatic plants is needed to develop proper control strategies in case of disease outbreaks. Thus, this study was aimed to identify *Cytospora* species associated with diseased woody plants by applying DNA barcoding approach and morphological (culture) characterization.

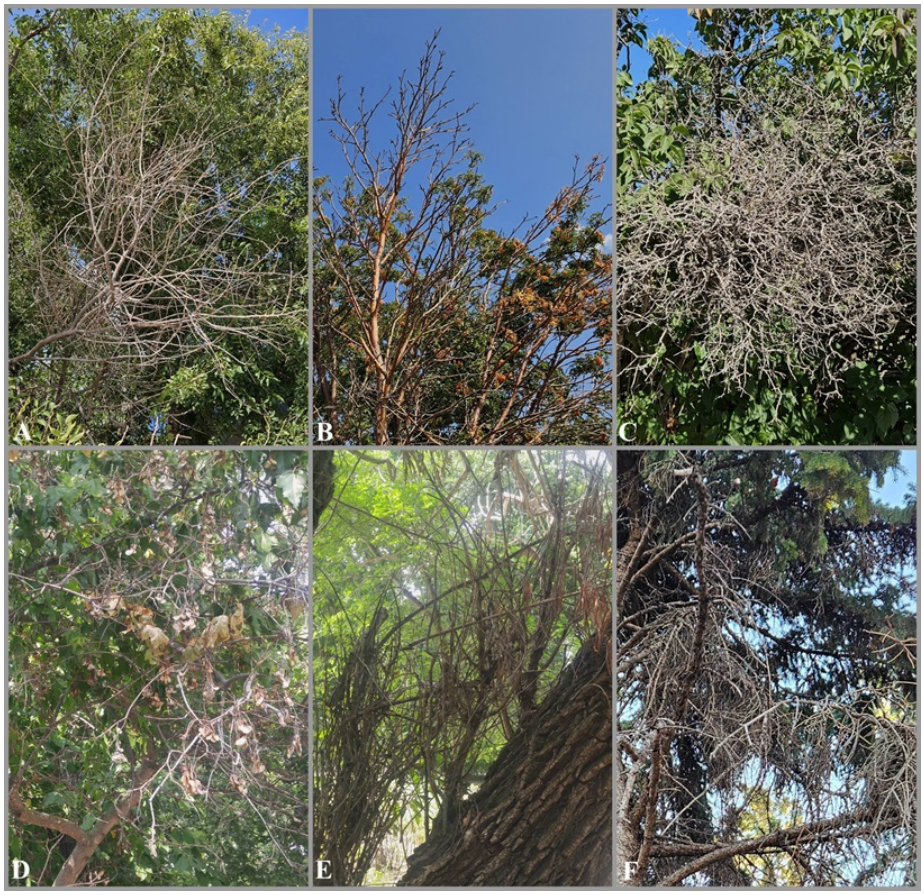

Figure 1. Symptoms of branch dieback and canker diseases observed in: (**a**) *Ulmus glabra*; (**b**) *Sorbus aucuparia*; (**c**) *Syringa vulgaris*; (**d**) *Acer ginnala*; (**e**) *Salix alba*; and (**f**) *Picea glauca*.

2. Materials and Methods

2.1. Sample Collection and Fungus Isolation

Branches and twigs with symptoms of dieback and canker were sampled for isolation and identification of *Cytospora* spp. associated with the diseases. The isolates were mainly obtained using the single spore isolation technique [16]. To isolate pathogen species from necrotic plant tissue, small wood pieces (0.5-1cm) were surface sterilized with 70% ethanol for 30s, following sterilization with 0.5% sodium hypochlorite for 2m, rinsed three times with sterile water, and plated on malt extract agar (MEA). *Cytospora*-like colonies were further purified by transferring hyphal-tips to new MEA

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plates. The obtained isolates were grouped in morphotypes and representative isolates for each morphotype were further selected for morphological (culture) characterization and sequencing.

2.2. Morphological Characterization

Fungus fruiting structures (conidio- or ascomata) were observed and sectioned under a dissecting microscope (AmScope SE306R-PZ). To confirm sample affiliation with *Cytospora* species, microstructures (conidia, spores) were examined using a compound microscope (AmScope B120C-E5). Radial colony growth and color (both adverse and reverse sides) were accessed after 7d of incubation at room temperature in the dark on MEA. Growth rate was defined as either slow growing (up to 4.5cm) or fast growing (up to 9cm). Pycnidia formation was checked after 21d of incubation.

2.3. DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA (gDNA) was extracted from 7-11d old pure cultures using DNeasy Plant ProKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The primers ITS1/ITS4 [17] were used to amplify internal transcribed spacer (ITS) region. Additionally, the partial actin (*act1*) gene region was amplified with the primer pair ACT-512F and ACT-783R [18] for the selected strains to improve species resolution. The quality of PCR products was examined using electrophoresis in 1% agarose gel. Sanger sequencing were carried out at the Genome Quebec Innovation Centre (Montreal, QC, Canada).

