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ORIGINAL ARTICLE

Cytospora from *Ulmus pumila* in Northern China

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Abstract *Cytospora* species are cosmopolitan, and commonly associated with dieback and canker diseases of numerous hosts. In the present study, isolates were collected and identified from diseased branches or twigs of *Ulmus pumila* in northern China. The morphological characteristics and multilocus phylogeny (act1, ITS, LSU, tefA and tubB) indicate four distinct lineages with high branch support, i.e., *C. carbonacea*, *C. chrysosperma*, *C. ribis* and *C. pruinopsis* sp. nov. *Cytospora pruinopsis* is distinguishable from the other *Cytospora* spp. on *Ulmus* by its single conidiomatal locule with one ostiole per disc, and its smaller conidia. This study represents the first attempt to clarify the taxonomy of *Cytospora* spp. associated with canker and dieback symptoms of *Ulmus pumila* in northern China.

Keywords Ascomycota · Canker disease · Diaporthales · Systematics

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Introduction

The genus *Cytospora* (Sordariomycetes, Diaporthales, Valsaceae), which was introduced by Ehrenberg (1818), is presently regarded as a major causal agent of canker diseases in dicots and monocots (Adams et al. 2005). *Cytospora* has been regarded as the asexual morph of *Valsa* Fr., *Valsella* Fuckel, *Valseutypella* Höhn. and *Leucostoma* (Nitschke) Höhn. (Fries 1823; Saccardo 1884; Spielman 1985; Adams et al. 2005). Following the end of dual nomenclature for pleomorphic fungi (Wingfield et al. 2012; Crous et al. 2015), the older and more commonly encountered genus *Cytospora* (1818) was chosen over that of its sexual morph, *Valsa* (1849), for placement on the list of protected fungi (McNeill et al. 2012; Fan et al. 2015a, b; Rossman et al. 2015). Morphologically, the *Cytospora* sexual morph is characterized by a diaporthalean-like perithecial ascoma, clavate to elongate obovoid asci, and allantoid, hyaline, aseptate ascospores (Spielman 1983, 1985; Adams et al. 2005). The asexual morph is characterized by single or labyrinthine locules, filamentous conidiophores, and allantoid, hyaline, aseptate conidia (Spielman 1983, 1985; Adams et al. 2005). Species identification in *Cytospora* was previously based on host affiliations and morphology (Deng 1963; Tai 1979; Wei 1979), but because these characters have limited value, molecular phylogenetic data were needed to accurately distinguish *Cytospora* spp. (Adams et al. 2002, 2005). Several recent papers have subsequently provided updated phylograms for the genus based on multigene phylogenies using ex-type or reference strains (Zhang et al. 2014a, b; Fan et al. 2015a, b; Wang et al. 2015).

Elms (*Ulmus* L.) are temperate plants with worldwide distribution. In China, *Ulmus pumila* L. is a component of natural forests, with some specimens also planted as ornamental trees in streets, gardens and parks. Although *Cytospora* spp. have

been recorded from a range of disease symptoms on *Ulmus* spp., the identities of the species involved have never been confirmed based on molecular techniques (Spaulding 1961; Conway and Morrison 1983; Chen 2002; Dudka et al. 2004; Zhuang 2005; Fotouhifar et al. 2010; Zhang et al. 2014a, b).

Previous studies have reported several *Cytospora* spp. from *Ulmus* spp. worldwide, i.e., *C. ambiens* Pers., *C. carbonacea* Fr., *C. chrysosperma* (Pers.) Fr., *C. leucostoma* (Pers.) Sacc., *C. pulchella* Sacc., and *C. sacculus* (Schwein.) Gvrit. (Gilman et al. 1957; Conway and Morrison 1983; Zhuang 2005; Mulenko et al. 2008; Fotouhifar et al. 2010). Thus far, three *Cytospora* spp. have been recorded from this host in China: *C. ambiens*, *C. carbonacea* and *C. pulchella* (Zhuang 2005; Zhang et al. 2014a, b). However, these species were recorded without detailed morphological or multi-gene phylogenetic analyses, and the phytopathogenic taxa causing Cytospora canker disease of *Ulmus* have not been clarified. In order to confirm the identities of *Cytospora* species that occur on *Ulmus* spp. in China, a total of 37 specimens were collected from symptomatic trees in six provinces in northern China. The objectives of this study were to 1) isolate the *Cytospora* spp. from symptomatic twigs and branches of *Ulmus* spp. and compare them to reference strains; and to 2) generate a multi-gene DNA phylogeny of the taxa concerned, and describe, illustrate and compare the new *Cytospora* species from *Ulmus pumila*.

Materials and methods

Isolates

Fresh specimens of *Cytospora* spp. were collected from infected branches or twigs of *Ulmus pumila* during collecting trips in Heilongjiang, Jilin, Ningxia, Qinghai, Shaanxi and Tibet Provinces in China (Table 1). Single conidial isolates were established from fruiting bodies by removing a mucoid conidial mass from pycnidial ostioles, and spreading the suspension on the surface of 1.8 % potato dextrose agar (PDA), incubated at 25 °C for up to 24 h. Single germinating conidia were removed and plated onto fresh PDA plates. Ten representative strains (selected based on morphology and cultural characteristics) were used in the phylogenetic analysis (Table 1). Specimens are deposited in the Museum of Beijing Forestry University (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Center (CFCC). Isolates of the new species are maintained in the China Center for Type Culture Collection (CCTCC).

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from colonies grown on cellophane-covered PDA using a modified CTAB [cetyltrimethylammonium bromide] method (Doyle and Doyle 1990). DNA was estimated by electrophoresis in 1 % agarose gel, and the quality was measured using the NanoDrop™ 2000 (Thermo Scientific, Waltham, MA, USA), following the user manual (Desjardins et al. 2009). PCR amplifications were performed in a DNA Engine Peltier Thermal Cycler (PTC-200; Bio-Rad Laboratories, Hercules, CA, USA). The ITS region was amplified using primers ITS1 and ITS4 (White et al. 1990). The partial large nuclear ribosomal RNA subunit (LSU) region was amplified using primers NL1 and NL4 (O'Donnell 1993). The partial actin (act1) region was amplified using primers ACT512F and ACT783R (Carbone and Kohn 1999). The partial translation elongation factor 1-alpha (tefA) gene region was amplified using primers EF1-728F and EF1-986R (Carbone and Kohn 1999), and tubB was amplified using primers Bt2a and Bt2b (Glass and Donaldson 1995). The PCR amplification products were estimated visually by electrophoresis in 2 % agarose gel. DNA sequencing was performed using an ABI PRISM® 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye® Terminator Kit v.3.1 (Invitrogen, Carlsbad, CA, USA) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequence analysis

DNA sequences generated by forward and reverse primers were used to obtain consensus sequences using SeqMan v.7.1.0 in the DNASTAR Lasergene Core Suite software program (DNASTAR Inc., Madison, WI, USA). Sequences were aligned using MAFFT v.6 (Katoh and Toh 2010) and edited manually using MEGA5 (Tamura et al. 2011). Phylogenetic analysis was performed using PAUP* v.4.0b10 for maximum parsimony (MP) analysis (Swofford 2003), MrBayes v.3.1.2 for Bayesian analysis (Ronquist and Huelsenbeck 2003), and PhyML v.7.2.8 for maximum likelihood (ML) analysis (Guindon et al. 2010). The first analysis was performed on the combined multi-gene dataset (act1, ITS, LSU, tefA and tubB). A second analysis using ITS sequence data was performed to compare *Cytospora* species from the current study with other ex-type strains in GenBank (Supplementary Table 1). *Phomopsis vaccinii* Shear was selected as outgroup (Adams et al. 2005). Trees were shown using FigTree v.1.3.1 (Rambaut and Drummond 2010).

