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Dieback of *Euonymus alatus* (Celastraceae) Caused by *Cytospora haidianensis* sp. nov. in China

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Abstract: *Euonymus alatus* (Celastraceae) is widely cultivated in China for its economic value and landscape benefits. *Euonymus alatus* dieback occurs due to members of *Cytospora* and has become one of the most severe diseases affecting its cultivation in China. In this study, we examined the causal agent of bough dieback on campuses of University Road, Beijing, China. Among the strains, three were morphologically consistent with *Cytospora*, showing hyaline and allantoid conidia. Based on phylogenetic analyses of the concatenated actin (ACT), internal transcribed spacer (ITS), RNA polymerase II second largest subunit (RPB2), translation elongation factor 1-alpha (TEF1-α) and beta-tubulin (TUB2) gene sequences, along with morphological and physiological features, we propose *C. haidianensis* as a novel species. It was confirmed as a causal agent of dieback of *E. alatus* by pathogenicity tests. Mycelial growth of *Cytospora haidianensis* occurred at pH values ranging from 3.0 to 11.0, with optimum growth at 8.3, and at temperatures from 5 to 35 °C, with optimum growth at 19.8 °C. We also tested the growth of *C. haidianensis* in the presence of six carbon sources. Sucrose, maltose and glucose were highly efficient and xylose was the least. The ability of *C. haidianensis* to grow at 19.8 °C may help to explain its occurrence causing dieback of *E. alatus* in Beijing during the autumn season.

Keywords: Cytosporaceae; Diaporthales; mycelial growth; pathogenicity; phylogeny; taxonomy

1. Introduction

Euonymus alatus (Celastraceae) has been widely cultivated for ornamental landscaping in China because of its tolerance to many environmental conditions [1]. At present, the related research on the fungal diseases of *Euonymus* is mainly on anthracnose caused by *Colletotrichum gloeosporioides*, powdery mildew by *Oidium euonymi-japonici* and dieback by *Cytospora euonymicola* and *C. euonymina* [2,3].

The genus *Cytospora* has wide distribution and has often been regarded as comprising phytopathogens, endophytes or saprobes occurring on a broad range of hosts [3,4]. Several species have been reported as pathogens causing severe branch or trunk dieback disease on monocotyledonous, dicotyledonous and gymnosperm hosts (e.g., Anacardiaceae, Elaeagnaceae, Fabaceae, Juglandaceae, Myrtaceae, Rosaceae, Salicaceae and Ulmaceae) [5,6]. The symptoms of *Cytospora* canker are elongate, slightly sunken and discoloured areas in the bark at first, then the forming of several prominent black fruit bodies [5]. Conidia emerge from the fructifications in the form of yellow to orange or red gelatinous tendrils under moist conditions [3]. *Cytospora* species have single or multiple locules (and/or diaporthalean-like perithecia), filamentous conidiophores (and/or clavate to elongate obovoid asci) and allantoid hyaline conidia (and/or ascospores) [5]. As plant pathogens, *Cytospora* species have also been reported to be associated with other diseases, such as root rot of Chinese jujube and collar rot of pomegranate [7,8].

In the past, it was difficult to name *Cytospora* species because of their morphological overlap, causing confusion in species delimitation. Previously, identification of *Cytospora* species was mainly based on host affiliations, often with unclear morphological descriptions. Since the advent of molecular analysis, morphology and phylogeny using internal transcribed spacer (ITS) sequence data were combined to describe 28 species of *Cytospora* from *Eucalyptus*, of which 11 species were new to science [5]. Later, similar methods were used to describe 14 species from South Africa [6]. However, only ITS sequences are available for most known *Cytospora* species, ex-type sequence data are available for only a few species and many taxa need epitypification. Thus, recent studies have subsequently emphasized only part of *Cytospora* species using a polyphasic approach to solve the confusion in species recognition [3,4,7,8].

Stem and branch dieback have occurred on *Euonymus alatus* growing on the streets of campuses of University Road in Beijing, China. Typical symptoms of the disease are stem blight and dieback, with lesions extending along the entire branch. Infected stems have light brown to brown pigmentation. According to our observation, the disease seriously affects the colour of *Euonymus* plants and growth status, along with significant damage to the landscape. The aim of this study was to identify the causal agent causing *E. alatus* dieback disease based on molecular, morphological and physiological data.

2. Materials and Methods

2.1. Collection and Isolation

Three diseased branches of *E. alatus* were selected from 20 infected plants observed during collecting trips on the campuses of University Road in Beijing, China. Part of the hymenium containing 3 to 4 fruiting bodies of fresh material was cut horizontally with a sterile blade and crushed in a drop of sterile water on a glass slide. The contents were agitated with the blade until a spore suspension was obtained. Half of the spore suspension was then spread over the surface of 1.8% potato dextrose agar (PDA) in a petri dish and incubated at 25 °C for up to 24 h, and a single germinating conidium was transferred to a fresh PDA plate. Specimens were deposited at the working collection of X.L. Fan (CF) housed at Beijing Forestry University (BJFC) and living cultures were deposited at the China Forestry Culture Collection Centre (CFCC).

2.2. Morphological Observation

Specimens were observed on infected plant tissues, and the structure and size of fruiting bodies, the presence or absence of a conceptacle, and the size and shape of the spores were recorded. Macro-morphological photographs were captured using a Leica stereomicroscope (M205), including size of conidiomata; the presence or absence of special structures such as conceptacle and central column; number and diameter of ostioles per ectostromatic disc; colour, shape and size of discs; and number of locules. Micro-morphological observations such as size and shape of conidiophores and conidia were determined under a Nikon Eclipse 80i microscope equipped with a Nikon digital sight DS-Ri2 high-definition colour camera with differential interference contrast (DIC). Over 30 conidiomata were sectioned and 50 conidia were selected randomly to measure their lengths and widths. Colony diameters were measured, and the colony colours were described after 3 and 14 days according to the colour charts of Rayner [9]. Adobe Bridge CS6 and Adobe Photoshop CS6 were used for manual editing.

2.3. DNA Extraction, PCR Amplification and Sequencing

Mycelium for DNA extraction was grown on PDA with cellophane for 3 days and obtained from the surface of the cellophane by scraping. Genomic DNA was extracted using the modified CTAB method [10]. DNA concentrations were estimated visually by electrophoresis in 1% agarose gel by comparing band intensity with a DNA marker 1 kbp (Takara Bio USA, Inc., Mountain View, CA, USA). PCR amplifications were performed in a DNA Engine (PTC-200) Peltier Thermal Cycler

(Bio-Rad Laboratories, Hercules, CA, USA). DNA was amplified from actin (ACT), internal transcribed spacer (ITS), RNA polymerase II second largest subunit (RPB2), translation elongation factor 1-alpha (TEF1-α) and beta-tubulin (TUB2) following Fan et al. [3]. The ACT region was amplified using primers ACT-512F and ACT-1567R [11]. The ITS rDNA region was amplified and sequenced with primers ITS-1 and ITS-4 [12]. The RPB2 was amplified with primers RPB2-5F and fRPB2-7cR [13]. The TEF1-α was amplified with primers EF1-688F and EF1-986R [11,14]. The TUB2 was amplified with primers Bt-2a and Bt-2b [15]. The PCR amplification products were electrophoresed and visualized in gels. The DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminator Kit v.3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China). DNA sequences generated by each primer combination were used to obtain consensus sequences using Seqman v.7.1 and the DNASTAR Lasergene Core Suite software package (DNASTAR Inc., Madison, WI, USA).

2.4. Phylogenetic Analysis

The current isolates were initially identified as *Cytospora* sp. based on morphological observations and BLAST results. To clarify their further phylogenetic position, an analysis based on the 5 combined genes (ACT, ITS, RPB2, TEF1-α and TUB2) was constructed to compare *Cytospora* species from the current study with other strains in the GenBank database. *Diaporthe vaccinii* CBS 160.32 was selected as the outgroup in all analyses. Subsequent alignments for each gene were generated using MAFFT v.7 [16] and manually adjusted using MEGA v.6 [17]. Ambiguously aligned sequences were excluded from the analysis. Reference sequences were selected based on ex-type or ex-epitype sequences available from recently published literature [5,7,18–24] (Table 1).

Phylogenetic analyses were formed by PAUP v.4.0b10 for the maximum parsimony (MP) method [25], MrBayes v.3.1.2 for the Bayesian inference (BI) method [26] and RAxML v.7.2.8 for the maximum likelihood (ML) method [27]. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC) were calculated [25]. ML analysis was generated using a GTR+G+I model of site substitution following recent study [4], including estimation of gamma distributed rate heterogeneity and proportion of invariant sites [27]. Branch support was evaluated with a bootstrapping method of 1000 replicates [28]. BI analysis was performed using a Markov chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities [29]. A nucleotide substitution model was estimated by MrModeltest v.2.3 [30] and a weighted Bayesian analysis was considered. Two MCMC chains were run from random trees for 10,000,000 generations and trees were sampled each 100th generation. The first 40% of trees were discarded as the burn-in phase of each analysis and the Bayesian posterior probability (BPP) was calculated to assess the remaining trees [29]. The branch support from MP and ML analysis was evaluated with a bootstrapping (BS) method of 1000 replicates [28]. Phylograms were constructed using Figtree v.1.3.1 [31]. Sequence data were deposited in GenBank. The aligned matrices used for phylogenetic analysis were submitted through TreeBASE (www.treebase.org; study ID S26000).

