



## ***Cytospora* species associated with canker disease of three anti-desertification plants in northwestern China**

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### **Abstract**

*Cytospora* species are important phytopathogens causing severe canker disease with a worldwide distribution and broad host range. However, identification of taxa to species level is difficult due to poor phylogenetic understanding and lack of sequenced type species. Morphological and phylogenetic studies have been carried out on several important hosts such as *Eucalyptus* and *Malus* in China, Iran, and South Africa. In this study destructive canker diseases of the anti-desertification plants, *Elaeagnus angustifolia*, *Hippophae rhamnoides*, and *Salix psammophila*, were investigated in northwest China. Multilocus phylogenetic analyses of ITS, nrLSU, RPB2, and ACT gene regions, combined with detailed morphological analyses and comparison with ex-type strains revealed six *Cytospora* species, *C. chrysoesperma*, *C. elaeagni*, *C. hippophaes*, *C. nivea*, *C. populina* comb. nov. and *C. gigaspora* sp. nov. causing cankers on these hosts. The novel species *C. gigaspora* has flat multiple locules with a conceptacle and unusually long 12 µm conidia. Detailed descriptions and molecular data for the *Cytospora* species causing cankers on the three psammophilic host plants are provided. *Cytospora elaeagni* and *C. hippophaes* have previously been recorded from *Elaeagnus angustifolia* and *Hippophae rhamnoides*, whereas the other species causing *Cytospora* canker of *Elaeagnus angustifolia* and *Salix psammophila* are new records.

**Key words:** Ascomycota, Diaporthales, Morphology, New species, Phylogeny

### **Introduction**

The genus *Cytospora* (Ascomycota: Diaporthales) was established by Ehrenberg (1818). It includes important phytopathogens that cause dieback and canker disease on a wide range of plants, causing severe commercial and ecological damage and significant losses worldwide (Adams *et al.* 2005, 2006). *Cytospora* has been categorized under several coelomycetous genera in the dual-classification system, including asexual states of *Leucostoma*, *Valsa*, *Valsella*, and *Valseutypella* (Fries 1823; Saccardo 1884; Deng 1963; Tai 1979; Wei 1979; Spielman 1985; Wang *et al.* 2011; Adams *et al.* 2002). All the genera were recently combined under *Valsa*, either as subgenera or species with no additional infrageneric rank (Adams *et al.* 2005). The current International Code of Nomenclature for Algae, Fungi, and Plants (ICN) requires a single-name for pleomorphic taxa, and the dual-nomenclature system has become redundant (Hawksworth 2011). A single name for complex genera such as *Diaporthe/Phomopsis*, *Glomerella/Colletotrichum*, *Pestalotia/Pestalotiopsis*, and *Phyllosticta/Guignardia* have followed the oldest or the most conserved name (Hyde *et al.* 2009; Wikee *et al.* 2011; Huang *et al.* 2013; Wei *et al.* 2013; Udayanga *et al.* 2014). *Cytospora* (1818) is an older name than *Valsa* (1849) and the asexual state more common in nature; therefore, we chose to adopt *Cytospora* and treat *Valsa* species as synonyms for *Cytospora*. More than 560 species epithets named *Cytospora* have been recorded in Index Fungorum (2014) with an estimated 110 species in Kirk *et al.* (2008). Ex-type sequence data, is however, available for only a very few species and many taxa need epitypifying, thus identification to species level is difficult. Therefore, research towards a backbone tree for *Cytospora* species is needed so that backbone trees to species can be updated as in previous studies in other genera (Hyde *et al.* 2014).

The northwest region of China suffers from serious desertification (Zhenda 1998; Yang *et al.* 2005). A number of plants used for a tree-planting campaign to combat desertification were infected with canker disease which caused great ecological losses (Deng 1963; Tai 1979; Wei 1979; Chen 2002). In some areas, canker disease of *Artemisia desertorum* Spreng. and *Haloxylon ammodendron* Bge. was absent. However, *Cytospora* cankers in anti-desertification plants such as, *Elaeagnus angustifolia* L., *Hippophae rhamnoides* L. and *Salix psammophila* C. Wang & Chang Y. Yang were widespread in plantations and natural forests. However, the phytopathogenic taxa causing the canker disease have not been identified. Therefore, a study of taxa causing cankers of anti-desertification plants is needed in order to ascertain whether the fungi are host-specific or generalists.