MP analysis was performed by a heuristic search option of 1000 random-addition sequences with a tree bisection and reconnection (TBR) algorithm. Maxtrees were set to 5000, branches of zero length were collapsed, and all equally parsimonious trees were saved.

Table 1 Strains included in molecular phylogenetic analyses

Species	Strain*	Host	Origin	GenBank accession numbers				
				ITS	LSU	act1	tefA	tubB
C. atrocirrhata	CFCC 89615	*Juglans regia*	Xining, Qinghai, China	KF225610	KF225624	KF498673	KP310858	KF498688
	CFCC 89616	*Juglans regia*	Xining, Qinghai, China	KF225611	KF225625	KF498674	KP310859	KF498689
C. carbonacea	**CFCC 50055**	*Ulmus pumila*	Qiqihar, Heilongjiang, China	KP281262	KP310808	KP310838	KP310851	KP310821
	CFCC 50056	*Ulmus pumila*	Huzhu, Qinghai, China	KP281263	KP310809	KP310839	KP310852	KP310822
	CFCC 50058	*Ulmus pumila*	Ping'an, Qinghai, China	KP281264	KP310810	KP310840	KP310853	KP310823
	CFCC 50059	*Ulmus pumila*	Xining, Qinghai, China	KP281265	KP310811	KP310841	KP310854	KP310824
	CFCC 89947	*Ulmus pumila*	Xining, Qinghai, China	KP281266	KP310812	KP310842	KP310855	KP310825
C. chrysosperma	**CFCC 89982**	*Ulmus pumila*	Shigatse, Xizang, China	KP281261	KP310805	KP310835	KP310848	KP310818
	CFCC 89600	*Sophora japonica*	Gansu, China	KC880150	KP310806	KP310834	KP310847	KP310817
	CFCC 89630	*Salix psammophila*	Yulin, Shaanxi, China	KF765674	KF765690	KF765722	KP321972	KP321973
C. hippophaes	CFCC 89636	*Hippophae rhamnoides*	Guyuan, Ningxia, China	KF765678	KF765694	KF765726	KP310864	KP310830
	CFCC 89640	*Hippophae rhamnoides*	Gannan, Gansu, China	KF765682	KF765698	KF765730	KP310865	KP310831
C. nivea	CFCC 89642	*Salix psammophila*	Yulin, Shaanxi, China	KF765684	KF765700	KF765732	KP310862	KP310828
	CFCC 89643	*Salix psammophila*	Yulin, Shaanxi, China	KF765685	KF765701	KF765733	KP310863	KP310829
C. pruinosa	CFCC 50036	*Syzygium aromaticum*	Ping'an, Qinghai, China	KP310800	KP310802	KP310832	KP310845	KP310815
	CFCC 50037	*Syzygium aromaticum*	Ping'an, Qinghai, China	KP310801	KP310803	KP310833	KP310846	KP310816
C. pruinopsis	**CFCC 50034**	*Ulmus pumila*	Harbin, Heilongjiang, China	KP281259	KP310806	KP310836	KP310849	KP310819
	CFCC 50035	*Ulmus pumila*	Harbin, Heilongjiang, China	KP281260	KP310807	KP310837	KP310850	KP310820
C. ribis	**CFCC 50026**	*Ulmus pumila*	Yulin, Shaanxi, China	KP281267	KP310813	KP310843	KP310856	KP310826
	CFCC 50027	*Ulmus pumila*	Yulin, Shaanxi, China	KP281268	KP310814	KP310844	KP310857	KP310827
C. sacculus	CFCC 89624	*Juglans regia*	Gannan, Gansu, China	KF225615	KM401886	KM401888	KP310860	KM401890
	CFCC 89625	*Juglans regia*	Gannan, Gansu, China	KF225616	KM401887	KM401889	KP310861	KM401891

* Ex-type strains used in this study are indicated in *bold*

Clade stability was assessed with bootstrap analysis of 1000 replicates (Hillis and Bull 1993). Other calculated parsimony scores included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was also performed with a generalised time-reversible (GTR) site substitution model, according to previous studies (Guindon et al. 2010; Fan et al. 2015a). The branch support was evaluated with a bootstrapping (BS) method of 1000 replicates (Hillis and Bull 1993).

Bayesian inference (BI) analysis employing a Markov chain Monte Carlo (MCMC) algorithm was performed (Rannala and Yang 1996). A nucleotide substitution model was estimated by MrModeltest v.2.3 (Posada and Crandall 1998). Two MCMC chains were run from random trees for 1000,000 generations, and trees were sampled every 100th generation, resulting in a total of 10,000 trees. The first 25 % of trees were discarded as the burn-in phase of each analysis. Branches with significant Bayesian posterior probabilities (BPP) were estimated in the remaining 7500 trees.

Sequence data were deposited in GenBank (Table 1). The multilocus and ITS sequence alignment files were deposited in TreeBASE (www.treebase.org) as accession S16893. The taxonomic novelty was deposited in MycoBank (Crous et al. 2004).

Morphology

Species identification was based on morphological features of the fruiting bodies produced on infected plant tissues, supplemented by cultural characteristics. Hence, thin cross-sections were prepared by hand using a double-edged blade. Morphological characteristics of the fruiting bodies including size and arrangement of stromata, presence or absence of conceptacle in stromata, number and diameter of ostioles per disc, shape and size of discs, arrangement of locules, and size and shape of conidiophores and conidia (asci and ascospores) were determined under a light microscope. More than 20 fruiting bodies were sectioned, and 50 spores were randomly selected for measurement using a Leica compound microscope (DM2500; Leica Microsystems, Wetzlar, Germany). Cultural characteristics of isolates incubated on PDA in the dark at 25 °C were recorded, including colony colour and pycnidium structure.

Results

Molecular phylogeny

A total of 37 specimens associated with *Cytospora* infections were collected from *Ulmus pumila* in six provinces in China. Of these, ten strains representing four species were added to 12 reference sequences for further phylogenetic analyses. The phylogram (Fig. 1) generated here indicated 22 ingroup taxa, including 2863 characters, of which 1827 characters were constant; 138 variable characters were parsimony-uninformative and 898 were parsimony-informative. The heuristic search using maximum parsimony (MP) generated two parsimonious trees (TL=2332, CI=0.673, RI=0.804, RC=0.541), from which one was selected and is shown in Fig. 1.