2.4. Sequence Alignment and Phylogenetic Analysis

The initial identification was performed using the BLASTn tool against the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI). Sequence data of the related reference strains [6] were downloaded from the GenBank database. The sequences were initially aligned employing CLUSTAL-X2 v.2.1 [19] and manually edited with MEGA-X [20]. Phylogenetic analyses were executed using randomized accelerated maximum likelihood (RAxML) v. 8.0 method [21] for maximum likelihood (ML) analysis. Bayesian posterior (BP) probabilities were defined with MrBayes v.3.2.7 [22] using the TrEase web server [23]. The ML analysis was performed using the transition (TIM) substitution model with gamma-distributed rate of heterogeneity selected with ModelTest-NG v.0.1.7 [24]. The statistical support values were estimated with bootstrapping of 1,000 replicates [25]. The general time reversible (GTR) model was chosen for the BI analysis. The Markov chain Monte Carlo (MCMC) algorithm was used to estimate Bayesian posterior probabilities (BPP). Six simultaneous Markov chains were run for 1,000,000 generations. A burn-in was implemented with discarding the first 30% of generated trees. The phylograms were visualized using FigTree v. 1.4.4 [26]. The newly generated sequences were deposited in GenBank (Table 1). The final alignment used in the analysis was submitted to TreeBase (www.treebase.org; ID: S31906).

Table 1. Strains of *Cytospora* species used in phylogenetic analysis with their GenBank accession numbers. Reference strains are marked in bold. Ex-type strains are marked with^{T}. NA - data not available.

				GenBank accession numbers	
Species	**Strain**	**Country**	**Host**	**ITS**	***act1***
Cytospora chrysosperma	**CFCC 89982**	**China**	***Ulmus pumila***	**KP281261**	**KP310835**
	EI-101	Canada	Unknown tree	PQ356607	PQ728056
	EI-351	Canada	*Rhus* sp.	PQ385601	NA
	EI-437	Canada	*Populus tremuloides*	PQ678997	NA

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	EI-SK-71	Canada	*Populus deltoides*	PQ678998	NA
	EI-SK-90	Canada	*Salix bebbiana*	PQ679047	NA
	EI-SK-93(A)	Canada	*Populus tremula*	PQ679032	NA
	EI-SK-127	Canada	*Populus* sp.	PQ679040	NA
	EI-SK-196	Canada	*Salix alba*	PQ679042	NA
Cytospora curvata	**MFLUCC 15-0865^{T}**	**Russia**	***Salix alba***	**KY417728**	**KY417694**
	EI-132	Canada	*Aronia* sp.	PQ677273	PQ728060
	EI-203	Canada	*Syringa vulgaris*	PQ677316	PQ728061
Cytospora euonymina	**CFCC 89993^{T}**	**China**	***Euonymus kiautschovicus***	**MH933630**	**MH933537**
	EI-250	Canada	*Salix* sp.	PQ678925	NA
	EI-316	Canada	*Berberis vulgaris*	PQ383411	NA
Cytospora hoffmannii	**CFCC 89641**	**China**	***Elaeagnus angustifolia***	**KF765683**	**KU711006**
	EI-SK-231	Canada	*Salix bebbiana*	PQ677104	PQ728057
	EI-SK-237	Canada	*Salix* sp.	PQ677112	PQ728058
Cytospora kantschavelii	**MFLUCC 15-0857^{T}**	**Russia**	***Populus* × *sibirica***	**KY417738**	**KY417704**
	EI-SK-33	Canada	*Ulmus glabra*	PQ678995	PQ728067
	EI-SK-44	Canada	*Acer spicatum*	PQ678996	PQ728068
Cytospora leucosperma	**MFLUCC 18-1199^{T}**	**Russia**	***Galega officinalis***	**MK912128**	**MN685810**
	EI-54(A)	Canada	*Berberis vulgaris*	PQ281438	NA
Cytospora leucostoma	**CFCC 50022**	**China**	***Prunus padus***	**MH933627**	**MH933534**
	EI-78	Canada	*Malus* sp.	PP751512	NA
	EI-223	Canada	*Vaccinium* sp.	PQ368601	PQ728059
Cytospora nitschkeana	**CBS. 118.22**	**Netherlands**	***Salix alba***	**MH854712**	**KX964746**
	EI-170	Canada	*Salix babylonica*	PQ356735	NA
	EI-193	Canada	*Berberis vulgaris*	PQ362651	NA
	EI-429	Canada	*Fraxinus americana*	PQ421750	NA
	EI-454	Canada	Unknown tree	PQ425077	NA
	EI-470	Canada	*Fraxinus nigra*	PQ678921	NA
	EI-SK-195	Canada	*Salix alba*	PQ678915	NA
Cytospora piceae	**CFCC 52841^{T}**	**China**	***Picea crassifolia***	**MH820398**	**MH820406**
	EI-273	Canada	*Picea pungens*	ON352565	NA
	EI-SK-36	Canada	*Rhus* sp.	PQ671332	NA
	EI-SK-110	Canada	*Picea* sp.	PQ671333	NA
	EI-SK-154(A)	Canada	*Fraxinus* sp.	PQ666762	NA