2.5. Pathogenicity Test

Three *Cytospora* strains (CFCC 54184, CFCC 54056 and CFCC 54057) obtained in this study were used to conduct the pathogenicity test. The pathogenicity test was performed on 1-year-old *E. alatus* plants obtained from seeds kept in a greenhouse at constant 28 °C and 99% relative humidity. On healthy plants, twigs to be used for inoculation were surface disinfected with 75% ethanol for 1 min. The bark surface of each disinfected twig was scalded with a sterilized inoculating loop within a region 5 mm in length to a depth of 2 mm. For mycelial inoculation, a 5 mm diameter PDA plug with mycelium was taken from a 3-day-old colony and inoculated onto the wounded twigs. Three replicates were conducted for each isolate. Non-colonized PDA plugs and sterile water were used as negative controls. Pathogenicity was determined by the length of the necrotic lesion caused by the tested isolates, which was measured 3 weeks after inoculation. Fungal isolates were re-isolated from the infected tissue, and morphological characterization and DNA sequence comparisons were conducted to follow Koch's postulates.

Table 1. Strains and GenBank accession numbers of *Cytospora* species used in the phylogenetic analyses in this study.

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora ailanthicola	CFCC 89970^{T}	*Ailanthus altissima*	China	MH933526	MH933618	MH933592	MH933494	MH933565
Cytospora leucosperma	CFCC 89622	*Pyrus bretschneideri*	China	KU710988	KR045616	KU710944	KU710911	KR045657
	CFCC 89894	*Pyrus bretschneideri*	China	KU710989	KR045617	KU710945	KU710912	KR045658
Cytospora ampulliformis	MFLUCC 16-0583^{T}	*Sorbus intermedia*	Russia	KY417692	KY417726	KY417794	NA	NA
	MFLUCC 16-0629	*Acer platanoides*	Russia	KY417693	KY417727	KY417795	NA	NA
Cytospora amygdali	CBS 144233^{T}	*Prunus dulcis*	USA	MG972002	MG971853	NA	MG971659	MG971718
Cytospora atrocirrhata	CFCC 89615	*Juglans regia*	China	KF498673	KR045618	KU710946	KP310858	KR045659
	CFCC 89616	*Juglans regia*	China	KF498674	KR045619	KU710947	KP310859	KR045660
Cytospora beilinensis	CFCC 50493^{T}	*Pinus armandii*	China	MH933527	MH933619	NA	MH933495	MH933561
	CFCC 50494	*Pinus armandii*	China	MH933528	MH933620	NA	MH933496	MH933562
Cytospora berberidis	CFCC 89927^{T}	*Berberis dasystachya*	China	KU710990	KR045620	KU710948	KU710913	KR045661
	CFCC 89933	*Berberis dasystachya*	China	KU710991	KR045621	KU710949	KU710914	KR045662
Cytospora bungeana	CFCC 50495^{T}	*Pinus bungeana*	China	MH933529	MH933621	MH933593	MH933497	MH933563
	CFCC 50496	*Pinus bungeana*	China	MH933530	MH933622	MH933594	MH933498	MH933564
Cytospora californica	CBS 144234^{T}	*Juglans regia*	USA	MG972083	MG971935	NA	MG971645	NA
Cytospora carbonacea	CFCC 89947	*Ulmus pumila*	China	KP310842	KR045622	KU710950	KP310855	KP310825
Cytospora carpobroti	CMW 48981^{T}	*Carpobrotus edulis*	South Africa	NA	MH382812	NA	MH411212	MH411207
Cytospora castanae	DBT 183^{T}	*Castanea sativa*	North India	NA	KC963921	NA	NA	NA
Cytospora celtidicola	CFCC 50497^{T}	*Celtis sinensis*	China	MH933531	MH933623	MH933595	MH933499	MH933566
	CFCC 50498	*Celtis sinensis*	China	MH933532	MH933624	MH933596	MH933500	MH933567
Cytospora centrivillosa	MFLUCC 16-1206^{T}	*Sorbus domestica*	Italy	NA	MF190122	MF377600	NA	NA
	MFLUCC 17-1660	*Sorbus domestica*	Italy	NA	MF190123	MF377601	NA	NA

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora ceratosperma	CFCC 89624	*Juglans regia*	China	NA	KR045645	KU710976	KP310860	KR045686
	CFCC 89625	*Juglans regia*	China	NA	KR045646	KU710977	KP31086	KR045687
Cytospora ceratospermopsis	CFCC 89626^{T}	*Juglans regia*	China	KU711011	KR045647	KU710978	KU710934	KR045688
	CFCC 89627	*Juglans regia*	China	KU711012	KR045648	KU710979	KU710935	KR045689
Cytospora chrysosperma	CFCC 89629	*Salix psammophila*	China	NA	KF765673	KF765705	NA	NA
	CFCC 89981	*Populus alba* subsp. *pyramidalis*	China	MH933533	MH933625	MH933597	MH933501	MH933568
	CFCC 89982	*Ulmus pumila*	China	KP310835	KP281261	NA	KP310848	KP310818
Cytospora coryli	CFCC 53162T	*Corylus mandshurica*	China	NA	MN854450	MN850751	MN850758	MN861120
Cytospora cotini	MFLUCC 14-1050^{T}	*Cotinus coggygria*	Russia	NA	KX430142	KX430144	NA	NA
Cytospora curvata	MFLUCC 15-0865^{T}	*Salix alba*	Russia	KY417694	KY417728	KY417796	NA	NA
Cytospora davidiana	CXY 1350^{T}	*Populus davidiana*	China	NA	KM034870	NA	NA	NA
	CXY 1374	*Populus davidiana*	China	NA	KM034869	NA	NA	NA
Cytospora diopuiensis	MFLUCC 18-1419^{T}	Undefined wood	Thailand	MN685819	MK912137	NA	NA	NA
Cytospora leucostoma	MFLUCC 15-0864	*Crataegus monogyna*	Ukraine	KY417729	KY417729	KY41769	KY417797	NA
Cytospora elaeagni	CFCC 89632	*Elaeagnus angustifolia*	China	KU710995	KR045626	KU710955	KU710918	KR045667
	CFCC 89633	*Elaeagnus angustifolia*	China	KU710996	KF765677	KU710956	KU710919	KR045668
Cytospora elaeagnicola	CFCC 52882^{T}	*Elaeagnus angustifolia*	China	MK732344	MK732341	MK732347	NA	NA
	CFCC 52883	*Elaeagnus angustifolia*	China	MK732345	MK732342	MK732348	NA	NA
	CFCC 52884	*Elaeagnus angustifolia*	China	MK732346	MK732343	MK732349	NA	NA
Cytospora erumpens	CFCC 50022	*Prunus padus*	China	MH933534	MH933627	NA	MH933502	MH933569
	MFLUCC 16-0580^{T}	*Salix* × *fragilis*	Russia	KY417699	KY417733	KY417801	NA	NA
Cytospora eucalypti	CBS 144241	*Eucalyptus globulus*	USA	MG972056	MG971907	NA	MG971617	MG971772

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora euonymicola	CFCC 50499^{T}	*Euonymus kiautschovicus*	China	MH933535	MH933628	MH933598	MH933503	MH933570
	CFCC 50500	*Euonymus kiautschovicus*	China	MH933536	MH933629	MH933599	MH933504	MH933571
Cytospora euonymina	CFCC 89993^{T}	*Euonymus kiautschovicus*	China	MH933537	MH933630	MH933600	MH933505	MH933590
	CFCC 89999	*Euonymus kiautschovicus*	China	MH933538	MH933631	MH933601	MH933506	MH933591
Cytospora fraxinigena	MFLUCC 14-0868^{T}	*Fraxinus ornus*	Italy	NA	MF190133	NA	NA	NA
	MFLU 17-0880	*Fraxinus ornus*	Italy	NA	MF190134	NA	NA	NA
Cytospora fugax	CXY 1371	*Populus simonii*	China	NA	KM034852	NA	NA	KM034891
	CXY 1381	*Populus ussuriensis*	China	NA	KM034853	NA	NA	KM034890
Cytospora galegicola	MFLUCC 18-1199^{T}	*Galega officinalis*	Italy	MN685810	MK912128	MN685820	NA	NA
Cytospora germanica	CXY 1322	*Elaeagnus oxycarpa*	China	NA	JQ086563	NA	NA	NA
Cytospora gigalocus	CFCC 89620^{T}	*Juglans regia*	China	KU710997	KR045628	KU710957	KU710920	KR045669
	CFCC 89621	*Juglans regia*	China	KU710998	KR045629	KU710958	KU710921	KR045670
Cytospora gigaspora	CFCC 50014	*Juniperus procumbens*	China	KU710999.	KR045630	KU710959	KU710922	KR045671
	CFCC 89634^{T}	*Salix psammophila*	China	KU711000	KF765671	KU710960	KU710923	KR045672
Cytospora granati	CBS 144237^{T}	*Punica granatum*	USA	MG971949	MG971799	NA	MG971514	MG971664
Cytospora haidianensis	**CFCC 54056**	***Euonymus alatus***	**China**	**MT363978**	**MT360041**	**MT363987**	**MT363997**	**MT364007**
	CFCC 54057^{T}	***Euonymus alatus***	**China**	**MT363979**	**MT360042**	**MT363988**	**MT363998**	**MT364008**
	CFCC 54184	***Euonymus alatus***	**China**	**MT363980**	**MT360043**	**MT363989**	**MT363999**	**MT364009**
Cytospora hippophaës	CFCC 89639	*Hippophaë rhamnoides*	China	KU711001	KR045632	KU710961	KU710924	KR045673
	CFCC 89640	*Hippophaë rhamnoides*	China	KF765730	KF765682	KU710962	KP310865	KR045674
Cytospora japonica	CFCC 89956	*Prunus cerasifera*	China	KU710993	KR045624	KU710953	KU710916	KR045665
	CFCC 89960	*Prunus cerasifera*	China	KU710994	KR045625	KU710954	KU710917	KR045666
Cytospora joaquinensis	CBS 144235^{T}	*Populus deltoides*	USA	MG972044	MG971895	NA	MG971605	MG971761