Phylogenetic analysis of ITS sequence data of representative *Cytospora* sequences, including sequences from available ex-type strains, comprised 101 sequences. The *Cytospora* dataset included a total of 584 base pairs used for analyses after alignment. Of these, 352 characters were constant; 62 variable characters were parsimony-uninformative and 170 were parsimony-informative. A heuristic MP search generated 500 parsimonious trees, with the best tree with short tree length (TL=820, CI=450, RI=0.829, RC=0.373) shown in Fig. 2. All trees of ML and Bayesian analysis are in agreement, and do not significantly differ from the MP tree.

Taxonomy

Cytospora pruinopsis C.M. Tian & X.L. Fan, **sp. nov.** (Fig. 3)
MycoBank 813225

Etymology: Named after its morphological similarity to *C. pruinosa*.

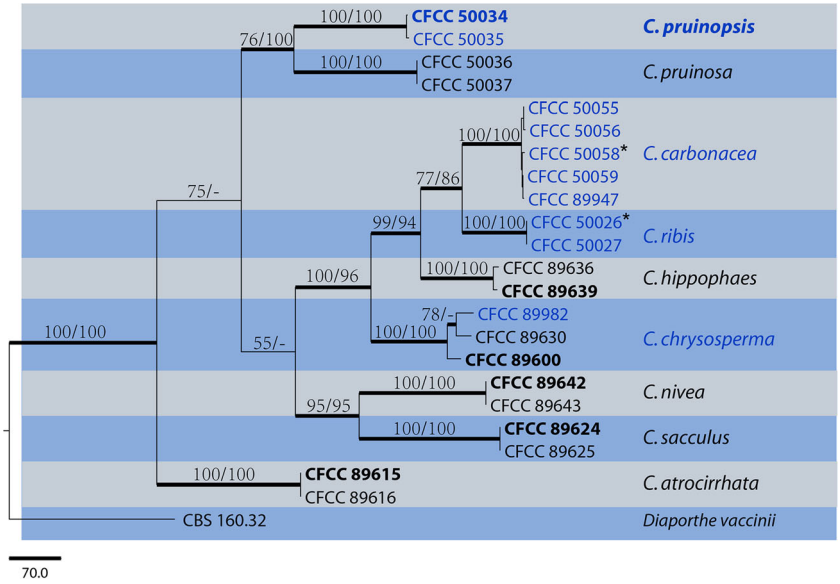


Fig. 1 Phylogram of combined act1, ITS, LSU, tefA, and tubB genes generated from maximum parsimony (MP) analyses. Values above the branches indicate maximum parsimony bootstrap (MP BP≥50 %) and maximum likelihood bootstrap (ML BP≥50 %). Thickened branches represent posterior probabilities (BI PP≥0.90) from Bayesian inference. Scale bar=70 nucleotide substitutions. Ex-type strains are in *bold*

Fig. 2 Phylogram of ITS regions generated from maximum parsimony (MP) analyses. Values above the branches indicate maximum parsimony bootstrap (MP BP≥50 %) and maximum likelihood bootstrap (ML BP≥50 %). Values below branches represent posterior probabilities (BI PP≥0.90) from Bayesian inference. Scale bar=20 nucleotide substitutions. The new sequences resulting from the current study are in *blue*. Ex-type strains are in *bold* ▶

Differs from *C. pruinosa* in wing-like ectostroma around ostiole and smaller conidial size, 2–4×1 μm.

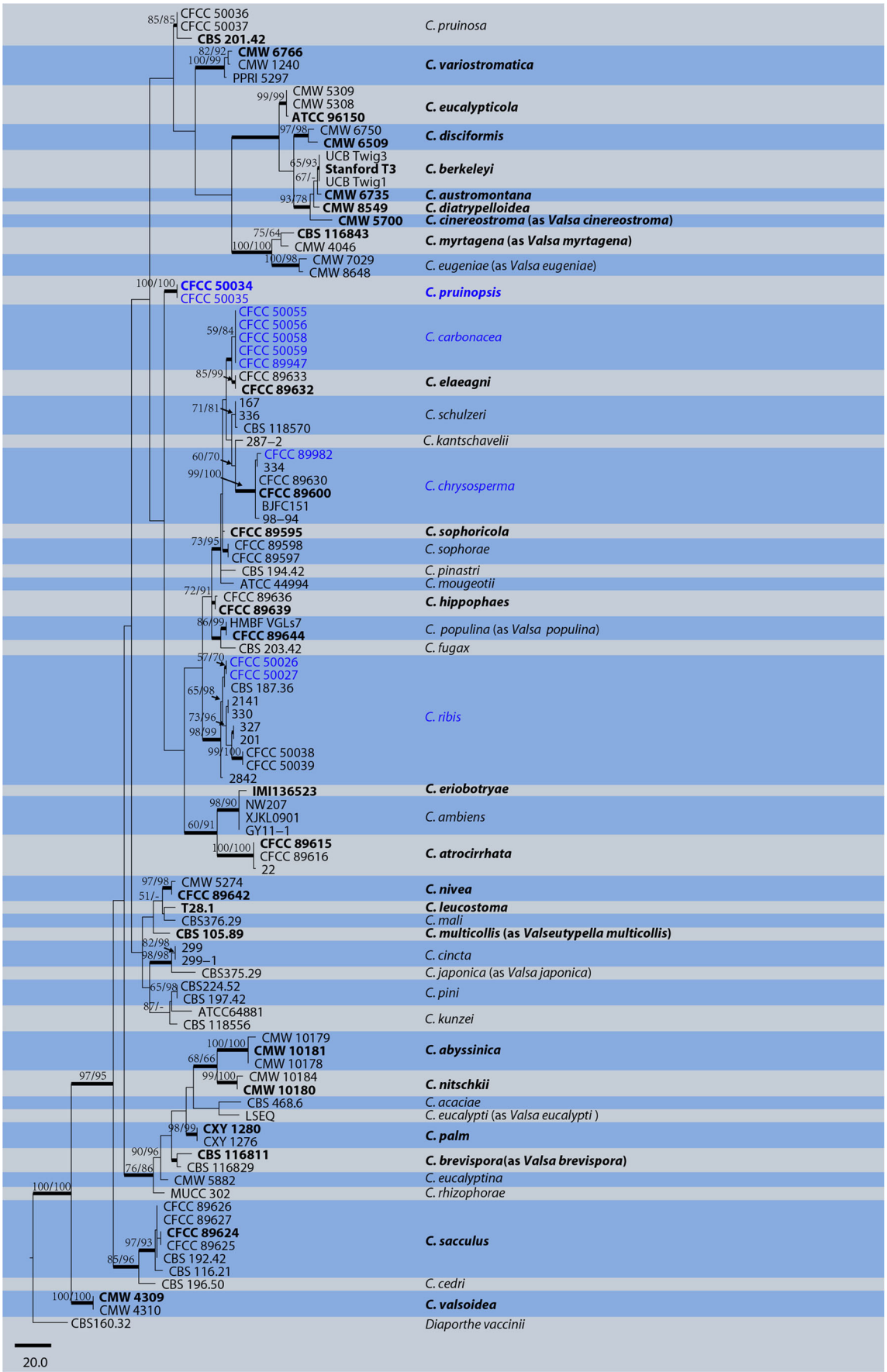
Holotype: BJFC-S1073. **CHINA**, **Shaanxi Province**: Yulin City, Yuyang, 37°26′50.46″N, 116°20′39.44″E, 1120 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 14 May 2013 (BJFC-S1073, *holotype*; living ex-type culture, CFCC50034, CCTCC AF2014026);

Host/Distribution: Pathogen on twigs and branches of *Ulmus pumila* in China.