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Cytospora populina	**CFCC 89644^{T}**	**China**	***Salix psammophila***	**KF765686**	**KU711007**
	EI-434	Canada	*Magnolia* sp.	PQ422182	NA
	EI-477(B)	Canada	*Sorbus* sp.	PQ683229	PQ728055
	EI-478	Canada	*Acer platanoides*	PQ425482	NA
	EI-SK-184	Canada	*Aronia* sp.	PQ683280	NA
Cytospora pruinopsis	**CFCC 50034^{T}**	**China**	***Ulmus pumila***	**KP281259**	**KP310836**
	EI-SK-38	Canada	*Populus deltoides*	PQ678306	PQ728066
	EI-SK-75	Canada	*Ulmus americana*	PQ678839	NA
	EI-SK-94(A)	Canada	*Malus* sp.	PQ678815	NA
	EI-SK-223	Canada	Unknown shrubs	PQ678307	NA
Cytospora pruinosa	**CFCC 50036**	**China**	***Syringa oblata***	**KP310800**	**KP310832**
	EI-SK-133(A)	Canada	*Aronia sp.*	PQ677817	PQ728065
	EI-SK-152	Canada	*Syringa reticulata*	PQ677975	NA
	EI-SK-155	Canada	Unknown shrubs	PQ680075	NA
	EI-SK-211	Canada	*Syringa* sp.	PQ677872	NA
	EI-SK-215	Canada	*Fraxinus* sp.	PQ677818	NA
	EI-SK-221	Canada	Unknown shrubs	PQ680079	NA
Cytospora ribis	**CFCC 50026**	**China**	***Ulmus pumila***	**KP281267**	**KP310843**
	EI-396	Canada	*Fraxinus americana*	PQ393077	PQ728070
	EI-SK-60	Canada	*Syringa vulgaris*	PQ683333	NA
Cytospora schulzeri	**CFCC 53173**	**China**	***Berberis* sp.**	**MK673070**	**MK673040**
	EI-378	Canada	*Sorbaria sorbifolia*	PQ392014	NA
	EI-414	Canada	*Malus* sp.	PQ421083	NA
	EI-480	Canada	*Acer platanoides*	PQ432426	PQ728069
	EI-SK-101	Canada	*Malus* sp.	PQ728069	PQ683213
Cytospora sorbina	**CF 20197660^{T}**	**China**	***Sorbus tianschanica***	**MK673052**	**MK673022**
	EI-SK-25	Canada	*Malus* sp.	PQ677620	PQ728063
	EI-SK-31	Canada	*Aronia* sp.	PQ677471	PQ728062
	EI-SK-67	Canada	*Aronia* sp.	PQ677674	PQ728064
	EI-SK-76	Canada	*Prunus* sp.	PQ736325	NA
	EI-SK-82	Canada	*Sorbus aucuparia*	PQ677675	NA
	EI-SK-92	Canada	*Prunus padus*	PQ677676	NA
	EI-SK-95(A)	Canada	*Aronia* sp.	PQ680078	NA
	EI-SK-131(A)	Canada	Unknown shrubs	PQ677688	NA
	EI-SK-148	Canada	*Sorbus* sp.	PQ680076	NA
	EI-SK-157(A)	Canada	*Viburnum* cf. *trilobum*	PQ680077	NA

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3. Results

3.1. Phylogenetic Analysis

The ML and BP analyses of the combined ITS and *act1* sequence data produced phylogenetic trees with similar topologies. The best-scoring ML tree with a log-likelihood value of −3511.991652 is depicted in Figure 2. Estimated base frequencies were as follows: A, C, G, T = 0.250000; substitution rates: AC = 2.518389, AG = 4.873235, AT = 2.518389, CG = 1.000000, CT = 8.755476, GT = 1.000000.