*Cytospora* canker disease in China has been attributed to 31 species, although the descriptions did not essentially differ between species; they also lacked illustrations and molecular data (Deng 1963; Tai 1979; Wei 1979). The *Cytospora* species associated with apple and pagoda canker disease have, however, been clarified (Wang *et al.* 2007, 2011; Fan *et al.* 2014).

The objectives of the current study in northern China was (i) to clarify species associated with canker disease of *Elaeagnus angustifolia*, *Hippophae rhamnoides* and *Salix psammophila*; (ii) to define the pathogenic species with detailed illustrations and descriptions; (iii) to provide a multilocus phylogenetic analysis based on combined ITS, nrLSU, RPB2, ACT sequences, and an ITS compared with other reference sequences; and (iv) to define the specificity or relationship between fungal species and host plants.

## Materials and methods

### *Sampling and fungal isolation*

Fresh specimens of *Cytospora* spp. (asexual state) on infected stems were collected from *Elaeagnus angustifolia*, *Hippophae rhamnoides*, and *Salix psammophila* exhibiting dieback during collecting trips in Gansu, Ningxia, Qinghai, and Shaanxi Provinces in China. Isolations were made directly from conidiomata or ascomata (if sexual state was present) on the host whenever possible. Single spore isolation follows a slightly modified protocol as described in Fan *et al.* (2014). Part of hymenium containing three to four sporocarps 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 % of potato dextrose agar (PDA) in a petri-dish, incubated at 25 °C for up to 24 h, and a single germinating conidium or ascospore was transferred to a fresh PDA plate. The remaining spore suspension was used for conidia (ascospores) measurements. This method established clear linkage between the two states, and the corresponding pure cultures were used for phylogenetic study.

Sixteen representative strains were used in the phylogenetic analysis (Table 1). Specimens are deposited in the Museum of Beijing Forestry University (BJFC). Single-spore cultures are maintained in the China Forestry Culture Collection Center (CFCC), with duplicate isolates of the new species in the China Center for Type Culture Collection (CCTCC).

### *Morphology*

Specimens were observed on infected plant tissues, and the structure and size of fruiting bodies, presence or absence of a conceptacle, and size and shape of spores were recorded. Dilutions were performed with the spore masses obtained as above, and drops of suspensions were placed on microscope slides. More than 20 fruiting bodies were sectioned, and 50 spores were selected randomly for measurement using a Leica light microscope (LM, DM 750). Cultural characteristics of isolates incubated on PDA in the dark at 25 °C were recorded. This included colony characters and pigment production, at 3, 7, and 30-days.

### *DNA extraction, PCR amplification, and sequencing*

Genomic DNA was extracted from colonies grown for one week on PDA with cellophane using a modified CTAB method (Doyle and Doyle 1990). DNA were estimated by electrophoresis in 1 % agarose gels, and the quality was measured by NanoDrop™ 2000 (Thermo, USA) following the user manual (Desjardins *et al.* 2009). PCR amplifications were performed in DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA). The ITS region was amplified using primers ITS1 and ITS4 (White *et al.* 1990). The partial large nuclear ribosomal RNA subunit (nrLSU) region was amplified using primers NL1 and NL4 (O'Donnell 1993). The partial RNA polymerase II subunit

(RPB2) region was amplified using primers bRPB-6F and bRPB-7.1R (Matheny 2005). The partial actin (ACT) region was amplified using primers ACT512F and ACT783R (Carbone and Kohn 1999). The PCR amplification products were estimated visually by electrophoresis in 2 % agarose gels. 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).