Conidiomata immersed in bark, slightly erumpent through the bark surface, discoid, with a single locule; superficially resembling cytophomoid conidiomata. *Disc* grey to black, nearly flat, circular to ovoid, (280–)290–310(−330) μm (av.=300 μm, *n*=20) diam, with one ostiole per disc. *Ostiole* medium grey to black, prominent, (106–)120.5–145.5(−152.5) μm (av.=130.5 μm, *n*=20) diam. *Locule* undivided, irregular, (470–)520–720(−790) μm (av.=600 μm, *n*=20) diam. *Conidiophores* hyaline, unbranched or occasionally branched at base and commonly branched above the base, (13–)14–20(−21) μm (av.=18 μm, *n*=20). *Conidia* hyaline, eguttulate, elongate-allantoid, aseptate, (2–)2.5–3.5(−4)×1 μm (av.=3×1 μm, *n*=50).

Cultures: Colony growth on PDA initially white, becoming grey after 7–10 days. Colonies flat, felt-like, with a uniform texture. Conidiomata irregularly dispersed over agar surface.

Materials examined: **CHINA**, **Jilin Province**: Tonghua City, 41°73′56.12″N, 125°96′85.84″E, 225 m.a.s.l., on twigs


85/85 CFCC 50036
CFCC 50037
CBS 201.42
C. pruinosa
82/92 CMW 6766
100/99 CMW 1240
PPRI 5297
C. variostromatica
99/99 CMW 5309
CMW 5308
ATCC 96150
C. eucalypticola
97/98 CMW 6750
CMW 6509
C. disciformis
65/93 UCB Twig3
Stanford T3
67/- UCB Twig1
C. berkeleyi
93/78 CMW 6735
C. austromontana
CMW 8549
C. diatrypelloidea
CMW 5700
C. cinereostroma (as Valsa cinereostroma)
75/64 CBS 116843
100/100 CMW 4046
C. myrtagena (as Valsa myrtagena)
100/98 CMW 7029
CMW 8648
C. eugeniae (as Valsa eugeniae)
100/100 CFCC 50034
CFCC 50035
C. pruinopsis
CFCC 50055
CFCC 50056
59/84 CFCC 50058
CFCC 50059
CFCC 89947
C. carbonacea
85/99 CFCC 89633
CFCC 89632
C. elaeagni
71/81 167
336
CBS 118570
C. schulzeri
287–2
C. kantschavelii
60/70 CFCC 89982
99/100 334
CFCC 89630
CFCC 89600
BJFC151
98–94
C. chrysosperma
CFCC 89595
C. sophoricola
73/95 CFCC 89598
CFCC 89597
C. sophorae
CBS 194.42
C. pinastri
ATCC 44994
C. mougeotii
72/91 CFCC 89636
CFCC 89639
C. hippophaes
86/99 HMBF VGLs7
CFCC 89644
C. populina (as Valsa populina)
CBS 203.42
C. fugax
57/70 CFCC 50026
CFCC 50027
65/98 CBS 187.36
2141
73/96 330
98/99 327
201
99/100 CFCC 50038
CFCC 50039
2842
C. ribis
IMI136523
C. eriobotryae
98/90 NW207
XJKL0901
60/91 GY11–1
C. ambiens
100/100 CFCC 89615
CFCC 89616
22
C. atrocirrhata
97/98 CMW 5274
CFCC 89642
C. nivea
51/- T28.1
C. leucostoma
CBS376.29
C. mali
CBS 105.89
C. multicollis (as Valseutypella multicollis)
82/98 299
98/98 299–1
C. cincta
CBS375.29
C. japonica (as Valsa japonica)
65/98 CBS224.52
CBS 197.42
C. pini
87/- ATCC64881
CBS 118556
C. kunzei
100/100 CMW 10179
CMW 10181
68/66 CMW 10178
C. abyssinica
97/95 99/100 CMW 10184
CMW 10180
C. nitschkii
CBS 468.6
C. acaciae
LSEQ
C. eucalypti (as Valsa eucalypti)
98/99 CXY 1280
CXY 1276
C. palm
90/96 CBS 116811
76/86 CBS 116829
C. brevispora(as Valsa brevispora)
CMW 5882
C. eucalyptina
100/100 MUCC 302
C. rhizophorae
CFCC 89626
CFCC 89627
CFCC 89624
97/93 CFCC 89625
85/96 CBS 192.42
CBS 116.21
C. sacculus
CBS 196.50
C. cedri
100/100 CMW 4309
CMW 4310
C. valsoidea
CBS160.32
Diaporthe vaccinii
20.0