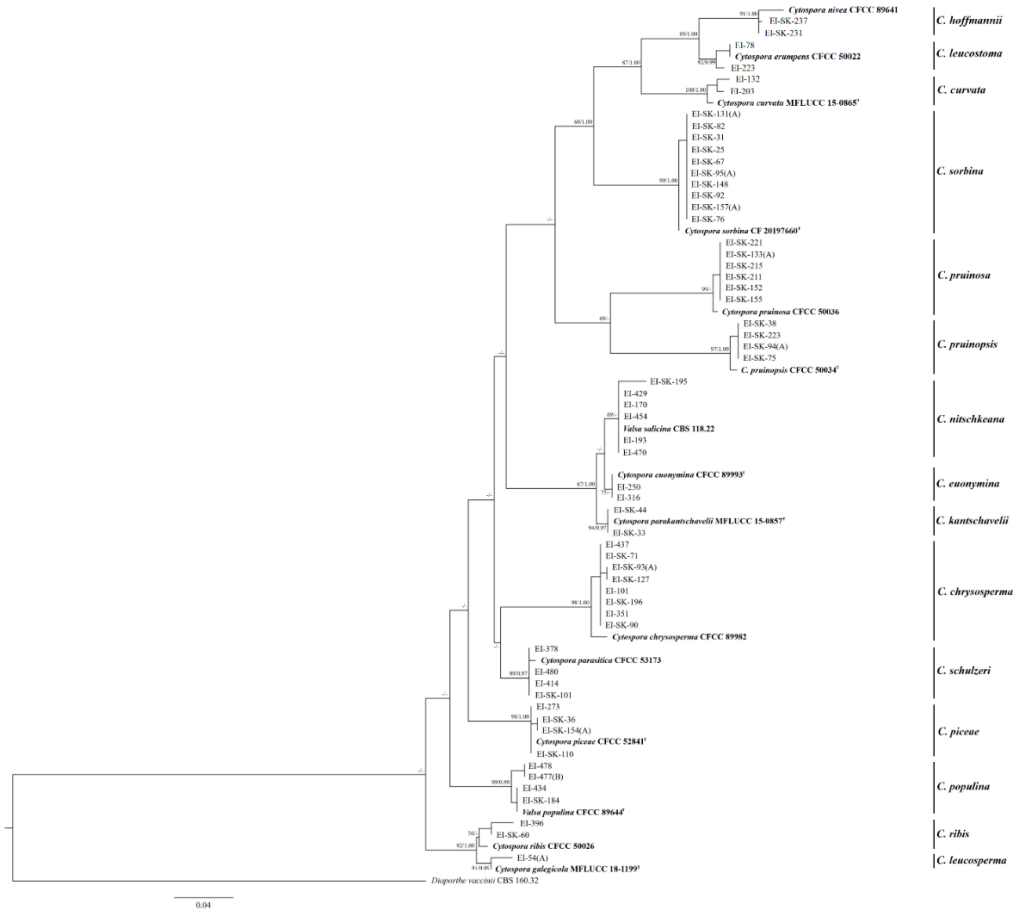

Figure 2. Phylogram of RAxML tree generated based on the analysis of ITS and *act1* sequence data of selected *Cytospora* strains. Bootstrap support values for ML ≥ 50% and BP ≥ 0.90 are shown as ML/ BP above or below the nodes. Reference strains are marked in bold. The tree is rooted to *Diaporthe vaccinii* (CBS 160.32).

The obtained strains clustered into 15 clades with high support values were assigned to the following known species: *C. chrysosperma*, *C. curvata*, *C. euonymina*, *C. hoffmannii*, *C. kantschavelii*, *C. leucosperma*, *C. leucostoma*, *C. nitschkeana*, *C. piceae*, *C. populina*, *C. pruinopsis*, *C. pruinosa*, *C. ribis*, *C. schulzeri*, and *C. sorbina*.

3.2. Taxonomy

Cytospora chrysosperma (Pers.) Fr., Syst. Mycol. (Lundae) 2(2): 542. 1823. Figure 3A.

Description: See [7].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, regular texture and sparce aerial mycelium, becoming brownish in adverse and beige in reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°13'15.8"N 79°13'32.2"W, from fallen branches of unknown tree, May 2020, *E. Ilyukhin* (EI-101); 43°03'51.1"N 79°17'10.1"W, from branches of *Rhus*

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sp., September 2020, *E. Ilyukhin* (EI-351); 43°08'21.7"N 79°10'09.3"W, from fallen branches of *Populus tremuloides*, July 2021, *E. Ilyukhin* (EI-437). Southwest Saskatchewan: 50°16'59.6"N 107°45'49.4"W, from fallen branches of *Populus deltoides*, July 2023, *E. Ilyukhin* (EI-SK-71); 50°38'50.8"N 108°00'18.2"W, from twigs of *Salix bebbiana*, August 2023, *E. Ilyukhin* (EI-SK-90); 50°39'35.2"N 108°02'55.5"W, from branches of *Populus tremula*, August 2023, *E. Ilyukhin* (EI-SK-93(A)); 50°40'24.6"N 107°57'16.2"W, from fallen branches of *Populus* sp., August 2023, *E. Ilyukhin* (EI-SK-127); 50°16'35.1"N 108°24'47.8"W, from twigs of *Salix alba*, September 2023, *E. Ilyukhin* (EI-SK-196).

Cytospora curvata Norph., Bulgakov, T.C. Wen & K.D. Hyde, Mycosphere 8 (1): 57. 2017. Figure 3B.

Description: See [27].