**TABLE 1.** Species and strain information used for the molecular phylogenetic analyses (ex-type strain is bolded).

Species	Strain	Host	Location <sup>a</sup>	GenBank accession numbers			
				ITS	nrLSU	RPB2	ACT
<i>Cytospora gigaspora</i>	<b>CFCC 89634</b>	<i>Salix psammophila</i>	Yulin, Shaanxi	KF765671	KF765687	KF765703	KF765719
	CFCC 89635		Yulin, Shaanxi	KF765672	KF765688	KF765704	KF765720
<i>Cytospora chrysosperma</i>	CFCC 89629	<i>Salix psammophila</i>	Yulin, Shaanxi	KF765673	KF765689	KF765705	KF765721
	CFCC 89630		Yulin, Shaanxi	KF765674	KF765690	KF765706	KF765722
<i>Cytospora elaeagni</i>	CFCC 89631	<i>Elaeagnus angustifolia</i>	Haidong, Qinghai	KF765675	KF765691	KF765707	KF765723
	CFCC 89632*		Guyuan, Ningxia	KF765676	KF765692	KF765708	KF765724
	CFCC 89633		Guyuan, Ningxia	KF765677	KF765693	KF765709	KF765725
<i>Cytospora hippophaes</i>	CFCC 89636	<i>Hippophae rhamnoides</i>	Guyuan, Ningxia	KF765678	KF765694	KF765710	KF765726
	CFCC 89637		Guyuan, Ningxia	KF765679	KF765695	KF765711	KF765727
	CFCC 89638		Yulin, Shaanxi	KF765680	KF765696	KF765712	KF765728
	CFCC 89639*		Gannan, Gansu	KF765681	KF765697	KF765713	KF765729
	CFCC 89640		Gannan, Gansu	KF765682	KF765698	KF765714	KF765730
	CFCC 89641	<i>Elaeagnus angustifolia</i>	Guyuan, Ningxia	KF765683	KF765699	KF765715	KF765731
<i>Cytospora nivea</i>	CFCC 89642*	<i>Salix psammophila</i>	Yulin, Shaanxi	KF765684	KF765700	KF765716	KF765732
	CFCC 89643		Yulin, Shaanxi	KF765685	KF765701	KF765717	KF765733
<i>Cytospora populina</i>	CFCC 89644*	<i>Salix psammophila</i>	Yulin, Shaanxi	KF765686	KF765702	KF765718	KF765734

<sup>a</sup>: Cities and provinces of China of strains origin.

\*: Strains designated as epitype in this study.

### 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 (DNASTAR Inc., Madison, WI, USA). Sequences were aligned using MAFFT v.6 (Kato 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 *et al.* 2003), MrBayes v.3.1.2 for Bayesian analysis (Ronquist and Huelsenbeck 2003), and RAxML v.7.2.8 for maximum likelihood (ML) analysis (Stamatakis 2006). The first analysis was performed on the multilocus alignment (ITS, nrLSU, RPB2, ACT) and for each gene separately. A second analysis using ITS sequence data was performed to compare *Cytospora* species from the current study with other strains in GenBank. *Phomopsis vaccinii* Shear was selected as outgroup in this analysis (Adams *et al.* 2005). Trees are shown using Figtree v.1.3.1 (Rambaut and Drummond 2010).

MP analysis was run using a heuristic search option of 1,000 random-addition sequences with a tree bisection and reconnection (TBR) branch swapping algorithm. The branches of zero length were collapsed and all equally parsimonious trees were saved. Clade stability was assessed with a bootstrap analysis of 1,000 replicates (Hillis and Bull 1993). Other calculated parsimony scores calculated were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC).

ML analysis was also performed using RAxML v.7.2.8 with a GTR model of site substitution, including estimation of gamma-distributed rate heterogeneity and a proportion of invariant sites (Stamatakis 2006). The branch support was evaluated with a bootstrapping method of 1,000 replicates (Hillis and Bull 1993).

Bayesian analysis was performed using a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities (Rannala and Yang 1996). A nucleotide substitution model was estimated by MrModeltest v.2.3 (Posada and Crandall 1998), and a weighted Bayesian analysis was considered. Two MCMC chains were run from random trees for 1,000,000 generations, and trees were sampled every 100<sup>th</sup> generation, resulting in 10,000 total trees. The first