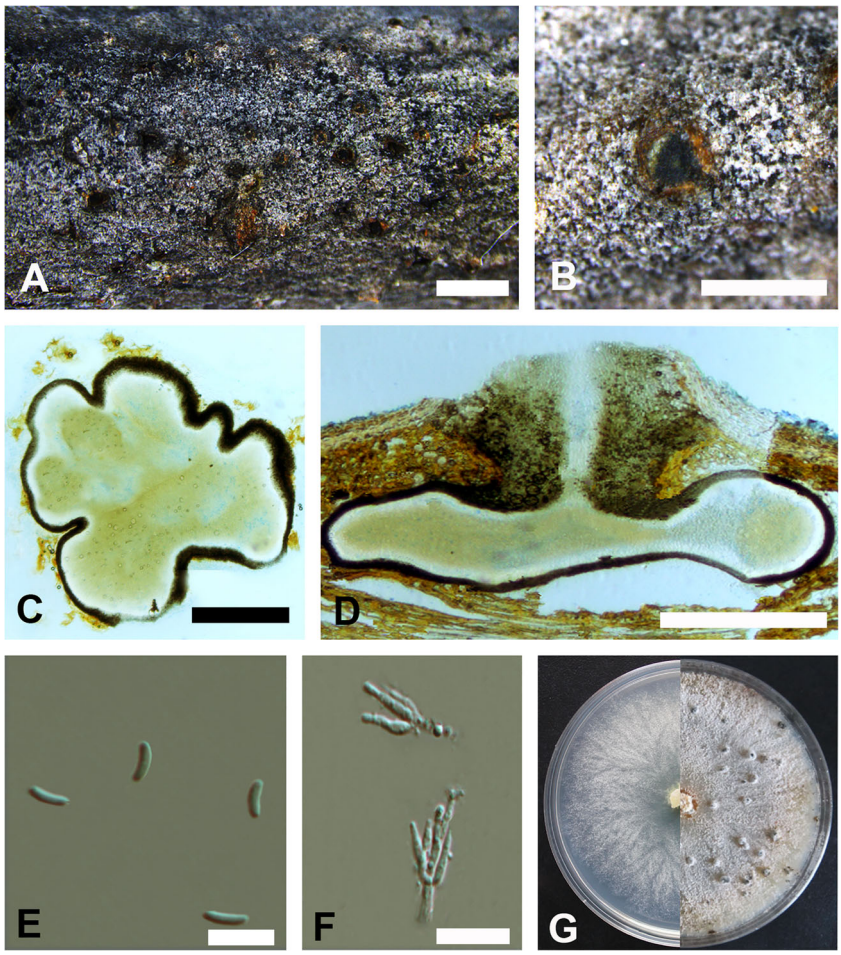


Fig. 3 *Cytospora pruinopsis* from *Ulmus pumila* (BJFC-S1073, ***holotype***). **a**, **b** Habit of conidiomata on a twig in vivo. **c** Transverse sections through conidiomata. **d** Longitudinal sections through conidiomata. **e** Conidia. **f** Conidiophores. **g** Colonies on PDA at 3 days (*left*) and 30 days (*right*). Scale bars: A=1 mm; B=500 μm; C, D=200 μm; E, F=5 μm

and branches of *U. pumila*, coll. X.L. Fan, 7 June 2012 (BJFC-S334, *paratype*; ex-paratype culture, CFCC50035).

Cytospora chrysosperma (Pers.) Fr., Syst. Mycol. 2: 542, 1823. (Fig. 4),

Host/Distribution: Pathogen on twigs and branches of *U. pumila*. Known from *Armeniaca vulgaris*, *Crataegus azarolus*, *Ficus carica*, *Ligustrum latifolium*, *Malus pumila*, *Morus alba*, *Olea sativa*, *Persica vulgaris*, *Prunus domestica*, *Robinia pseudoacacia* and *Thuja orientalis* in Iran; *Fraxinus* in Europe and Iran; *Juglans regia* in China and Iran; Salicaceae in China, Iran, Netherlands, South Africa, Switzerland, UK and USA; *Sophora japonica* in China; *Triticum* in Germany; and *Ulmus* in USA. (Saccardo 1884; Adams et al. 2005; Gadgil 2005; Fotouhifar et al. 2010; Fan et al. 2014a, 2015a).

Ascostromata immersed in the bark, erumpent, scattered, circular to ovoid, (800–)870–1090(−1120) μm (av.=900 μm, *n*=20) diam. *Disc* usually obscured by tightly ostiolar necks, when apparent pale brown to black, nearly hemispherical, circular to ovoid, (300–)330–370(−400) μm (av.=350 μm, *n*=20) diam, with 4–8 ostioles arranged circinately in a disc, brown to black, (65–)68.5–81(−89.5) μm (av.=75.5 μm, *n*=20) diam; 4–8 perithecia arranged circinately in black entostromata, flask-shaped to spherical, (420–)460–550(−610) μm (av.=500 μm, *n*=20) diam. *Asci* free, clavate to elongate obovoid, 25–33.5(−34)×4–4.5 μm (av.=31×4 μm, *n*=20), 8-spored. *Ascospores* elongate-allantoid, thin-walled, hyaline, aseptate, (9–)9.5–11.5(−12)×(2–)2.5–3 μm (av.=10×2.5 μm, *n*=50). *Conidiomata* immersed in bark, erumpent, discoid, flask-shaped to conical, with a large multiple locules. *Disc* grey to black, nearly flat, circular to ovoid, (200–)230–310(−340) μm (av.=300 μm, *n*=20) diam, with one ostiole per disc. *Ostiole* medium grey, prominent, (61.5–)65–81(−86.5) μm (av.=75.5 μm, *n*=20) diam. *Locules* complex multi-loculed, subdivided frequently by invaginations, sharing common walls, (620–)650–1250(−1280) μm (av.=1110 μm, *n*=20) diam. *Conidiophores* hyaline, unbranched or occasionally branched at the base, (15.5–)16–23 μm (av.=17.5 μm, *n*=20). *Conidia* hyaline, eguttulate, elongate-allantoid, aseptate, (4–)4.5–5(−5.5)×1–1.5 μm (av.=4.5×1.5 μm, *n*=50).

Cultures: Culture initially white, becoming partially pale yellow after 6–7 days, chiefly white. Colonies flat, felt-like, with uniform texture. Conidiomata formed randomly over agar surface.