Culture characteristics: Colonies on MEA initially white, relatively slow growing, with thick, irregular texture without aerial mycelium, becoming dark green in adverse and dark grey in reverse. Abundant pycnidia appear after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°06'36.3"N 79°15'04.7"W, from branches of *Aronia* sp., May 2020, *E. Ilyukhin* (EI-132); 43°11'59.5"N 79°13'37.1"W, from twigs of *Syringa reticulata*, May 2020, *E. Ilyukhin* (EI-203).

Cytospora euonymina X.L. Fan & C.M. Tian, Persoonia 45: 21. 2019. Figure 3C.

Description: See [28].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thick, irregular texture without aerial mycelium, becoming brown in adverse and light brown in reverse. Rare pycnidia appear after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°11'27.3"N 79°15'53.2"W, from twigs of *Salix* sp., June 2020, *E. Ilyukhin* (EI-250); 43°06'23.2"N 79°13'39.0"W, from twigs of *Berberis vulgaris*, August 2020, *E. Ilyukhin* (EI-316).

Cytospora hoffmannii L. Lin, X.L. Fan & Crous, Stud. Mycol. 109: 354. 2024. Figure 3D.

Description: See [27] (as *C. nivea*).

Culture characteristics: Colonies on MEA initially white, relatively slow growing, with thin, irregular texture and sparce aerial mycelium, becoming olivaceous in adverse and greyish in reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Canada, Southwest Saskatchewan: 50°59'43.0"N 106°25'45.1"W, from twigs of *Salix bebbiana*, October 2023, *E. Ilyukhin* (EI-SK-231); 50°59'34.9"N 106°25'33.5"W, from branches of *Salix* sp., October 2023, *E. Ilyukhin* (EI-SK-237).

Cytospora kantschavelii Gvrit., Mikol. Fitopatol. 7: 547. 1973. Figure 3E.

Description: See [27] (as *C. parakantschavelii*).

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, irregular texture without aerial mycelium, becoming light brown in both adverse and reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Canada, Southwest Saskatchewan: 50°17'05.5"N 107°47'06.0"W, from twigs of *Ulmus glabra* (plant tissues plated), September 2022, *E. Ilyukhin* (EI-SK-33); 50°16'00.8"N 107°46'53.5"W, from twigs of *Acer spicatum*, October 2022, *E. Ilyukhin* (EI-SK-44).

Cytospora leucosperma (Pers.) Fr., Syst. Mycol. (Lundae) 2(2): 543. 1823. Figure 3F.

Description: See [29] (as *C. galegicola*).

Culture characteristics: Colonies on MEA initially white, relatively slow growing, with thin, regular texture without aerial mycelium, becoming light brown in both adverse and reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°12'40.4"N 79°14'31.0"W, branches of *Bereberis vulgaris*, April 2020, *E. Ilyukhin* (EI-54(A)).

Cytospora leucostoma (Pers.) Sacc., Michelia 2(7): 264. 1881. Figure 3G.

Description: See [27] (as *C. erumpens*).

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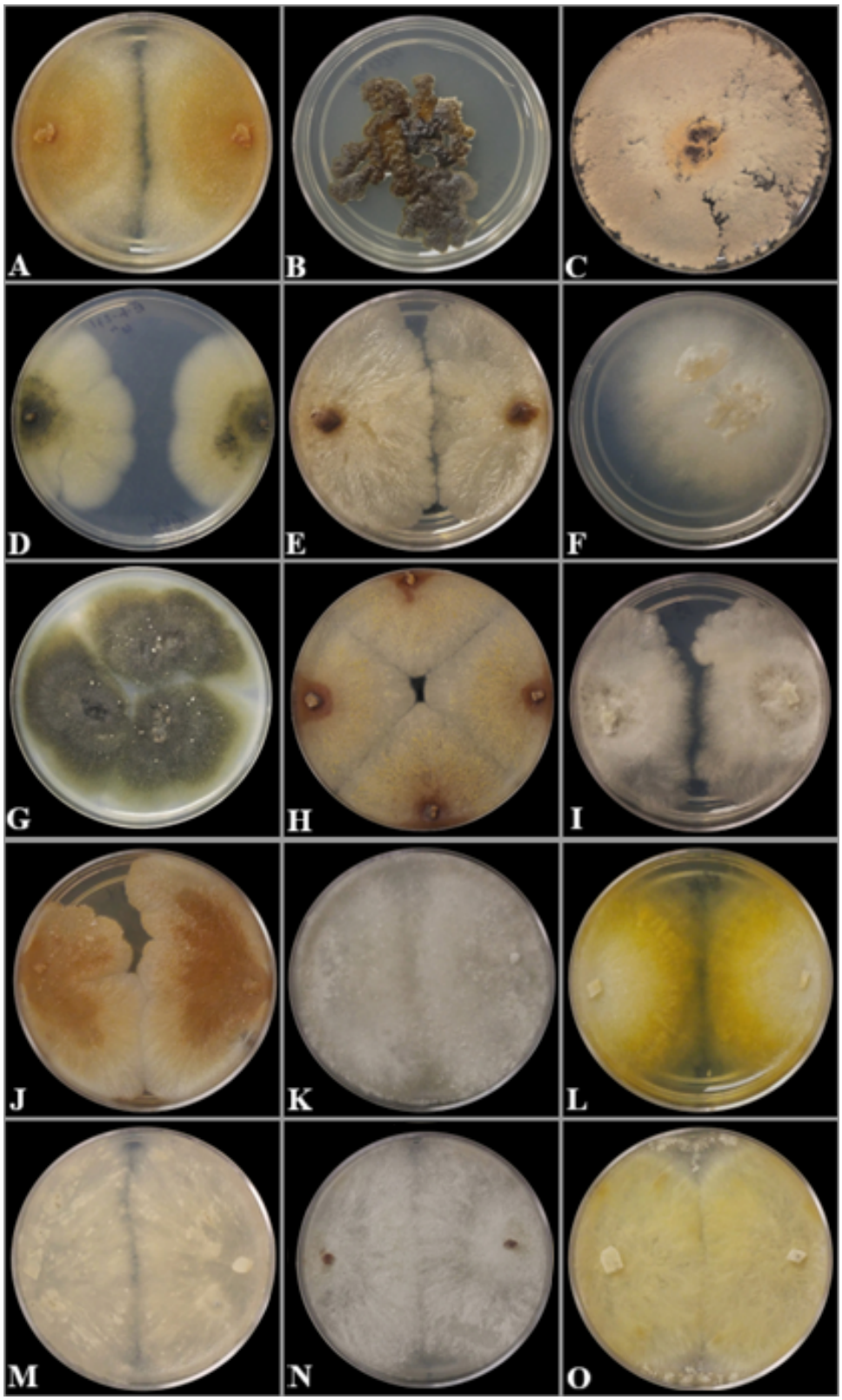