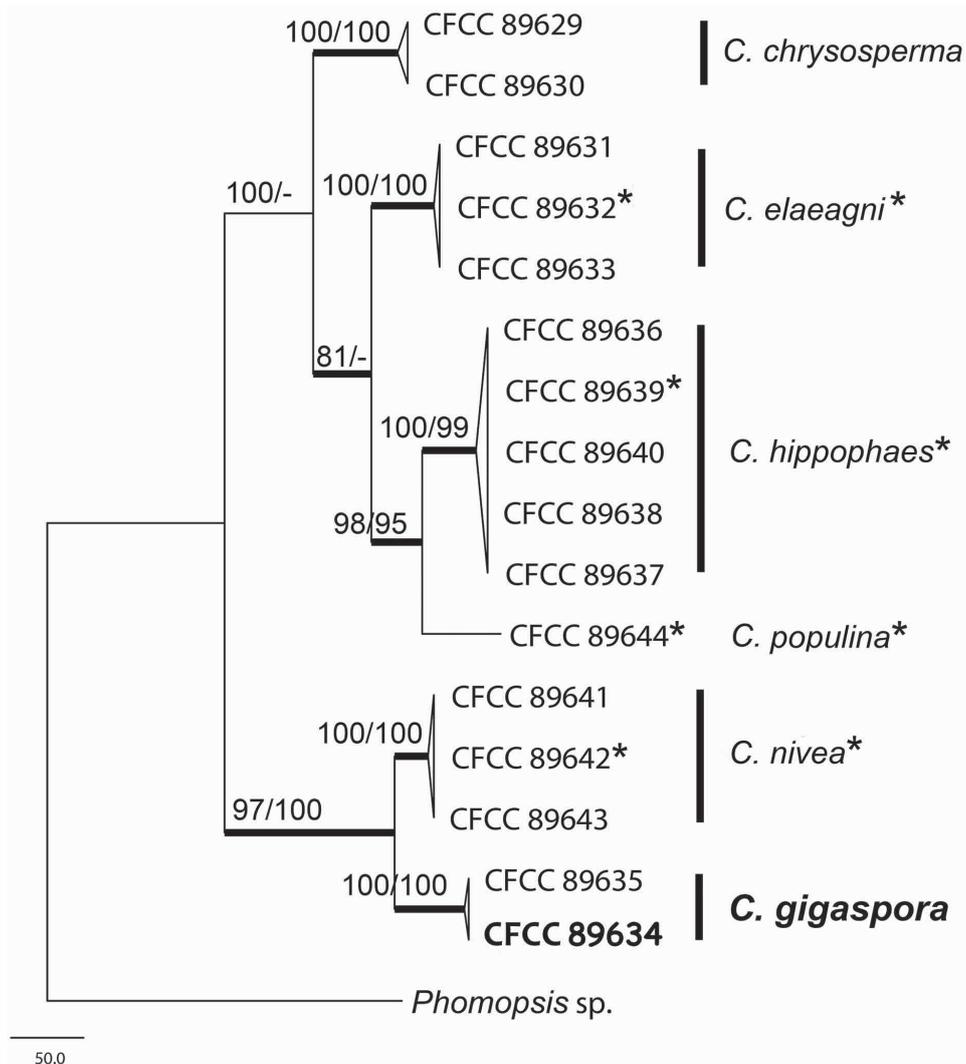
25 % of trees were discarded as the burn-in phase of each analysis and the posterior probabilities (PP) were calculated using the remaining 7,500 trees.

Sequences data is deposited in GenBank (Table 1). The multilocus sequences alignment file and ITS sequence-alignment file are deposited in TreeBASE ([www.treebase.org](http://www.treebase.org)) as accession S16154. The taxonomic novelty is deposited in MycoBank (Crous *et al.* 2004) and Face of Fungi ([www.facesoffungi.org](http://www.facesoffungi.org)).