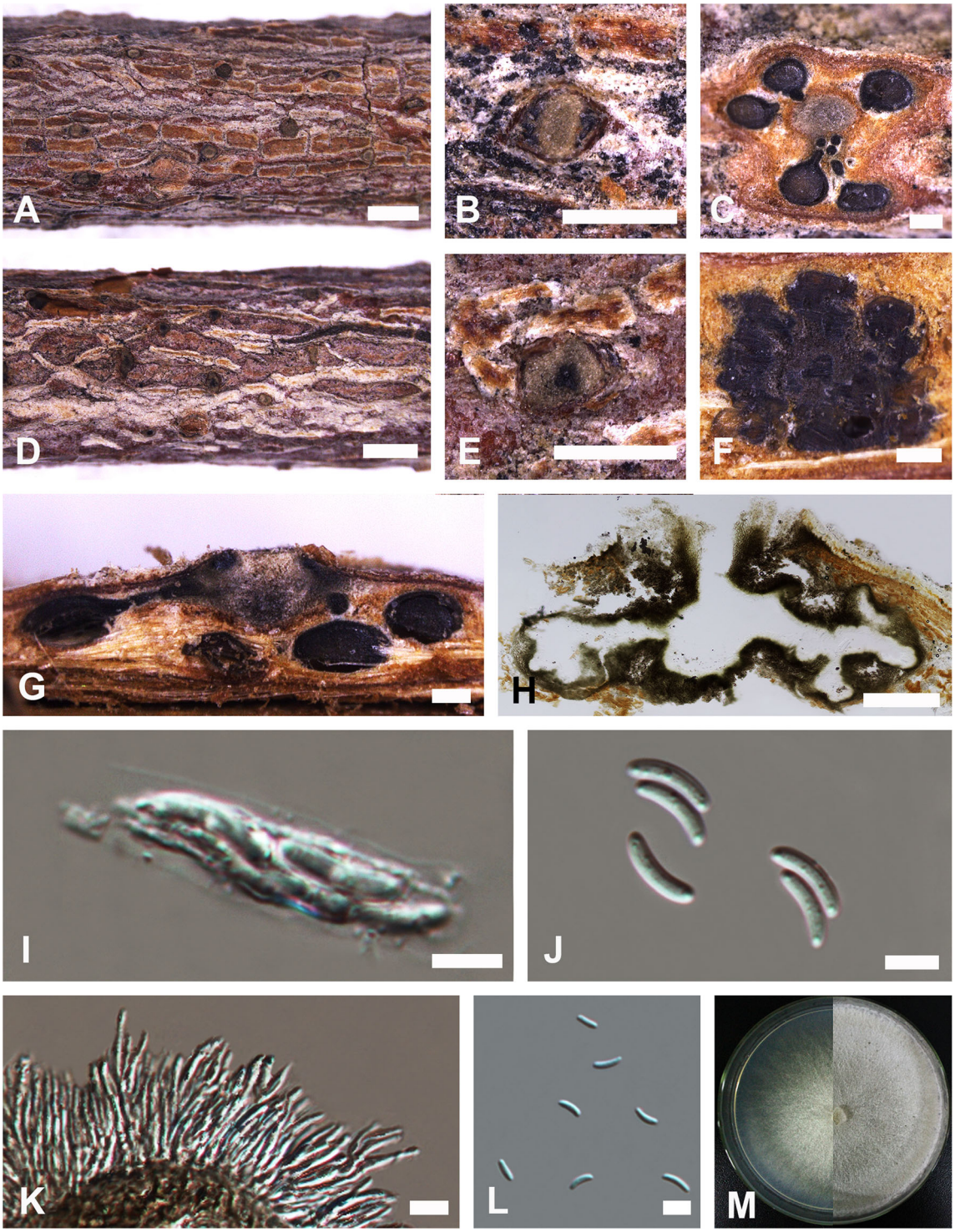


Fig. 4 Morphology of *Cytospora chrysosperma* from *Ulmus pumila* (BJFC-S788). **a**, **b** Habit of ascomata on twig. **c** Transverse sections through ascomata. **d**, **e** Habit of conidiomata on twig. **f** Transverse sections through conidiomata. **g** Longitudinal sections through ascomata. **h** Longitudinal sections through conidiomata. **i** Asci. **j** ascospores. **k** Conidiophores. **l** Conidia. **m** Colonies on PDA at 3 days (*left*) and 30 days (*right*). Scale bars: A, D=1 mm; B, E=500 μm; C, F – H=200 μm; I – L=5 μm

Materials examined: **CHINA**, **Tibet Province**: Shigatse City, 29°27′34.53″N, 89°90′23.08″E, elev. 3976 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 13 Feb. 2012, (BJFC-S788; living culture, CFCC89982).

Notes: *Cytospora chrysosperma* is the most commonly recorded *Cytospora* species, with a wide host range (Deng 1963; Tai 1979; Wei 1979; Chen 2002; Zhuang 2005; Adams et al. 2006; Fan et al. 2014b). Fan et al. (2014b) treated

BJFC-CGHs-10 (living culture CFCC89600) as a reference specimen, as it has not yet been epitypified. In the current study, isolate CFCC89982 was shown to be *C. chrysosperma* based on phylogenetic analyses and morphological characters. This is the first record of *C. chrysosperma* on *U. pumila* in China.

Cytospora carbonacea Fr., Syst. mycol. (Lundae) 2(2): 544 (1823). (Fig. 5)

Host/Distribution: Pathogen on twigs and branches of *U. pumila.* Known from *U. americana* and *U. campestris* in Germany, and *U. minor* in Iran (Fries 1823; Fotouhifar et al. 2010)

Ascostromata immersed in the bark, erumpent, not crowded, circular to ovoid, (840–)900–1180(−1230) μm (av.=980 μm, *n*=20) diam. *Disc* pale brown to black, circular to ovoid, (240–)260–330(−380) μm (av.=320 μm, *n*=20) diam, with 4–8 ostioles arranged circinately in a disc, brown to black, (76–)80–100.5(−106) μm (av.=95.5 μm, *n*=20) diam; 4–10 perithecia arranged circinately in black entostromata, flask-shaped to spherical, (300–)320–390(−410) μm (av.=350 μm, *n*=20) diam. *Asci* free, clavate to elongate obovoid, (49.5–)50–59.5(−60)×4–4.5(−5) μm (av.=54.5×4.5 μm, *n*=20), 8-spored. *Ascospores* elongate-allantoid, thin-walled, hyaline, aseptate, (14–)14.5–17.5(−18)×(4–)4.5–5 μm (av.=16×4.5 μm, *n*=50). *Conidiomata* immersed in bark, erumpent in a large area, discoid, with large multiple locules. *Disc* grey to black, nearly flat, circular to ovoid, (310–)350–380(−420) μm (av.=360 μm, *n*=20) diam, with multiple ostioles per disc, with columnar stroma crossed over the pycnidia. *Ostiole* medium grey, prominent, (46.5–)50–57(−62) (av.=54.5 μm, *n*=20) diam. *Locules* complex multi-loculed, subdivided frequently by invaginations with common walls, (940–)1100–1450(−1510) μm (av.=1270 μm, *n*=20) diam. *Conidiophores* hyaline, unbranched or occasionally branched at the base, (11–)12–20(−21.5) μm (av.=15 μm, *n*=20). *Conidia* hyaline, eguttulate, elongate-allantoid, aseptate, (8.5–)9–13(−13.5)×(1.5–)2–3 μm (av.=11×2 μm, *n*=50).

Cultures: Cultures initially white, slow-growing, becoming pale to dark brown after 7–10 days. The colony was flat, felt-like, with a uniform texture, compact. Conidiomata formed randomly on the agar surface.

Materials examined: **CHINA**, **Shaanxi Province**: Yulin City, 38°15′02.66″N, 109°44′31.79″E, elev. 1080 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 26 May 2012, (BJFC-S1071; living culture, CFCC50055); 38°15′01.73″N, 109°44′30.27″E, elev. 1071 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 26 May 2012, (BJFC-S1072; living culture, CFCC50056); **CHINA**, **Qinghai Province**: Haidong city, Pingan county, 36°28′50.48″N, 102°10′03.29″E, elev. 2208 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 15 August 2012, (BJFC-S630; living culture, CFCC89947); Haidong city, Huzhu county, Weiyuan, 36°50′54.64″N, 101°57′41.89″E, elev. 2601 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 15 August 2012, (BJFC-S631; living culture, CFCC50059); **CHINA**, **Heilongjiang Province**: Qiqihar City, Longsha Park, 47°35′06.68″N, 123°95′05.97″E, elev. 231 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 13 July 2011, (BJFC-S339; living culture, CFCC50058).

Note: *Cytospora carbonacea* has previously been recorded from *Ulmus americana*, *U. campestris* and *U. minor* in Germany and Iran (Fotouhifar et al. 2010). The first report of this species in China was from *Syzygium aromaticum* (Zhang et al. 2014a, b). The present study is the first report of *C. carbonacea* from *U. pumila* also providing detailed descriptions of both sexual and asexual morphs. In the present study, isolates CFCC50055, CFCC50056, CFCC50058, CFCC50059 and CFCC89947 were shown to be *C. carbonacea* based on phylogenetic analyses and morphological characters. Fresh collections of *C. carbonacea* are needed from *Ulmus* sp. in USA (Fries 1823) for epitypification purposes to fix the application of the name.