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora junipericola	BBH 42444	*Juniperus communis*	Italy	NA	MF190126	NA	MF377579	NA
	MFLU 17-0882^T	*Juniperus communis*	Italy	NA	MF190125	NA	MF377580	NA
Cytospora juniperina	CFCC 50501^T	*Juniperus przewalskii*	China	MH933539	MH933632	MH933602	MH933507	NA
	CFCC 50502	*Juniperus przewalskii*	China	MH933540	MH933633	MH933603	MH933508	MH933572
	CFCC 50503	*Juniperus przewalskii*	China	MH933541	MH933634	MH933604	MH933509	NA
Cytospora kantschavelii	CXY 1383	*Populus maximowiczii*	China	NA	KM034867	NA	NA	NA
	CXY 1386	*Populus maximowiczii*	China	NA	KM034867	NA	NA	NA
Cytospora kuanchengensis	CFCC 52464^T	*Castanea mollissima*	China	MK442940	MK432616	MK578076	NA	NA
	CFCC 52465	*Castanea mollissima*	China	MK442941	MK432617	MK578077	NA	NA
Cytospora leucostoma	CFCC 50015	*Sorbus aucuparia*	China	KU711002	KR045634	NA	KU710925	KR045675
	CFCC 50016	*Sorbus aucuparia*	China	MH820408	MH820400	NA	MH820404	MH820389
	CFCC 50017	*Prunus cerasifera*	China	MH933542	MH933635	NA	MH933510	MH933573
	CFCC 50018	*Prunus serrulata*	China	MH933543	MH933636	NA	MH933511	MH933574
	CFCC 50019	*Rosa helenae*	China	MH933544	MH933637	NA	NA	NA
	CFCC 50020	*Prunus persica*	China	MH933545	MH933638	NA	NA	NA
	CFCC 50021	*Prunus salicina*	China	MH933546	MH933639	NA	MH933512	MH933575
	CFCC 50023	*Cornus alba*	China	KU711003	KR045635	KU710964	KU710926	KR045676
	CFCC 50024	*Prunus pseudocerasus*	China	MH933547	MH933640	MH933605	NA	MH933576
	CFCC 50467	*Betula platyphylla*	China	NA	KT732948	NA	NA	NA
	CFCC 50468	*Betula platyphylla*	China	NA	KT732949	NA	NA	NA
	CFCC 53140	*Prunus sibirica*	China	MN850760	MN854445	MN850746	MN850753	MN861115
	CFCC 53141	*Prunus sibirica*	China	MN850761	MN854446	MN850747	MN850754	MN861116
	CFCC 53156	*Juglans mandshurica*	China	MN850762	MN854447	MN850748	MN850755	MN861117
	MFLUCC 16-0574	*Rosa* sp.	Russia	KY417696	KY417731	KY417798	NA	NA

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora longiostiolata	MFLUCC 16-0628^T	*Salix* × *fragilis*	Russia	KY417700	KY417734	KY417802	NA	NA
Cytospora longispora	CBS 144236^T	*Prunus domestica*	USA	MG972054	MG971905	NA	MG971615	MG971764
Cytospora lumnitzericola	MFLUCC 17-0508^T	*Lumnitzera racernosa*	Thailand	MH253457	MG975778	MH253453	NA	NA
Cytospora mali	CFCC 50028	*Malus pumila*	China	MH933548	MH933641	MH933606	MH933513	MH933577
	CFCC 50029	*Malus pumila*	China	MH933549	MH933642	MH933607	MH933514	MH933578
	CFCC 50030	*Malus pumila*	China	MH933550	MH933643	MH933608	MH933524	MH933579
	CFCC 50031	*Crataegus* sp.	China	KU711004	KR045636	KU710965	KU710927	KR045677
	CFCC 50044	*Malus baccata*	China	KU711005	KR045637	KU710966	KU710928	KR045678
Cytospora melnikii	CFCC 89984	*Rhus typhina*	China	MH933551	MH933644	MH933609	MH933515	MH933580
	MFLUCC 15-0851^T	*Malus domestica*	Russia	KY417701	KY417735	KY417803	NA	NA
	MFLUCC 16-0635	*Populus nigra* var. *italica*	Russia	KY417702	KY417736	KY417804	NA	NA
Cytospora myrtagena	CFCC 52454	*Castanea mollissima*	China	MK442938	MK432614	MK578074	NA	NA
	CFCC 52455	*Castanea mollissima*	China	MK442939	MK432615	MK578075	NA	NA
Cytospora nivea	MFLUCC 15-0860	*Salix acutifolia*	Russia	KY417703	KY417737	KY417805	NA	NA
	CFCC 89641	*Elaeagnus angustifolia*	China	KU711006	KF765683	KU710967	KU710929	KR045679
	CFCC 89643	*Salix psammophila*	China	NA	KF765685	KU710968	KP310863	KP310829
Cytospora notastroma	NE_TFR5	*Populus tremuloides*	USA	NA	JX438632	NA	JX438543	NA
	NE_TFR8	*Populus tremuloides*	USA	NA	JX438633	NA	JX438542	NA
Cytospora oleicola	CBS 144248^T	*Olea europaea*	USA	MG972098	MG971944	NA	MG971660	MG971752
Cytospora palm	CXY 1276	*Cotinus coggygria*	China	NA	JN402990	NA	KJ781296	NA
	CXY 1280^T	*Cotinus coggygria*	China	NA	JN411939	NA	KJ781297	NA
Cytospora parakantschavelii	MFLUCC 15-0857^T	*Populus* × *sibirica*	Russia	KY417704	KY417738	KY417806	NA	NA
	MFLUCC 16-0575	*Pyrus pyraster*	Russia	KY417705	KY417739	KY417807	NA	NA

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora parapistaciae	CBS 144506^{T}	*Pistacia vera*	USA	MG971954	MG971804	NA	MG971519	MG971669
Cytospora parasitica	MFLUCC 15-0507^{T}	*Malus domestica*	Russia	KY417706	KY417740	KY417808	NA	NA
	XJAU 2542-1	*Malus* sp.	China	NA	MH798884	NA	MH813452	NA
Cytospora paratranslucens	MFLUCC 15-0506^{T}	*Populus alba* var. *bolleana*	Russia	KY417707	KY417741	KY417809	NA	NA
	MFLUCC 16-0627	*Populus alba*	Russia	KY417708	KY417742	KY417810	NA	NA
Cytosporapiceae	CFCC 52841T	*Picea crassifolia*	China	MH820406	MH820398	MH820395	MH820402	MH820387
	CFCC 52842	*Picea crassifolia*	China	MH820407	MH820399	MH820396	MH820403	MH820388
Cytospora pingbianensis	MFLUCC 18-1204^{T}	Undefined wood	China	MN685817	MK912135	MN685826	NA	NA
Cytospora pistaciae	CBS 144238^{T}	*Pistacia vera*	USA	MG971952	MG971802	NA	MG971517	MG971667
Cytospora platanicola	MFLU 17-0327^{T}	*Platanus hybrida*	Italy	MH253449	MH253451	MH253450	NA	NA
Cytospora platyclada	CFCC 50504^{T}	*Platycladus orientalis*	China	MH933552	MH933645	MH933610	MH933516	MH933581
	CFCC 50505	*Platycladus orientalis*	China	MH933553	MH933646	MH933611	MH933517	MH933582
	CFCC 50506	*Platycladus orientalis*	China	MH933554	MH933647	MH933612	MH933518	MH933583
Cytospora platycladicola	CFCC 50038^{T}	*Platycladus orientalis*	China	MH933555	KT222840	MH933613	MH933519	MH933584
	CFCC 50039	*Platycladus orientalis*	China	KU711008	KR045642	KU710973	KU710931	KR045683
Cytospora plurivora	CBS 144239^{T}	*Olea europaea*	USA	MG972010	MG971861	NA	MG971572	MG971726
Cytospora populicola	CBS 144240^{T}	*Populus deltoides*	USA	MG972040	MG971891	NA	MG971601	MG971757
Cytospora populina	CFCC 89644^{T}	*Salix psammophila*	China	KU711007	KF765686	KU710969	KU710930	KR045681
Cytospora populinopsis	CFCC 50032^{T}	*Sorbus aucuparia*	China	MH933556	MH933648	MH933614	MH933520	MH933585
	CFCC 50033	*Sorbus aucuparia*	China	MH933557	MH933649	MH933615	MH933521	MH933586
Cytospora pruinopsis	CFCC 50034^{T}	*Ulmus pumila*	China	KP310836	KP281259	KU710970	KP310849	KP310819
	CFCC 50035	*Ulmus pumila*	China	KP310837	KP281260	KU710971	KP310850	KP310820
	CFCC 53153	*Ulmus pumila*	China	MN850763	MN854451	MN850752	MN850759	MN861121