## Results

### Molecular data analysis

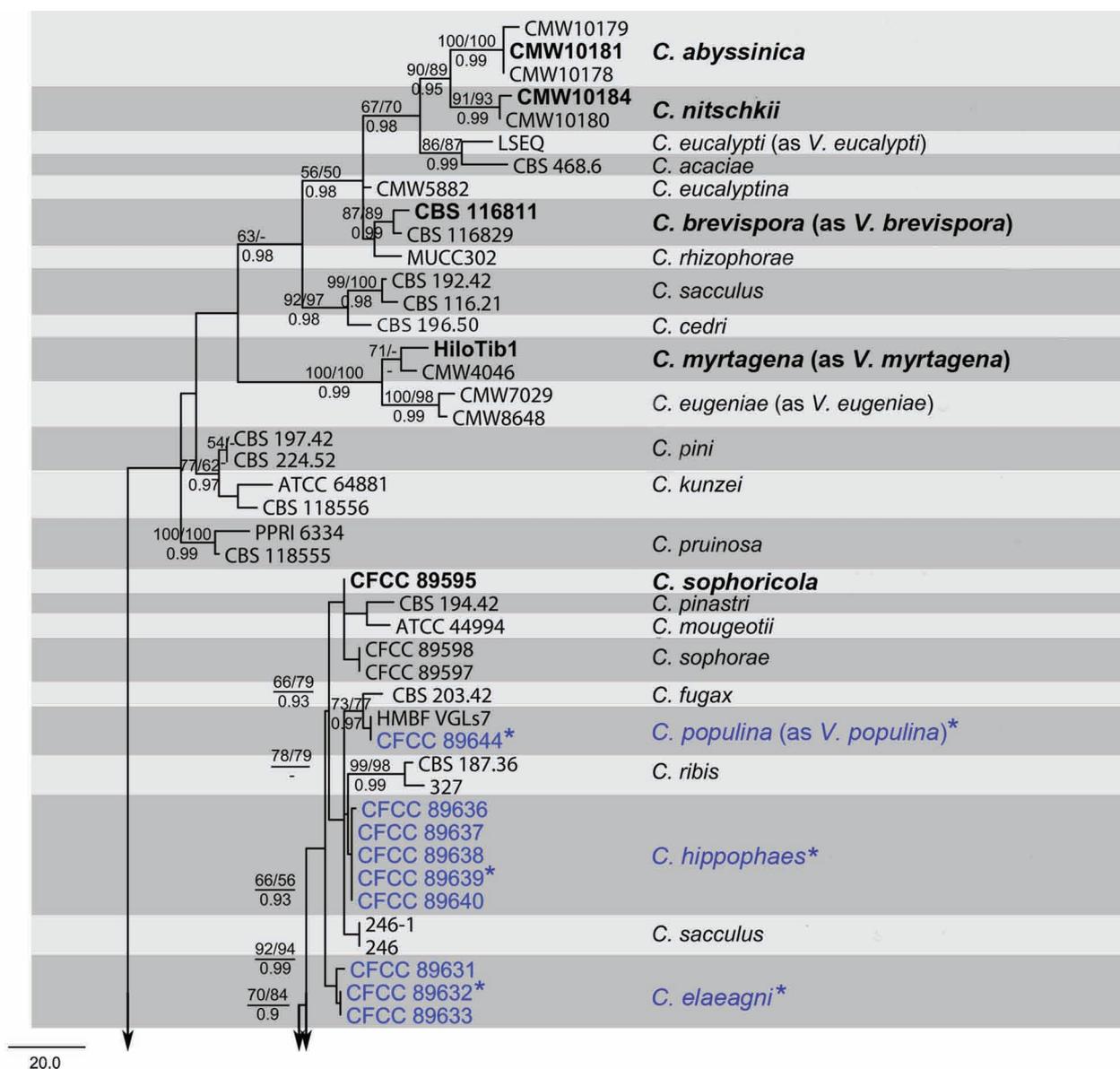
A total of 52 strains associated with *Cytospora* canker disease were isolated from *Salix psammophila*, *Elaeagnus angustifolia*, and *Hippophae rhamnoides* in four provinces of China, of which sixteen strains representing six species were used in the phylogeny. The sequence datasets for the ITS, nrLSU, RPB2, and ACT were analyzed individually and in combination. Tree topologies computed from the ML, MP, and Bayesian analysis was similar for the individual gene regions and in the combined analysis. The multi-locus analysis includes 2,257 characters, of which 1,730 characters are constant, 194 variable characters are parsimony-uninformative, and 333 are parsimony informative. Heuristic search generated one parsimonious tree (TL = 809, CI = 0.807, RI = 0.882, RC = 0.712) and is selected and shown in Fig. 1. All trees of ML method and Bayesian analysis are in agreement and not significantly different from with MP tree. MP bootstrap support (MP-BS) and ML bootstrap support (ML-BS) equal to or above 50 %. Branches with significant Bayesian posterior probability (BPP) equal to or above 0.90 were thickened in the phylogram.