Cytospora ribis Ehrenb., Sylv. mycol. berol. (Berlin): 28 (1818). (Fig. 6)

Host/Distribution: Pathogen on twigs and branches of *U. pumila*. Known from *Elaeagnus angustifolia*, *Lepidium latifolium*, *Platanus orientalis*, *Thuja orientalis* in Iran, *Platanus orientalis*, *Ribes mandshuricum* in Poland, and *Ribes rubrum* in the Netherlands (Saccardo 1884; Mulenko et al. 2008; Fotouhifar et al. 2010).

Conidiomata immersed in bark, erumpent in a large area, discoid, with large multiple locules. *Disc* grey to black, nearly flat, circular to ovoid, (350–)380–410(−470) μm (av.=400 μm, *n*=20) diam, with one to four ostioles per disc. *Ostiole* medium grey, prominent, (120–)130–160(−180) μm (av.=150 μm, *n*=20) diam. *Locules* complex multi-loculed, subdivided frequently by invaginations with common walls, (1440–)1470–1800(−1890) μm (av.=1650 μm, *n*=20) diam. *Conidiophores* hyaline, unbranched or occasionally branched at the bases, (17–)17.5–18(−18.5) μm (av.=17.5 μm, *n*=20), occasionally conidiophores 38.5–39.5(−40) μm (av.=39 μm, *n*=20). *Conidia* hyaline, eguttulate, elongate-allantoid, aseptate, (3.0–)3.5–4.5(−5)×1–1.5 μm (av.=4× 1 μm, *n*=50).

Cultures: Colonies remaining white, growing rapidly, covering the dish after 7–10 days. Colonies flat, felt-like, with a regular edge, texture uniform; conidiomata sparse, irregularly distributed over agar surface.

Materials examined: **CHINA**, **Qinghai Province**: Xining City, 36°38′32.51″N, 101°44′42.89″E, elev. 2419 m.a.s.l., on twigs and branches of *U. pumila*, coll. X.L. Fan, 16 Aug. 2012, (BJFC-S671; living culture, CFCC50026); *ibid.* (living culture, CFCC50027).

Notes: *Cytospora ribis* has been reported from Iran, Poland and the Netherlands (Mulenko et al. 2008; Fotouhifar et al. 2010). However, these records lacked

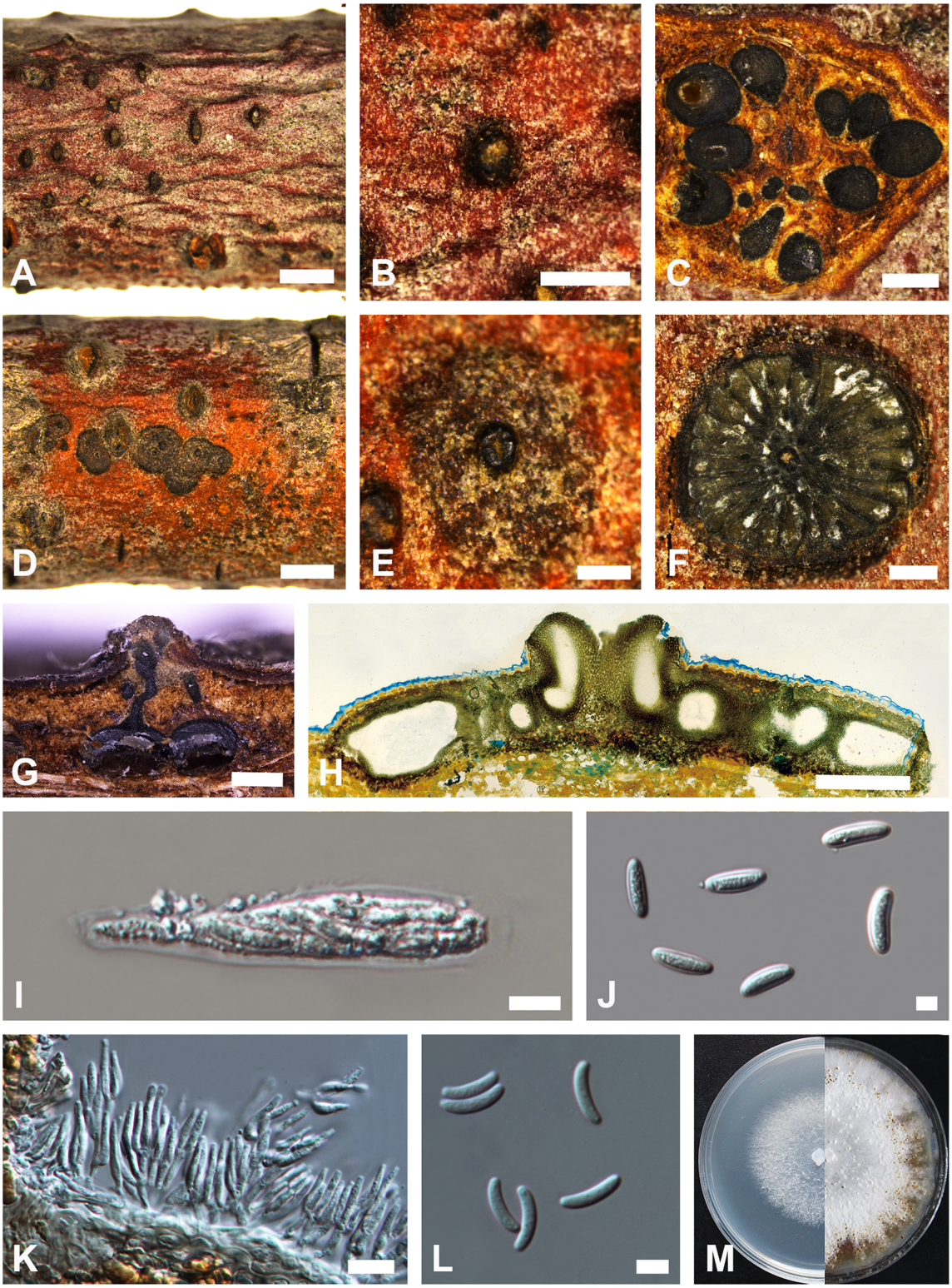


Fig. 5 Morphology of *Cytospora carbonacea* from *Ulmus pumila* (BJFC-S630). **a**, **b** Habit of ascomata on twig. **c** Transverse sections through ascomata. **d**, **e** Habit of conidiomata on twig. **f** Transverse sections through conidiomata. **g** Longitudinal sections through ascomata. **h** Longitudinal sections through conidiomata. **i** Asci. **j** Ascospores. **k** Conidiophores. **l** Conidia. **m** Colonies on PDA at 3 days (*left*) and 30 days (*right*). Scale bars: A, D=1 mm; B, E=500 μm; C, F–H=200 μm; I–L=5 μm

detailed descriptions and illustrations (Mulenko et al. 2008; Fotouhifar et al. 2010). Isolates CFCC50026, CFCC50027 were shown to be *C. ribis* based on phylogenetic analysis and morphological characters. This