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora predappioensis	MFLUCC 17-2458^{T}	*Platanus hybrida*	Italy	NA	MG873484	NA	NA	NA
Cytospora pruinosa	CFCC 50036	*Syringa oblata*	China	KP310832	KP310800	NA	KP310845	KP310815
	CFCC 50037	*Syringa oblata*	China	MH933558	MH933650	NA	MH933522	MH933589
Cytospora prunicola	MFLU 17-0995^{T}	*Prunus* sp.	Italy	MG742353	MG742350	MG742352	NA	NA
Cytospora pubescentis	MFLUCC 18-1201^{T}	*Quercus pubescens*	Italy	MN685812	MK912130	MN685821	NA	NA
Cytospora punicae	CBS 144244	*Punica granatum*	USA	MG972091	MG971943	NA	MG971654	MG971798
Cytospora quercicola	MFLUCC 14-0867^{T}	*Quercus* sp.	Italy	NA	MF190129	NA	NA	NA
	MFLU 17-0881	*Quercus* sp.	Italy	NA	MF190128	NA	NA	NA
Cytospora ribis	CFCC 50026	*Ulmus pumila*	China	KP310843	KP281267	KU710972	KP310856	KP310826
	CFCC 50027	*Ulmus pumila*	China	KP310844	KP281268	NA	KP310857	KP310827
Cytospora rosae	MFLU 17-0885	*Rosa canina*	Italy	NA	MF190131	NA	NA	NA
Cytospora rostrata	CFCC 89909^{T}	*Salix cupularis*	China	KU711009	KR045643	KU710974	KU710932	KR045684
	CFCC 89910	*Salix cupularis*	China	KU711010	KR045644	KU710975	KU710933	NA
Cytospora rusanovii	MFLUCC 15-0853	*Populus* × *sibirica*	Russia	KY417709	KY417743	KY417811	NA	NA
	MFLUCC 15-0854^{T}	*Salix babylonica*	Russia	KY417710	KY417744	KY417812	NA	NA
Cytospora salicacearum	MFLUCC 15-0861	*Salix* × *fragilis*	Russia	KY417711	KY417745	KY417813	NA	NA
	MFLUCC 15-0509^{T}	*Salix alba*	Russia	KY417712	KY417746	KY417814	NA	NA
	MFLUCC 16-0576	*Populus nigra* var. *italica*	Russia	KY417707	KY417741	KY417809	NA	NA
	MFLUCC 16-0587	*Prunus cerasus*	Russia	KY417708	KY417742	KY417810	NA	NA
Cytospora salicicola	MFLUCC 15-0866	*Salix alba*	Russia	KY417715	KY417749	KY417817	NA	NA
	MFLUCC 14-1052^{T}	*Salix alba*	Russia	KU982637	KU982636	NA	NA	NA
Cytospora salicina	MFLUCC 15-0862^{T}	*Salix alba*	Russia	KY417716	KY417750	KY417818	NA	NA
	MFLUCC 16-0637	*Salix* × *fragilis*	Russia	KY417717	KY417751	KY417819	NA	NA

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora schulzeri	CFCC 50040	*Malus domestica*	China	KU711013	KR045649	KU710980	KU710936	KR045690
	CFCC 50042	*Malus asiatica*	China	KU711014	KR045650	KU710981	KU710937	KR045691
Cytospora sibiraeae	CFCC 50045^{T}	*Sibiraea angustata*	China	KU711015	KR045651	KU710982	KU710938	KR045692
	CFCC 50046	*Sibiraea angustata*	China	KU711015	KR045652	KU710983	KU710939	KR045693
Cytospora sophorae	CFCC 50047	*Styphnolobium japonicum*	China	KU711017	KR045653	KU710984	KU710940	KR045694
	CFCC 50048	*Magnolia grandiflora*	China	MH820409	MH820401	MH820397	MH820405	MH820390
	CFCC 89598	*Styphnolobium japonicum*	China	KU711018	KR045654	KU710985	KU710941	KR045695
Cytospora sophoricola	CFCC 89595^{T}	*Styphnolobium japonicum* var. *pendula*	China	KU711019	KR045655	KU710986	KU710942	KR045696
	CFCC 89596	*Styphnolobium japonicum* var. *pendula*	China	KU711020	KR045656	KU710987	KU710943	KR045697
Cytospora sophoriopsis	CFCC 89600^{T}	*Styphnolobium japonicum*	China	KU710992	KR045623	KU710951	KU710915	KP310817
Cytospora sorbi	MFLUCC 16-0631^{T}	*Sorbus aucuparia*	Russia	KY417718	KY417752	KY417820	NA	NA
Cytospora sorbicola	MFLUCC 16-0584^{T}	*Acer pseudoplatanus*	Russia	KY417721	KY417755	KY417823	NA	NA
	MFLUCC 16-0633	*Cotoneaster melanocarpus*	Russia	KY417724	KY417758	KY417826	NA	NA
Cytospora spiraeae	CFCC 50049^{T}	*Spiraea salicifolia*	China	MG708196	MG707859	MG708199	NA	NA
	CFCC 50050	*Spiraea salicifolia*	China	MG708197	MG707860	MG708200	NA	NA
Cytospora spiraeicola	CFCC 53138^{T}	*Spiraea salicifolia*	China	NA	MN854448	MN850749	MN850756	MN861118
	CFCC 53139	*Tilia nobilis*	China	NA	MN854449	NA	NA	NA
Cytospora tamaricicola	CFCC 50507	*Rosa multifolora*	China	MH933559	MH933651	MH933616	MH933525	MH933587
	CFCC 50508^{T}	*Tamarix chinensis*	China	MH933560	MH933652	MH933617	MH933523	MH933588
Cytospora tanaitica	MFLUCC 14-1057^{T}	*Betula pubescens*	Russia	KT459413	KT459411	NA	NA	NA

Table 1. *Cont.*

Species	Strain[1]	Host	Origin	GenBank Accession Numbers				
				ACT	ITS	RPB2	TEF1-α	TUB2
Cytospora thailandica	MFLUCC 17-0262^{T}	*Xylocarpus moluccensis*	Thailand	MH253459	MG975776	MH253455	NA	NA
	MFLUCC 17-0263^{T}	*Xylocarpus moluccensis*	Thailand	MH253460	MG975777	MH253456	NA	NA
Cytospora tibouchinae	CPC 26333^{T}	*Tibouchina semidecandra*	France	NA	KX228284	NA	NA	NA
Cytospora translucens	CXY 1351	*Populus davidiana*	China	NA	KM034874	NA	NA	KM034895
Cytospora ulmi	MFLUCC 15-0863^{T}	*Ulmus minor*	Russia	NA	KY417759	NA	NA	NA
Cytospora vinacea	CBS 141585^{T}	*Vitis interspecific* hybrid 'Vidal'	USA	NA	KX256256	NA	KX256277	KX256235
Cytospora xinglongensis	CFCC 52458	*Castanea mollissima*	China	MK442946	MK432622	MK578082	NA	NA
	CFCC 52459	*Castanea mollissima*	China	MK442947	MK432623	MK578083	NA	NA
Cytospora viridistroma	CBS 202.36^{T}	*Cercis canadensis* Castigl.	USA	NA	MN172408	NA	MN271853	NA
Cytospora viticola	CBS 141586^{T}	*Vitis vinifera*	USA	NA	KX256239	NA	KX256260	KX256218
Cytospora xylocarpi	MFLUCC 17-0251^{T}	*Xylocarpus granatum*	Thailand	MH253458	MG975775	MH253454	NA	NA
Diaporthe vaccinii	CBS 160.32	*Vaccinium macrocarpon*	USA	JQ807297	KC343228	NA	KC343954	KC344196

BBH, BIOTEC Bangkok Herbarium, National Science and Technology Development Agency, Thailand; CBS, Westerdijk Fungal Biodiversity Institute (CBS-KNAW Fungal Biodiversity Centre), Utrecht, Netherlands; CFCC, China Forestry Culture Collection Centre, Beijing, China; CMW, culture collection of Michael Wingfield, University of Pretoria, South Africa; CPC, culture collection of Pedro Crous, Netherlands; MFLU, Mae Fah Luang University herbarium, Thailand; MFLUCC, Mae Fah Luang University Culture Collection, Thailand; XJAU, Xinjiang Agricultural University, Xinjiang, China; NA, not applicable. All new isolates used in this study are indicated in bold type and strains from generic type species are marked by a superscript T.

2.6. Temperature and pH Tests

The 3 *Cytospora* isolates showed similar growth characteristics, so we used the type strain of the new species (CFCC 54057) to evaluate the effects of temperature and pH on colony growth using PDA plates. Tested temperatures ranged from 0 to 40 °C at intervals of 5 °C (i.e., 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C). In order to clarify the effect of pH on radial mycelial growth, PDA medium was adjusted with 0.1 M NaOH and 0.1 M HCl to obtain pH values from 2.0 to 12.0 at intervals of 1.0 (i.e., 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0). A 5 mm diameter mycelial plug was placed in the centre of a 90 mm petri dish with PDA medium and incubated at 28 °C in the dark, with 3 replicates for each treatment. The effects of pH and temperature on mycelial growth were determined by measuring the colony diameter after 24, 48, 72 and 96 h of incubation and the data were converted to radial growth in millimetres [32]. Data were analysed in IBM SPSS Statistics v.22.0 (IBM Inc., Armonk, NY, USA) to select the model that best fit the individual data points, and SPSS was used to confirm the selected model. The optimal temperature and pH value of the regression curves were calculated based on the regression equations generated by IBM SPSS Statistics, and output figures with Origin v.8.0.