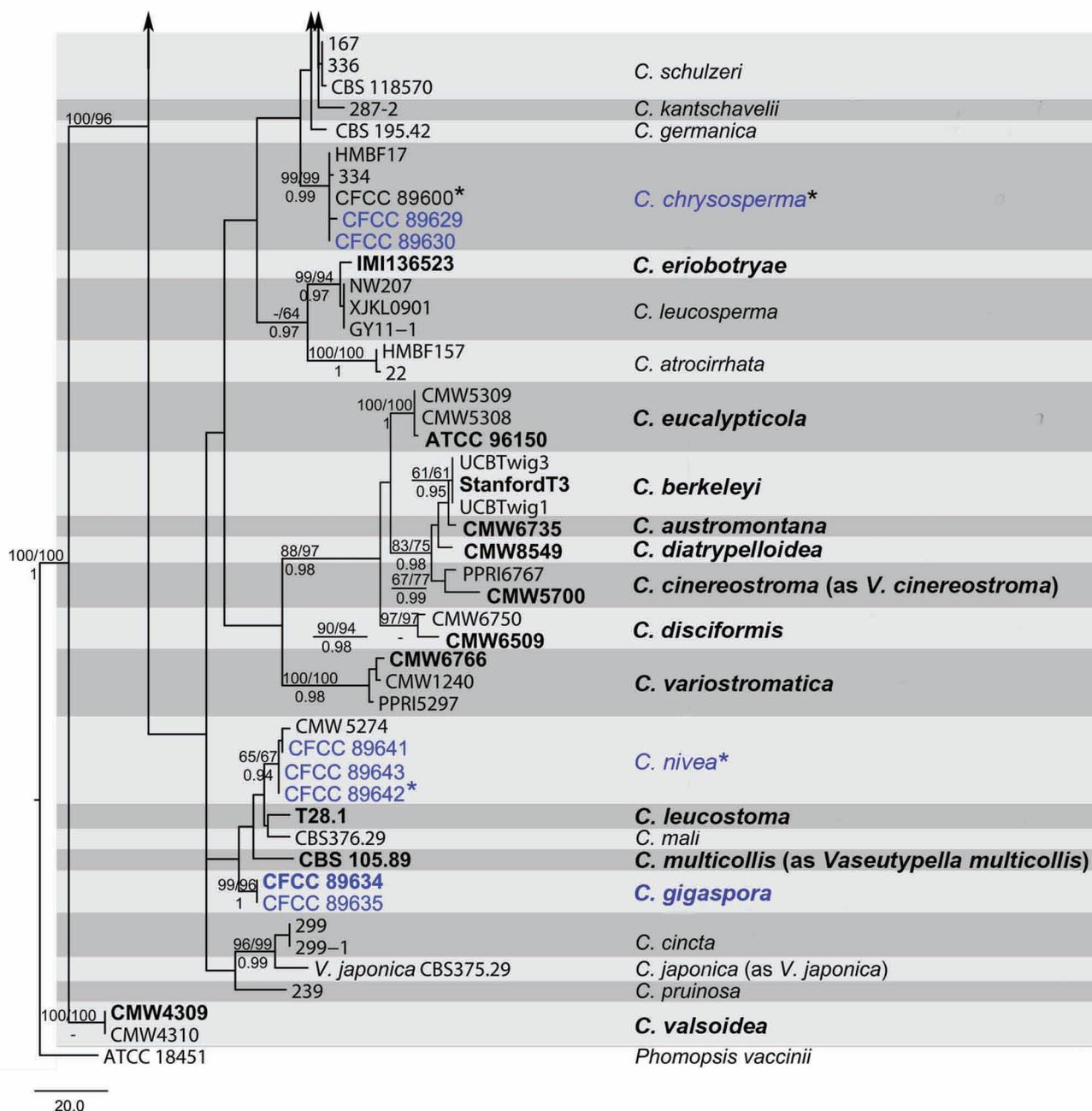


**FIGURE 1.** Phylogram of combined genes of ITS, nrLSU, RPB2, and ACT genes based on MP, ML and Bayesian analysis. Values above the branches indicate maximum parsimony bootstrap (MP BP  $\geq$  50 %) and maximum likelihood bootstrap (ML BP  $\geq$  50 %). Thickened branches represent posterior probabilities (BI PP  $\geq$  0.90) from Bayesian inference. Scale bar = 50 nucleotide substitutions. Ex-type strains are in bold. The strains designated as epitype are shown in asterisk.

A second analysis of ITS sequence data included representative *Cytospora* sequences, available ex-type strains and comprises 91 taxa. A total of 585 base pairs are used for analyses after alignment. Of these, 346 characters are constant, 64 variable characters are parsimony-uninformative and 175 characters are parsimony informative. A heuristic search generates 67 parsimonious trees, and the best tree (TL = 807, CI = 0.462, RI = 0.814, RC = 0.376) is shown in Fig. 2. The phylogenetic tree obtained from ML and Bayesian analyses with the MCMC algorithm was consistent with the previous MP tree.