Figure 3. 7-11d old pure cultures of (**a**) *C. chrysosperma*; (**b**) *C. curvata*; (**c**) *C. euonymina*; (**d**) *C. hoffmannii*; (**e**) *C. kantschavelii*; (**f**) *C. leucosperma*; (**g**) *C. leucostoma*; (**h**) *C. nitschkeana*; (**i**) *C. piceae*; (**j**) *C. populina*; (**k**) *C. pruinopsis*; (**l**) *C. pruinosa*; (**m**) *C. ribis*; (**n**) *C. schulzeri*; (**o**) *C. sorbina*.

Culture characteristics: Colonies on MEA initially white, relatively slow growing, with thick, regular texture and sparce aerial mycelium, becoming dark green in adverse and greyish green in reverse. Abundant pycnidia appear after 21d of incubation.

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Material examined: Canada, Southwest Ontario: 43°13'07.3"N 79°13'42.3"W, from twigs of *Malus* sp., May 2020, *E. Ilyukhin* (EI-78); 43°11'05.2"N 79°13'23.8"W, from branches of *Vaccinium* sp., May 2020, *E. Ilyukhin* (EI-223).

Cytospora nitschkeana L. Lin, X.L. Fan & Crous, Stud. Mycol. 109: 367. 2024. Figure 3H.

Description: See [6].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thick, regular texture and aerial mycelium, becoming brown in both adverse and reverse. Rare pycnidia appear after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°12'03.0"N 79°14'25.6"W, from twigs of *Salix babylonica*, May 2020, *E. Ilyukhin* (EI-170); 43°12'59.7"N 79°12'51.7"W, from branches of *Berberis vulgaris*, May 2020, *E. Ilyukhin* (EI-193); 43°08'29.3"N 79°10'09.0"W, from twigs of *Fraxinus americana*, July 2021, *E. Ilyukhin* (EI-429); 43°05'33.3"N 79°18'13.1"W, from branches of *Syringa* sp., October 2021, *E. Ilyukhin* (EI-454); 43°05'29.4"N 79°18'19.6"W, from branches of *Fraxinus nigra*, October 2021, *E. Ilyukhin* (EI-470). Southwest Saskatchewan: 50°16'35.1"N 108°24'47.8"W, from twigs of *Salix alba*, September 2023, *E. Ilyukhin* (EI-SK-195).

Note: The reference strain *Cytospora* (*Valsa*) *salicina* CBS 118.22 is currently designated as *C. nitschkeana* [6].

Cytospora piceae X.L. Fan, Phytotaxa 383 (2): 188. 2018. Figure 3I.

Description: See [30].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thick, irregular texture and dense aerial mycelium, becoming grey with brownish tint in both adverse and reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°11'36.8"N 79°16'02.4"W, from branches of *Picea pungens* (plant tissue plated), August 2020, *E. Ilyukhin* (EI-273). Southwest Saskatchewan: 50°16'50.6"N 107°47'09.1"W, from branches of *Rhus* sp., September 2022, *E. Ilyukhin* (EI-SK-36); 50°40'43.3"N 107°56'38.9"W, from branches of *Picea* sp. (plant tissue plated), August 2023, *E. Ilyukhin* (EI-SK-110); 50°16'30.5"N 108°25'02.6"W, from twigs of *Fraxinus* sp., September 2023, *E. Ilyukhin* (EI-154(A)).