2.7. Carbon Colony Growth Test

To investigate the utilization of carbon sources, the type strain of the new species (CFCC 54057) was incubated in the dark at 28 °C on PDA medium for 4 days. PDA medium was used as the base medium (potato 20 g, sucrose 20 g, agar 17 g, distilled water to complete 1000 mL). The 20 g of sucrose was replaced by 20 g of fructose, galactose, glucose, maltose, sucrose or xylose to test these compounds as carbon sources. A 5 mm diameter PDA plug of mycelium was transferred to the centre of each sole carbon source medium. Colony growth was determined by measuring the colony diameters after incubation for 24, 48, 72 and 96 h at 28 °C in the dark, and the results were subsequently converted to radial growth [32]. Mean comparisons were conducted using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) in SigmaPlot v.14.0.

3. Results

3.1. Phylogenetic Analyses

A combined matrix of five gene sequences of *Cytospora* species was constructed. The combined alignment matrices (ACT, ITS, RPB2, TEF1-α and TUB2) included 192 accessions (3 from this study and 189 retrieved from GenBank) and counted 3056 characters including gaps (350 characters for ACT, 631 for ITS, 726 for RPB2, 725 for TEF1-α and 624 for TUB2), of whih 1594 characters were constant, 130 variable characters were parsimony-uninformative and 1349 (44.14%) characters were variable and parsimony-informative. The MP analysis generated 200 parsimonious trees, the first of which is presented in Figure 1 (TL = 8,573, CI = 0.312, RI = 0.788, RC = 0.246). The tree topologies of ML and BI analyses were similar to that of the MP tree.

Based on the initial analysis, a second, more inclusive combined matrix was constructed using 27 accessions from the first dataset. The second combined alignment matrix counted 2531 characters including gaps (274 characters for ACT, 529 for ITS, 726 for RPB2, 553 for TEF1-α and 449 for TUB2). In total, 1,819 characters were constant, 182 variable characters were parsimony-uninformative and 547 (21.61%) characters were variable and parsimony-informative. The MP analysis generated one parsimonious tree and the best tree (TL = 1,225, CI = 0.768, RI = 0.853, RC = 0.656) is presented in Figure 2. The tree topologies of ML and BI analyses were similar to that of the MP tree.

Based on the multilocus phylogeny and morphology, all three strains were assigned to one new species, named *Cytospora haidianensis*, representing a monophyletic clade with high support value (MP/ML/BI = 100/100/1).

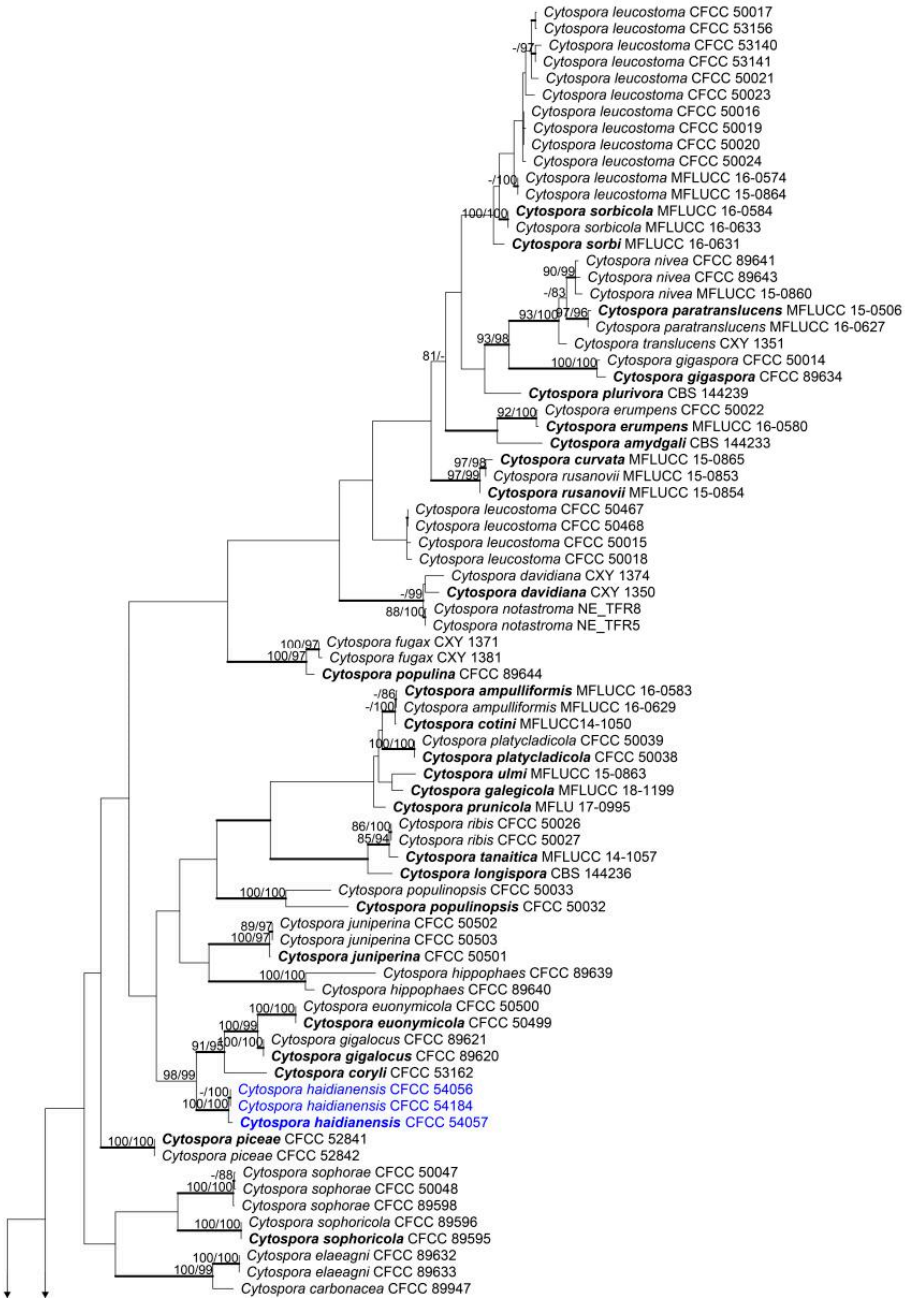

Figure 1. *Cont*.

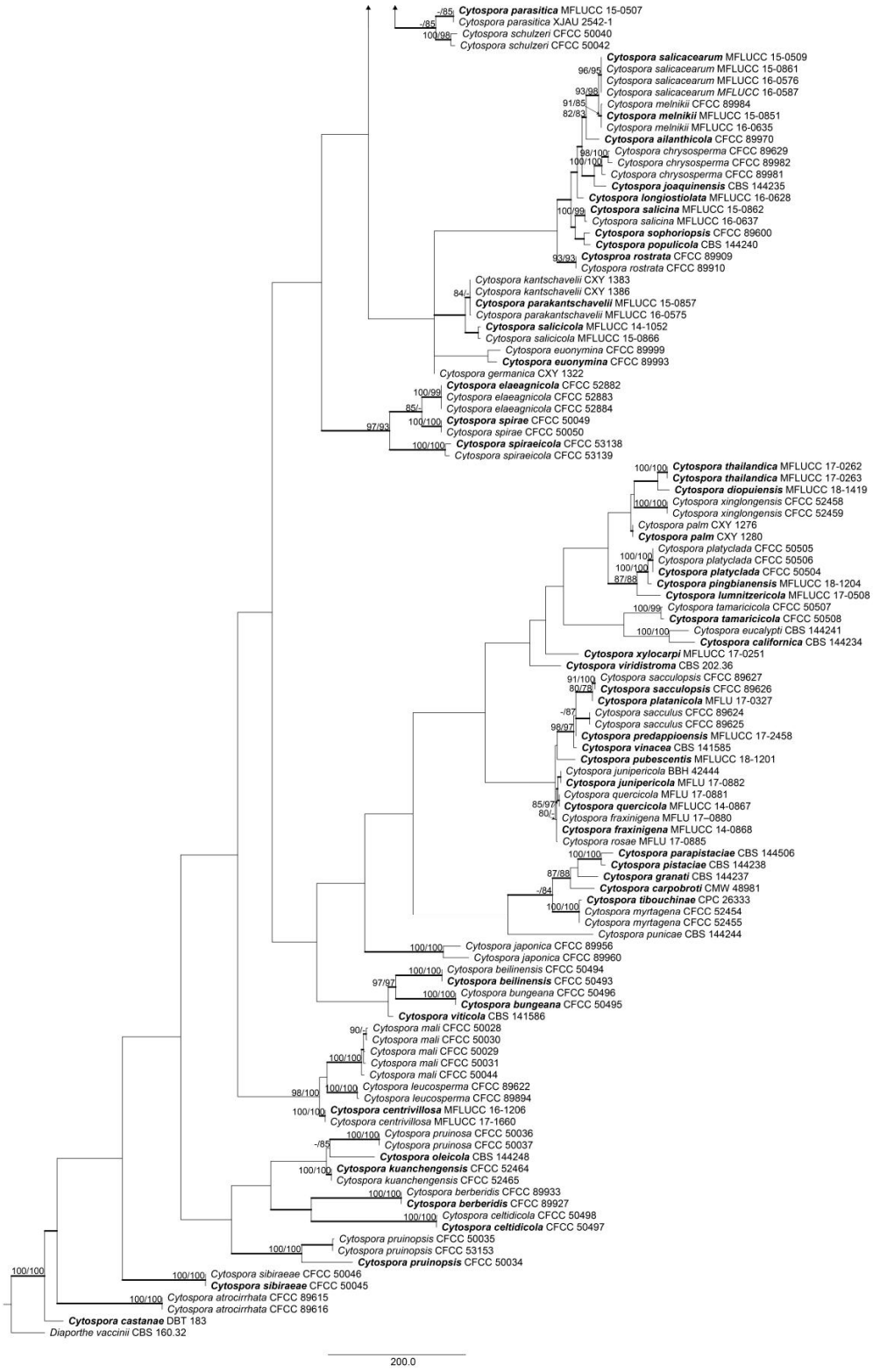

Figure 1. Phylogram of the best-parsimonious tree of *Cytospora* based on combined actin (ACT), internal transcribed spacer (ITS), RNA polymerase II second largest subunit (RPB2), translation elongation factor 1-alpha (TEF1-α) and beta-tubulin (TUB2) genes. Maximum parsimony (MP) and maximum likelihood (ML) bootstrap support values above 70% are shown at the first and second positions, respectively. Thickened branches represent posterior probabilities from Bayesian inference (BI) above 0.95. Scale bar = 200 nucleotide substitutions. *Diaporthe vaccinii* CBS 160.32 was used as the outgroup. Ex-type strains are in bold. Strains from the current study are in bold and blue.