There are 47 clades in Fig. 2 which are equivalent to 47 *Cytospora* species. The strains of *Cytospora* in this study clustered in six clades (Figs 1, 2) and represents *Cytospora chrysosperma*, *C. elaeagni*, *C. hippophaes*, *C. nivea*, *C. populina*, and a new clade named as *C. gigaspora* sp. nov. Four species were isolated from *Salix psammophila*, including *C. gigaspora* sp. nov., *C. chrysosperma*, *C. nivea*, and *C. populina*. *Cytospora elaeagni* was recorded from *Elaeagnus angustifolia* and *C. hippophaes* from *Hippophae rhamnoides*, indicating possible host-specificity. Only *C. nivea* occurred on both *Elaeagnus angustifolia* and *Salix psammophila*, indicating it may be a host generalist. As there is a lack of ex-type data for *Cytospora* species detailed descriptions and illustrations are provided for each species.





**FIGURE 2.** Phylogram of ITS regions based on MP, ML and Bayesian analysis. Values above the branches indicate maximum parsimony bootstrap (MP BP  $\geq 50\%$ ) and maximum likelihood bootstrap (ML BP  $\geq 50\%$ ). Values below branches represent posterior probabilities (BI PP  $\geq 0.90$ ) 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. The strains designated as epitype are shown in asterisk.

## Taxonomy

*Cytospora gigaspora* C.M. Tian, X.L. Fan & K.D. Hyde, *sp. nov.* (Fig. 3)

Mycobank MB 806044, Facesoffungi number: FOF00303

Differs from *Cytospora nivea* in flat locules and larger conidia size (8.1–8.9–12.1(–13.5)  $\times$  (1.7–)1.9–2.9(–3.3)  $\mu\text{m}$ , average in 10.4  $\times$  2.2  $\mu\text{m}$ .

Holotype:—BJFC-S975.

Host and Distribution:—Known from *Salix psammophila* in China.

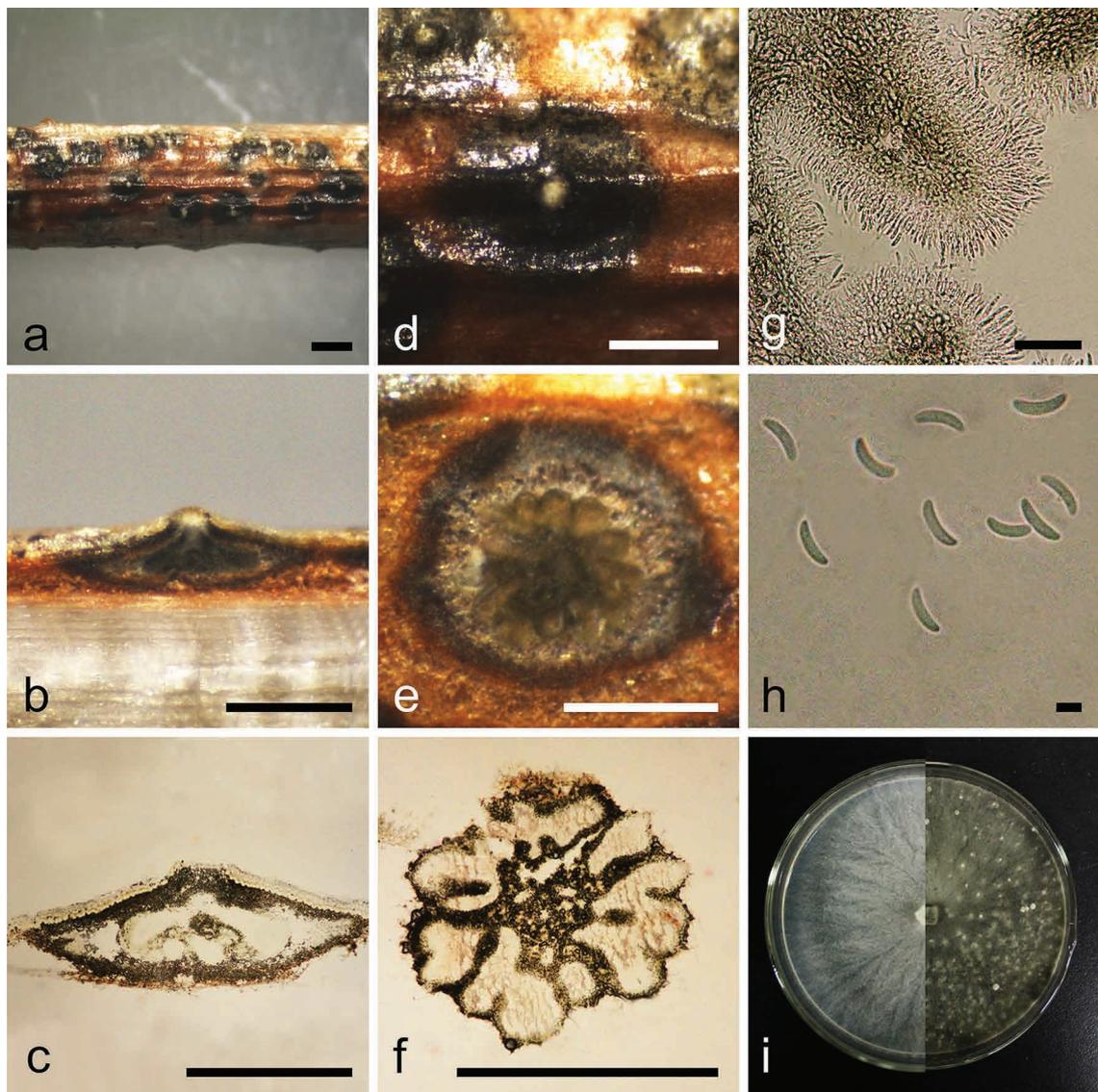
Etymology:—*gigaspora* (Lat.), referring to the unusual large size of conidia.