Cytospora populina (Pers.) Rabenh., Deutschl. Krypt.-Fl. (Leipzig) 1: 148. 1844. Figure 3J.

Description: See [31].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thick, irregular texture without aerial mycelium, becoming dark brown in adverse and light brown in reverse. Rare pycnidia appear after 21d of incubation.

Material examined: Canada, Southwest Ontario: 43°08'29.0"N 79°10'23.0"W, from branches of *Magnolia* sp., July 2021, *E. Ilyukhin* (EI-434); 43°05'23.6"N 79°18'25.1"W, from branches of *Sorbus* sp., October 2021, *E. Ilyukhin* (EI-477(B)); 43°05'26.5"N 79°18'21.7"W, from twigs of *Acer platanoides*, October 2021, *E. Ilyukhin* (EI-478). Southwest Saskatchewan: 50°16'36.6"N 108°24'52.3"W, from branches of *Aronia* sp., September 2023, *E. Ilyukhin* (EI-SK-184).

Cytospora pruinopsis C.M. Tian & X.L. Fan, Mycol. Progr. 14: 74. 2015. Figure 3K.

Description: See [32].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, regular texture and dense aerial mycelium, becoming light grey in adverse and grey in reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Southwest Saskatchewan: 50°16'44.5"N 107°47'10.0"W, from branches of *Populus deltoides*, October 2022, *E. Ilyukhin* (EI-SK-38); 50°16'51.8"N 107°45'37.2"W, from twigs of *Ulmus americana*, June 2023, *E. Ilyukhin* (EI-SK-75); 50°40'00.5"N 108°02'59.7"W, from twigs of *Malus* sp., August 2023, *E. Ilyukhin* (EI-SK-94(A)); 51°00'01.3"N 106°25'59.3"W, from branches of unknown shrubs, October 2023, *E. Ilyukhin* (EI-SK-223).

Cytospora pruinosa (Fr.) Sacc., Michelia 1(5): 519. 1879. Figure 3L.

Description: See [6].

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Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, regular texture and sparce aerial mycelium, becoming bright yellow in adverse and brownish in reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Southwest Saskatchewan: 50°38'58.5"N 107°56'52.1"W, from twigs of *Aronia* sp., August 2023, *E. Ilyukhin* (EI-SK-133(A)); 50°16'34.8"N 108°24'45.6"W, from twigs of *Syringa reticulata*, September 2023, *E. Ilyukhin* (EI-SK-152); 50°16'38.4"N 108°25'01.1"W, from twigs of unknown shrubs, September 2023, *E. Ilyukhin* (EI-SK-155); 50°59'42.2"N 106°25'22.6"W, from branches of *Syringa* sp., October 2023, *E. Ilyukhin* (EI-SK-211); 50°59'37.6"N 106°25'35.4"W, from twigs of *Fraxinus* sp., October 2023, *E. Ilyukhin* (EI-SK-215); 50°59'43.8"N 106°25'53.2"W, from branches of unknown shrubs, October 2023, *E. Ilyukhin* (EI-SK-221).

Cytospora ribis Ehrenb., Sylv. mycol. berol. (Berlin): 28. 1818. Figure 3M.

Description: See [6].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, regular texture and sparce aerial mycelium, becoming beige in both adverse and reverse. Rare pycnidia appear after 21d of incubation.

Material examined: Southwest Ontario: 43°08'01.3"N 79°22'50.9"W, from twigs of *Fraxinus americana*, May 2021, *E. Ilyukhin* (EI-396). Southwest Saskatchewan: 50°16'51.8"N 107°45'40.6"W, from twigs of *Syringa vulgaris*, June 2023, *E. Ilyukhin* (EI-SK-60).

Cytospora schulzeri Sacc. & P. Syd., Syll. fung. (Abellini) 14: 918. 1899. Figure 3N.

Description: See [33] (as *C. parasitica*).

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thin, regular texture and dense aerial mycelium, becoming greyish in both adverse and reverse. Rare pycnidia appear after 21d of incubation.

Material examined: Southwest Ontario: 43°04'00.5"N 79°17'06.3"W, from twigs of *Sorbaria sorbifolia*, September 2020, *E. Ilyukhin* (EI-378); 43°08'21.5"N 79°22'26.2"W, from branches of *Malus* sp., May 2021, *E. Ilyukhin* (EI-414); 43°05'26.5"N 79°18'21.0"W, from twigs of *Acer platanoides*, October 2021, *E. Ilyukhin* (EI-480). Southwest Saskatchewan: 50°40'16.2"N 108°00'11.3"W, from twigs of *Malus* sp., August 2023, *E. Ilyukhin* (EI-SK-101).