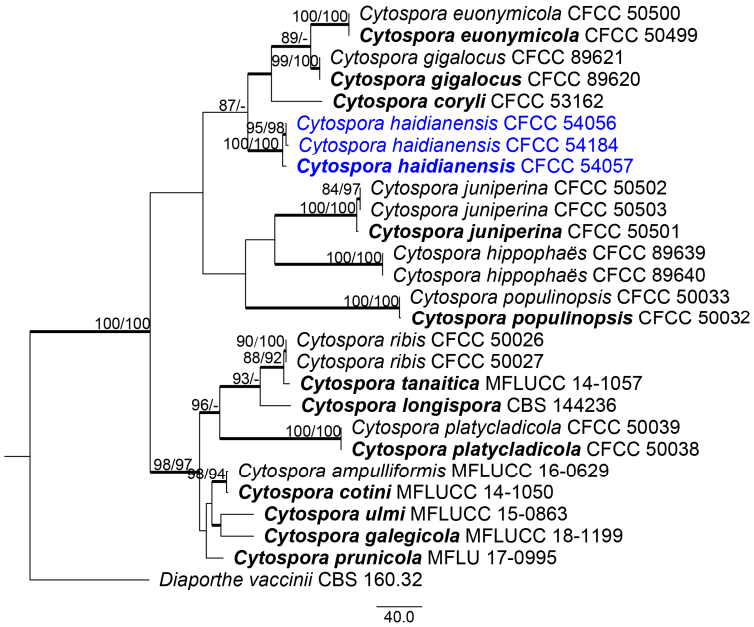

Figure 2. Phylogram of the best-parsimonious tree of *Cytospora* based on combined ACT, ITS, RPB2, TEF1-α and TUB2 genes. MP and ML bootstrap support values above 70% are shown at the first and second positions, respectively. Thickened branches represent posterior probabilities from BI above 0.95. Scale bar = 40 nucleotide substitutions. *Diaporthe vaccinii* CBS 160.32 was used as the outgroup. Ex-type strains are in bold. Strains from the current study are in bold and blue.

3.2. Taxonomy

Cytospora haidianensis X. Zhou & X.L. Fan, sp. nov. (Figure 3)

MycoBank MB 835121

Holotype: CF 20198643

Etymology: named after the place where it was first collected, Haidian

Host/Distribution: on cankered *Euonymus alatus* branches in China

Description: Sexual morph not observed. Pycnidial stromata ostiolate, immersed in bark, scattered, erumpent through the surface, with multiple locules. Conceptacle absent. Ectostromatic disc isabelline to dark brick, conspicuous, circular to ovoid, (330–)380–500(–520) µm ($\bar{x}$ = 460 ± 30 µm, n = 35) diam, with one ostiole per disc. Ostiole in the centre of the disc, black, conspicuous, (170–)179–195(–200) µm ($\bar{x}$ = 188 ± 3 µm, n = 10) diam. Numerous locules, subdivided frequently by invaginations with common walls, (650–)700–800(–1000) µm ($\bar{x}$ = 760 ± 30 µm, n = 30) diam. Conidiophores hyaline, branched at the base or unbranched, thin-walled, (9–)12–15(–16.5) × 1.0–1.5 µm ($\bar{x}$ = 13.5 ± 1.5 × 1.4 ± 0.1 µm, n = 50), embedded in a gelatinous layer. Conidiogenous cells enteroblastic, phialidic, subcylindrical to cylindrical, (8.5–)9–12.5(–13.5) × 1–1.5 µm ($\bar{x}$ = 11 ± 1.5 µm, n = 30), tapering towards the apices. Conidia hyaline, allantoid, smooth, aseptate, thin-walled, (6–)6.5–7.5 × 1–1.5 µm ($\bar{x}$ = 6.8 ± 0.2 × 1.2 ± 0.1 µm, n = 50).

Cultural characteristics: Colonies on PDA are initially white after 3 days, becoming light brown after 14 days. The colonies are thin with a uniform texture, lack aerial mycelium and grow up to 90 mm after 4 days. Pycnidia were randomly observed on the surface of the colony.

Material examined: CHINA, Beijing, Haidian, University Road, 116°20′19.11″ E, 40°00′16.21″ N, 51 m asl, on stems and branches of *Euonymus alatus*, Xinlei Fan, 12 November 2019 (CF 20198643, holotype; ex-type culture, CFCC 54057). Beijing, Haidian, University Road, 116°35′49.37″ E, 40°00′37.85″ N, 50 m asl, on stems and branches of *Euonymus alatus*, Xinlei Fan, 12 November 2019 (CF 20198644; living culture, CFCC 54056). Beijing, Haidian, University Road, 116°20′19.11″ E,

40°00′16.21′′ N, 51 m asl, on stems and branches of *Euonymus alatus*, Xinlei Fan, 12 November 2019 (CF 20198646; living culture, CFCC 54184).

Notes: *Cytospora haidianensis* differs from the phylogenetically related species *C. euonymicola* and *C. gigalocus* based on the sizes of the ectostromatic disc (240–350 µm diam in *C. euonymicola* and 330–620 µm diam in *C. gigalocus*), ostiole (60–120 µm diam in *C. euonymicola* and 130–190 µm diam in *C. gigalocus*), locules (1150–1400 µm diam in *C. euonymicola* and 1630–2180 µm diam in *C. gigalocus*), conidiophores (13–21.5 × 1.5–2 µm in *C. euonymicola* and 16.1–23.6 µm in *C. gigalocus*) and conidia (4.5–5 × 1 µm in *C. euonymicola* and 4.6–5.6 × 0.8–1.3 µm in *C. gigalocus*) [3,21]. Fan et al. [21] typified *C. gigalocus* based on material collected on the stems of *Juglans regia*, *C. euonymicola* and *C. euonymina* first found on twigs and branches of *Euonymus kiautschovicus* in China [3]. Similar to the other species, *C. haidianensis* also differs from the recently described species, *C. coryli*, based on macro- and micro-morphological characteristics [4]. At the molecular level, *C. haidianensis* differs from *C. euonymicola* by ACT (45/350), ITS (35/631), RPB2 (24/726), TEF1-α (47/725) and TUB2 (24/624), and differs from *C. gigalocus* by ACT (62/350), ITS (32/631), RPB2 (17/726), TEF1-α (41/725) and TUB2 (22/624).

Based on a BLAST search of the NCBI GenBank nucleotide database, the closest hits using the ACT sequence had distant hits with *Cytospora gigalocus* (strain CFCC 89620; GenBank KU710997; identities = 236/249 (94.78%), 3 gaps (1%)); *Cytospora carbonacea* (strain CFCC 50055; GenBank KP310838; identities = 237/252 (94.44%), 7 gaps (1%)). The closest hits using the ITS sequence had distant hits with *Cytospora populina* (strain CFCC 89644; GenBank KR045640; identities = 499/522 (95.59%), 10 gaps (1%)); *Cytospora cenisia* (strain CPC 28396; GenBank KY051983; identities = 489/521 (95.59%), 9 gaps (1%)). The closest hits using the RPB2 sequence had the highest similarity to *Cytospora gigalocus* (strain CFCC 89620; GenBank KU710957; identities = 690/711 (97.05%), 0 gaps (0%)); *Cytospora hippophaes* (strain CFCC 89637; GenBank KF765711; identities = 686/711 (96.48%), 0 gaps (0%)). The closest hits using the TEF1-α sequence had distant hits with *Cytospora coryli* (strain CFCC 53162; GenBank MN850758; identities = 397/423 (93.85%), 3 gaps (0%)); *Cytospora piceae* (strain CFCC 52842; GenBank MH820403; identities = 385/420 (91.67%), 12 gaps (2%)). The closest hits using the TUB2 sequence had distant hits with *Cytospora gigalocus* (strain CFCC 89620; GenBank KR045669; identities = 400/420 (95.24%), 11 gaps (2%)); *Cytospora leucostoma* (strain CFCC 53140; GenBank MN861115; identities = 395/419 (94.27%), 10 gaps (2%)).