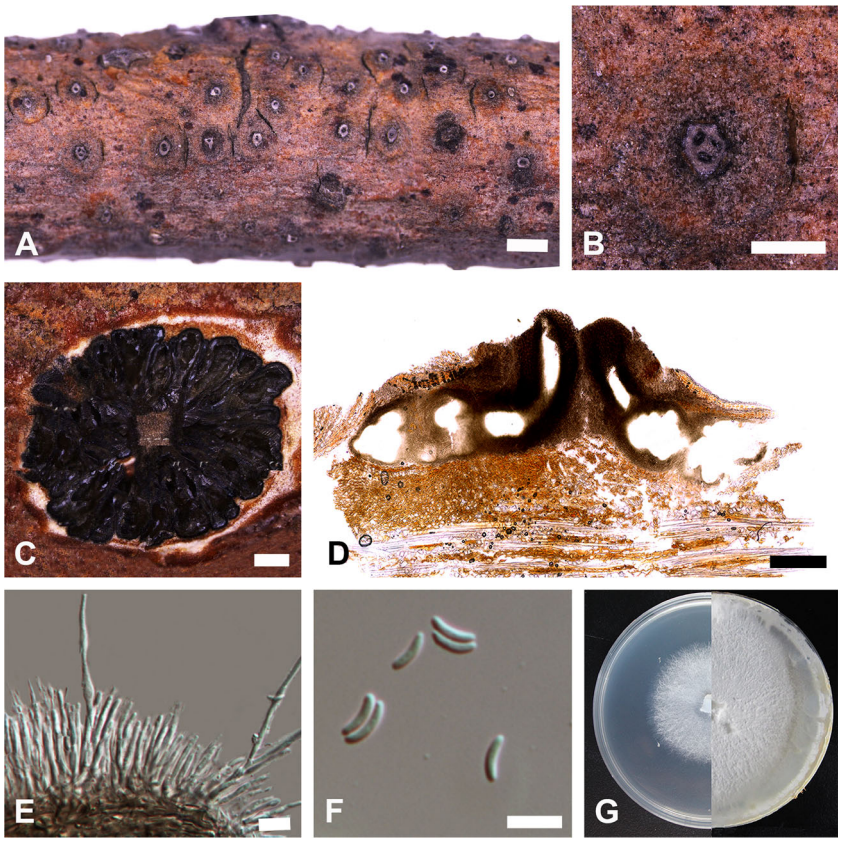


Fig. 6 Morphology of *Cytospora ribis* from *Ulmus pumila* (BJFC-S671). **a**, **b** Habit of conidiomata on a twig. **c** Transverse sections through conidiomata. **d** Longitudinal sections through conidiomata. **e** Conidiophores. **f** Conidia. **g** Colonies on PDA at 3 days (*left*) and 30 days (*right*). Scale bars: A=1 mm; B=500 μm; C, D=200 μm; E, F=5 μm

finding represents a new host record for China. *Cytospora ribis* has not been epitypified, and fresh collections are needed from *Ribes rubrum* L. in Germany for epitypification purposes.

Discussion

The current study identified four species (*C. carbonacea*, *C. chrysosperma*, *C. pruinopsis* sp. nov. and *C. ribis*) associated with canker disease on *Ulmus pumila* in northern China. *Cytospora carbonacea* and *C. chrysosperma* have been reported previously from *Ulmus* spp., but without detailed morphological observations (Conway and Morrison 1983; Fotouhifar et al. 2010; Zhang et al. 2014a, b). In addition, two other species were isolated from *Ulmus pumila*, namely *C. pruinopsis* sp. nov. and *C. ribis*. In the phylogenetic analysis, *C. ribis* formed a distinct clade with high support values (MP-BS/ML-BS/BPP=98/99/100) (Fig. 2). *Cytospora pruinopsis* sp. nov. has a single conidiomatal locule, with one ostiole per disc, and is thus easily distinguishable from the other three species. Furthermore, based on phylogenetic analyses, these four species also proved to be distinct (Fig. 2).

Although species of *Cytospora* have been commonly reported from *Ulmus* spp. in several countries, these records have largely been lacking in detailed morphological and molecular data. The species can be differentiated, however, based on the characteristics of their conidiomata and conidial dimensions. Six species (*C. ambiens*, *C. carbonacea*, *C. chrysosperma*, *C. leucostoma*, *C. pulchella* and *C. sacculus*) associated with Cytospora canker disease of *Ulmus* spp. can be distinguished. *Cytospora carbonacea* has multiple conidiomatal ostioles, and can thus easily be differentiated from the other species in this study. *C. chrysosperma* has multi-loculate conidiomata, subdivided by invaginations into irregular chambers sharing common walls, which distinguishes it from *C. sacculus*. *Cytospora pulchella* has larger conidia than *C. ambiens* and *C. sacculus* (6–8×1.5–2 vs. 4.5–4.9×1–1.2 μm and 3.6–5.2×0.9–1.2 μm). *Cytospora leucostoma* can be separated based on the presence of a conceptacle compared with other *Cytospora* spp. from *Ulmus*.

The new species *C. pruinopsis* is similar to *C. pruinosa* (Fr.) Sacc. in that it has a single locule with a central ostiole. It differs from *C. pruinosa*, however, in its wing-like ectostroma around the ostiole and smaller conidia (3×0.8 vs. 5–6×1.2 μm in *C. pruinosa*) (Adams et al. 2006). Based on phylogenetic analyses, *C. carbonacea* is most closely related to *C. elaeagni*, which has been recorded from *Elaeagnus angustifolia* in China, Germany and North America (Saccardo 1889; Chen 2002; Zhuang 2005). *Cytospora pruinopsis* can be distinguished from *C. elaeagni* based on

its ostiolar morphology (multiple ostioles vs. a single ostiole), larger mean conidial size (11×2 vs. 7.7×2.6 μm) and smaller mean conidiophore length (14.9 vs. 22.5 μm).

Cytospora ribis was newly reported from *Ulmus pumila* in this study, which also represents a new host association for this species. *C. carbonacea* has previously been reported from *U. americana* L. and *U. campestris* L. in Germany and *U. minor* Mill. in Iran (Fotouhifar et al. 2010). In the present study *C. carbonacea* was isolated from *U. pumila*, suggesting that *Ulmus* spp. may be its primary host. *C. chrysosperma* is the type species of *Cytospora*, and occurs globally on a wide range of hosts (Adams et al. 2005; Fan et al. 2014a). Previous records show five plant genera listed as hosts of this species in China—chiefly, *Salix* and *Populus*, but also *Castanea*, *Morus*, and less frequently, *Ulmus* (Deng 1963; Tai 1979; Wei 1979; Chen 2002; Zhuang 2005). The present study clarified the presence of four *Cytospora* species isolated from *U. pumila* in northern China, of which one species proved to be new to science. To fully elucidate the *Cytospora* spp. that occur on woody hosts, a more exhaustive sampling of other hosts from other regions of the world will be needed to help clarify the host range and distribution of these important canker pathogens.

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