Cytospora sorbina M. Pan & X.L. Fan, Adverseiers in Plant Science 11 (no. 690): 13. 2020. Figure 3N.

Description: See [34].

Culture characteristics: Colonies on MEA initially white, relatively fast growing, with thick, regular texture and sparce aerial mycelium, becoming light orange in adverse and brownish in reverse. Sterile mycelium, withouth fruiting structures after 21d of incubation.

Material examined: Southwest Saskatchewan: 50°17'26.2"N 107°47'17.3"W, from branches of *Malus* sp., September 2022, *E. Ilyukhin* (EI-SK-25); 50°17'11.5"N 107°47'24.6"W, from branches of *Aronia* sp., September 2022, *E. Ilyukhin* (EI-SK-31); 50°16'55.4"N 107°45'43.9"W, from twigs of *Aronia* sp., June 2023, *E. Ilyukhin* (EI-SK-67); 50°16'51.8"N 107°45'37.2"W, from twigs of *Prunus* sp., June 2023, *E. Ilyukhin* (EI-SK-76); 50°38'45.8"N 107°58'42.0"W, from branches of *Sorbus aucuparia*, August 2023, *E. Ilyukhin* (EI-SK-82); 50°38'50.8"N 108°00'18.2"W, from branches of *Prunus padus*, August 2023, *E. Ilyukhin* (EI-SK-92); 50°39'52.4"N 108°02'38.0"W, from branches of *Aronia* sp., August 2023, *E. Ilyukhin* (EI-SK-95(A)); 50°38'59.6"N 107°56'52.0"W, from twigs of unknown shrubs, August 2023, *E. Ilyukhin* (EI-SK-131(A)); 50°16'42.4"N 108°24'36.0"W, from twigs of *Sorbus* sp., September 2023, *E. Ilyukhin* (EI-SK-148); 50°16'38.2"N 108°24'52.3"W, from twigs of *Viburnum* cf. *trilobum*, September 2023, *E. Ilyukhin* (EI-SK-157(A)).

4. Discussion

Cytospora species associated with branch dieback and canker diseases of economically important fruit trees have been recently reported in North America [35,36]. In case of disease outbreaks, it can lead to significant yield losses for the growers. Since *Cytospora* is not host-specific [7,28], the fungus may switch from hosts occurring in natural habitats to fruit trees growing in agricultural systems.

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This study was conducted to reveal *Cytospora* species associated with diseased woody plants in non-agricultural terrains in Canada. DNA barcoding approach and morphological (culture) characterization were applied to properly identify the obtained *Cytospora* isolates. The analysis based on combined ITS and *act1* sequence data resolved phylogenies of the selected *Cytospora* strains. The results highlighted the relatively rich species diversity of *Cytospora* isolated from symptomatic plants (15 species amongst 59 isolates). But only four species (*C. leucostoma, C. pruinopsis, C. schulzeri* and *C. sorbina*) were isolated from affected *Malus* spp. in the surveyed areas while 24 species of *Cytospora* were found to be related to apple tree diseases worldwide [12,34]. It indicates that more studies employing different techniques (e.g., metabarcoding) should be conducted to fully uncover pathogenic species in such a diverse genus. Most of the identified *Cytospora* have previously been reported as causal agents of tree diseases. The species of *C. chrysosperma*, *C. kantchavelii*, *C. nivea*, *C. piceae*, *C. populina*, and *C. sorbina* were found to be associated with canker disease of common forest-forming tree species such as *Juglans nigra*, *Picea crassifolia*, *Populus alba*, *Salix* spp., *Sorbus tianschanica*, and *Ulmus pumila* [5,30,32,34,37,38]. Host specificity can be attributed to a group of *Cytospora* species (incl., *C. piceae*) affiliated with conifers [4,30]. Meantime, *C. piceae* was isolated from symptomatic deciduous trees (ash) and shrubs (staghorn) in this study. This evidence additionally supports a lack of specific host affiliations among *Cytospora* spp. Other tree species widely cultivated in agricultural systems (e.g., *Malus* spp., *Prunus persica*, *Olea europaea*) can also be significantly affected by *C. leucostoma*, *C. parasitica*, *C. pruinosa*, and *C. pruinopsis* [14,15,39] found in the surveyed areas.

Multiple *in-vivo* pathogenicity assays have been recently conducted to show that *Cytospora* spp. are able to cause canker symptoms on related hosts [14,40,41]. It was also shown that the species of *Cytospora* may infect healthy (not stressed) trees maintained under proper growing conditions [12]. It points out that regular monitoring of trees (incl., asymptomatic) growing in the surroundings of fruit tree orchards should be implemented for early detection of pathogenic *Cytospora* species.

Overall, the obtained results revealed a strong association of *Cytospora* species with diseased woody plants, as well as the emergence of new hosts in southwestern Ontario and Saskatchewan, Canada. This study will contribute to the further research of fungal tree pathogens and help to develop effective disease prevention and control strategies.

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