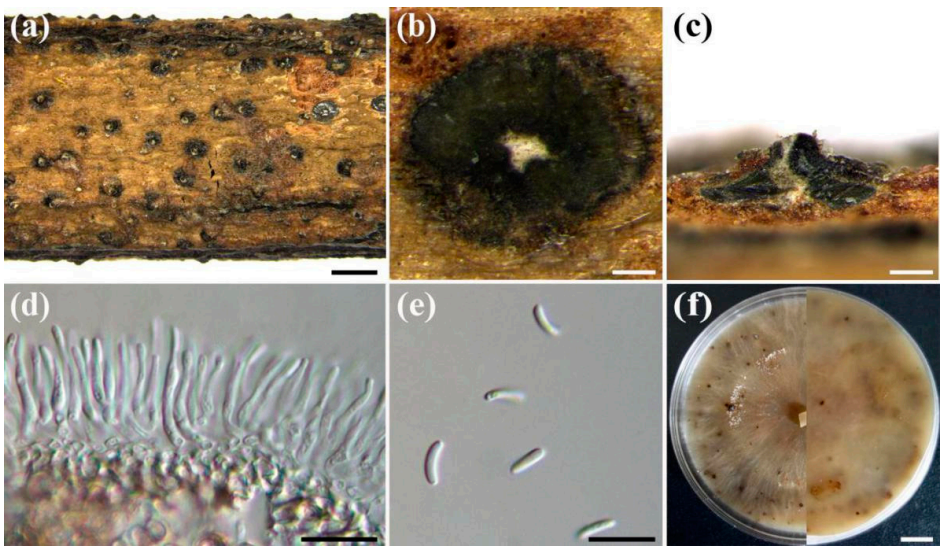

Figure 3. *Cytospora haidianensis* (CF 20198643). (**a**) Habitat of conidiomata on stems and branches of *Euonymus alatus*. (**b**) Transverse section of conidioma. (**c**) Longitudinal section through conidioma. (**d**) Conidiophores and conidiogenous cells. (**e**) Conidia. (**f**) Top (left) and bottom (right) sides of colonies on potato dextrose agar (PDA) after 30 days. Scale bars: **a**: 1 mm; **b**: 100 µm; **c**: 200 µm; **d**,**e**: 10 µm; **f**: 1 cm.

3.3. Pathogenicity Test

The three *Cytospora haidianensis* strains (CFCC 54184, CFCC 54056 and CFCC 54057) tested in this study were pathogenic on the *Euonymus alatus* twigs. No symptoms were observed in the non-inoculated controls. Brown lesions appeared at the inoculated points after 7 days of inoculation. The diseased spots turned brown and lesion areas were up to 16 mm long at 14 days after inoculation. By the third week after inoculation, the length of the brown necrotic lesions ranged from 36 to 45 mm (Figure 4). Koch's postulates were performed by successful re-isolation of fungal strains from all necrotic twigs inoculated with *Cytospora haidianensis*. The morphology and DNA sequences of the isolates retrieved from the inoculated twigs were consistent with those of the strains used for inoculation.

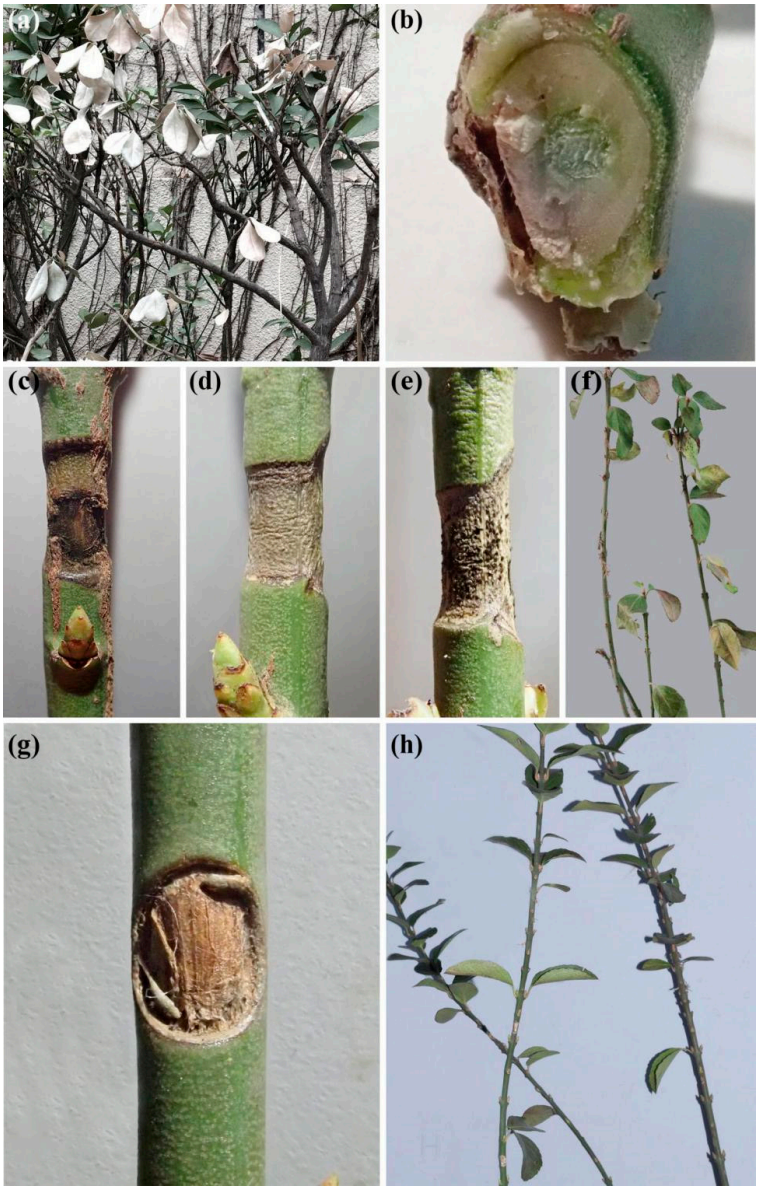

Figure 4. Stem blight symptoms on *Euonymus alatus* caused by *Cytospora haidianensis*. (**a**) Death of the whole plant caused by *C. haidianensis* on University Road, Beijing, China. (**b**) Stem blight caused by *C. haidianensis* in the greenhouse. Symptoms after (**c**) one week, (**d**) two weeks and (**e**) three weeks after inoculation of *C. haidianensis*. (**f**) Symptoms on *Euonymus alatus* twigs three weeks after inoculation of *Cytospora haidianensis*. (**g**,**h**) No symptoms on *Euonymus alatus* twigs after three weeks of inoculation with agar block (control).

3.4. Effects of Temperature and pH on Mycelial Growth

Colonies of *C. haidianensis* grew on PDA in the temperature range from 5 to 35 °C but not at 0 and 40 °C after 48 h of incubation. The fastest mycelial growth occurred at 19.8 °C, reaching 20 mm after 24 h and 86 mm after 96 h, and the least growth occurred at 5 and 35 °C. The data conform to the regression equations $Y = 4.535 + 0.986X - 0.13X^2$ ($p < 0.0001$, $R^2 = 0.846$) at 24 h, $Y = 4.747 - 2.868X - 0.64X^2$ ($p < 0.0001$, $R^2 = 0.883$) at 48 h, $Y = 6.667 + 4.821X - 0.132X^2$ ($p < 0.0001$, $R^2 = 0.868$) at 72 h and $Y = 6.263 + 8.055X - 0.239X^2 + 0.001X^3$ ($p < 0.0001$, $R^2 = 0.914$) at 96 h (X = temperature (°C), Y = growth (colony diameter, mm)). Based on the regression analysis, the optimal growth for *C. haidianensis* after incubation was estimated to occur at 19.8 °C (Figure 5).

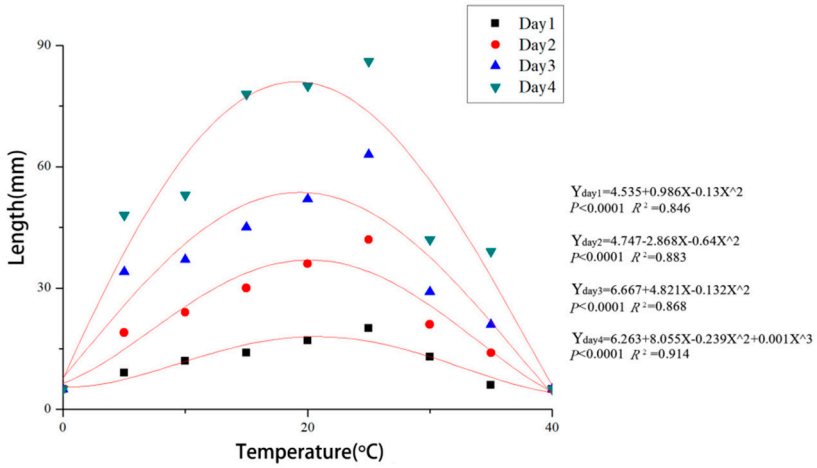

Figure 5. Regression curves and equations for mycelial growth of *Cytospora haidianensis* after incubation for 24, 48, 72 and 96 h at 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C on PDA medium (X = temperature (°C), Y = growth (colony diameter, mm)). Optimal mycelial growth temperature was estimated to be 19.8 °C.

Colonies of *C. haidianensis* grew on PDA in the pH range from 3.0 to 10.0, but not at pH 2.0 and 12.0. After 48 h, the mycelium of *C. haidianensis* grew on PDA in the pH range from 3.0 to 10.0, but not at pH 2.0 or 12.0. Mycelium grew most rapidly at pH 9.0 after 24 h, reaching 14 mm, followed by pH 8.0 and 10.0, which gave colony diameters of 13 mm and 12 mm, respectively. The mycelia almost covered the 90 mm dishes after 96 h incubation at pH 8.0 and 9.0, while they grew more slowly at pH 3.0, 4.0, 5.0 and 11.0, attaining colony diameters of no more than 45 mm after 96 h. The data fit the regression equations $Y = 5.788 - 2.075X + 0.795X^2 - 0.53X^3$ ($p < 0.0001$, $R^2 = 0.837$) at 24 h, $Y = 10.848 - 7.209X + 2.328X^2 - 0.148X^3$ ($p < 0.0001$, $R^2 = 0.955$) at 48 h, $Y = 9.576 - 7.340X + 3.080X^2 - 0.210X^3$ ($p < 0.0001$, $R^2 = 0.964$) at 72 h and $Y = 20.424 - 17.750X + 6.382X^2 - 0.420X^3$ ($p < 0.0001$, $R^2 = 0.948$) at 96 h incubation (X = pH, Y = growth (colony diameter, mm)) (Figure 6). Based on these regression equations, the optimal growth of *C. haidianensis* after 24 and 48 h incubation was estimated to be at pH 8.3.

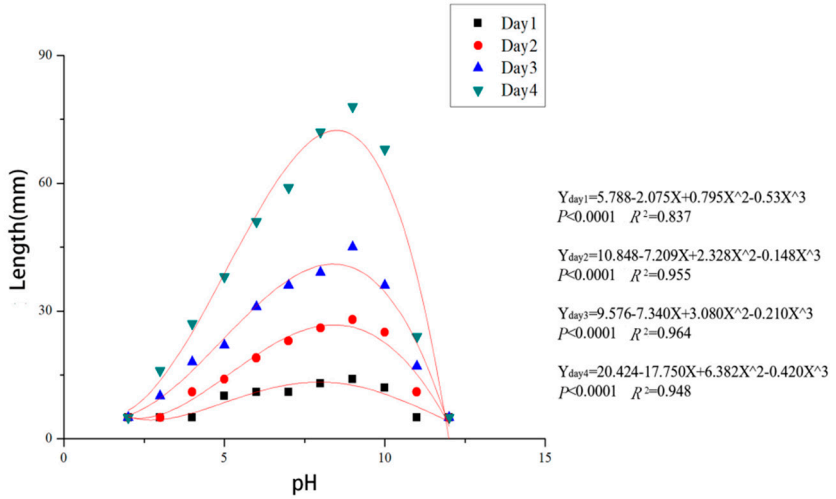

Figure 6. Regression curves and equations for mycelial growth of *Cytospora haidianensis* after incubation for 24, 48, 72 and 96 h at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 on PDA medium (X = pH, Y = growth (colony diameter, mm)). Optimum mycelial growth was estimated to be at pH 8.3.

3.5. Effects of Carbon Sources on Mycelial Growth

Cytospora haidianensis was able to grow using all six carbon sources tested. After 24 h, the utilization of sucrose was significantly greater than galactose, while there was no difference among fructose, glucose, xylose and maltose, which were slightly less well utilized than the other three carbon sources. The utilization of galactose was significantly lower than that of all other carbon sources tested. However, after 96 h, sucrose utilization was significantly higher than galactose and xylose, while there was no difference between fructose and glucose. Galactose had the lowest level of carbon utilization (Figure 7).

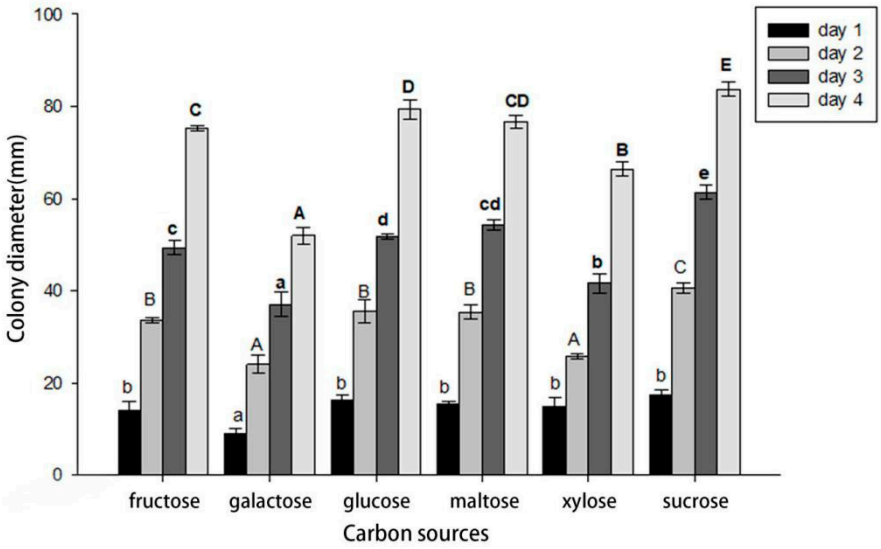

Figure 7. Effects of carbon source on growth of *Cytospora haidianensis*. Bars with uppercase or lowercase letters represent significant differences after, respectively, 24, 48, 72 and 96 h incubation, according to HSD tests at the $p < 0.05$ level.

4. Discussion

In the present study, three specimens were collected from symptomatic branches and twigs associated with dieback disease of *Euonymus alatus* in Beijing, China. A novel fungal species,

C. haidianensis, was introduced based on molecular, morphological and physiological data, and confirmed as the causal agent after pathogenic tests.

According to our multilocus phylogenetic analysis, *C. haidianensis* is a lineage well supported (MP-BS = 100, ML-BS = 100 and BPP = 1.0) and placed in a clade related to *C. euonymicola*, *C. gigalocus* and *C. coryli* (Figure 3). In a recent study, Fan et al. [3] described *C. euonymicola* and *C. euonymina* from twigs and branches of *Euonymus kiautschovicus* in Shaanxi Province, China. Comparing these species with the novel species *C. haidianensis*, *C. euonymicola* mainly has small ectostromatic discs (240–350 µm diam) and conidia (4.5–5 × 1 µm) and *C. euonymina* mainly has small ectostromatic discs (200–230 µm diam) and slightly larger conidia (6.5–7.5 × 1.5–2 µm), but the latter is not phylogenetically related to the new species. *Cytospora gigalocus* was described by Fan et al. [22] on stems of *Juglans regia* in Qinghai Province, China, mainly having slightly large ectostromatic discs (330–620 µm diam) and small conidia (4.6–5.6 × 0.8–1.3 µm), differing from *C. haidianensis* based on these morphological features (see notes for *C. haidianensis*). *Cytospora coryli* was recently proposed by Zhu et al. [4] as necrotrophic on branches of *Corylus mandshurica* in Mount Dongling (China), differing from *C. haidianensis* based on the size of ectostromatic discs (270–340 µm diam), large locules (1550–1710 µm diam), conidiophores (15.5–18.5 × 1–2 µm), conidiogenous cells (7.5–14 × 1–2 µm) and conidia (5–7 × 1–2 µm), and culture characteristics.

Pathogenicity tests were conducted on 1-year potted *E. alatus* plants in a greenhouse. The results indicated that *C. haidianensis* was pathogenic on *E. alatus* twigs. According to Pan et al. [7], *Cytospora* species invade the xylem and cause mortality of the whole branch, similar to the results obtained in this study within three weeks, showing the typical stem blight that occurred in the sampled place (Figure 4). The growth temperature for phytopathogenic fungi is generally from 10 to 35 °C, optimally from 20 to 30 °C [33]. For instance, the optimal growth temperature of *Penicillium cellarum* causing rot in stored sugar beet roots was reported as 22 °C [34]; for *Diaporthe neotheicola* and *D. ambigua* causing dieback blueberry in Chile, it was 25 °C; for *Diaporthe* sp., it was 22 °C [35]; and for *Phoma sorghina*, which was found to cause twisted leaf disease in sugarcane in China, it was 20–25 °C [36]. The mycelia of *C. haidianensis* grew from 5 to 35 °C, with an optimal temperature of 19.8 °C (Figure 5).

Most phytopathogenic fungi grow optimally in a pH range between 5 and 6.5 [37]. For *Lasiodiplodia vaccinii*, the range was 5.0 to 7.0, though it could still grow slowly at pH of 4.0 or 10.0 [33]. Similar results have been reported for *L. theobromae*, which could grow on media with a pH range from 4.0 to 10.0, with the optimal pH in the range of 5.0 to 7.0 [36]. The optimal pH value for *C. haidianensis* was from 8.0 to 10.0, though it could still grow slowly at pH of 4.0 or 11.0 (Figure 6). All six carbon sources tested in this study contributed to the growth of *C. haidianensis*, with less utilization of xylose than all the other carbon sources used (Figure 7).

The dieback in *Euonymus alatus* caused by *C. haidianensis* damages the plants. *Cytospora haidianensis* blights many branches and leaves discolouration, causing gradual death of a large number of *E. alatus* (Figure 4). This phenomenon is not confined to Beijing; *Cytospora euonymicola* was also reported as a pathogenic fungus from *Euonymus* in Shaanxi Province, and *Cytospora euonymina* was also found in *Euonymus* in Shanxi Province [3]. A similar phenomenon also happens in other countries; *Cytospora euonymi* was also associated with the blight of *Euonymus* twigs in the USA and Europe. Other genera such as *Cercospora*, *Colletotrichum*, *Coniothyrium* and *Fusarium* were also reported to be pathogenic fungi in *Euonymus* [38].

To date, *C. haidianensis* has been found only from *Euonymus alatus* in Beijing. Management practices, including better ventilation and lighting, might help to alleviate the damage resulting from stem dieback caused by *C. haidianensis*. The distribution and host spectrum of *C. haidianensis* need further study.

5. Conclusions

A novel fungal species, *Cytospora haidianensis*, is an emerging pathogen on *Euonymus alatus* dieback disease in Beijing, China. The new species is the causal agent for *E. alatus* by Koch's postulates that

grows best at 19.8 °C, pH 8.3. All the six carbon sources tested support the growth of *C. haidianensis* with the sucrose utilization is significantly higher than others.

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