Pathogen on twigs and branches of *Salix psammophila*. Sexual state:—Unknown.

Asexual state:—Stromata immersed in bark. Conidiomata, slightly erumpent through the surface of bark, discoid, nearly flat, with multiple small locules. Conceptacle dark, surrounding the stromata. Disc white to light brown, circular to ovoid, (90–)110–150(–180)  $\mu\text{m}$  (average = 130  $\mu\text{m}$ , n = 20) in diameter, with one ostiole per disc. Ostiole in the center of the disc, inconspicuous, at the same level as the disc surface, area below disc a lighter entostroma, (12.6–)13.2–17.1(–19.3)  $\mu\text{m}$  (average = 15.6  $\mu\text{m}$ , n = 20) in diameter. Multi-locules arranged irregularly with common walls, (540–)670–810(–890)  $\mu\text{m}$  (average = 730  $\mu\text{m}$ , n = 20) in diameter. Conidiophores hyaline, unbranched or occasionally branched at the bases, (9.4–)12.5–19.7(–20.8)  $\mu\text{m}$  (average = 15.5  $\mu\text{m}$ , n = 20). Conidia hyaline, elongate-allantoid, eguttulate, aseptate, (8.1–)8.9–12.1(–13.5)  $\times$  (1.7–)1.9–2.9(–3.3)  $\mu\text{m}$  (average = 10.4  $\times$  2.2  $\mu\text{m}$ , n = 50).

Cultures:—colony originally white, and producing dark green to black pigment after 7–10 days, flat and thin, with a uniform texture, conidiomata irregular on medium surface.

Material examined:—**CHINA Shaanxi Province**: Yulin, Jichang East Road, 38°19'21.16" N, 109°39'54.73" E, 1124 m asl., on twigs and branches of *Salix psammophila*, collected by X.L. Fan, 1 Aug 2013 (BJFC-S975, *holotype*); living culture, CFCC 89634, CCTCC AF2013031; **Shaanxi Province**: Yulin, Hongshi Gorge, 38°19'32.43" N, 109°42'00.69" E, 1105 m asl., on twigs and branches of *Salix psammophila*, collected by X.L. Fan, 29 Jul 2013 (BJFC-S979, *paratype*); living culture, CFCC 89635, CCTCC AF2013032.



**FIGURE 3.** Morphology of *Cytospora gigaspora* from *Salix psammophila* (BJFC-S975, *holotype*). a, d: Habit of conidiomata on a twig. b, c: Longitudinal sections through conidiomata. e, f: Transverse sections through conidiomata. g: Conidiophores. h: Conidia. i: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: a = 1 mm; b–f = 0.5 mm; g = 20  $\mu\text{m}$ ; h = 5  $\mu\text{m}$ .

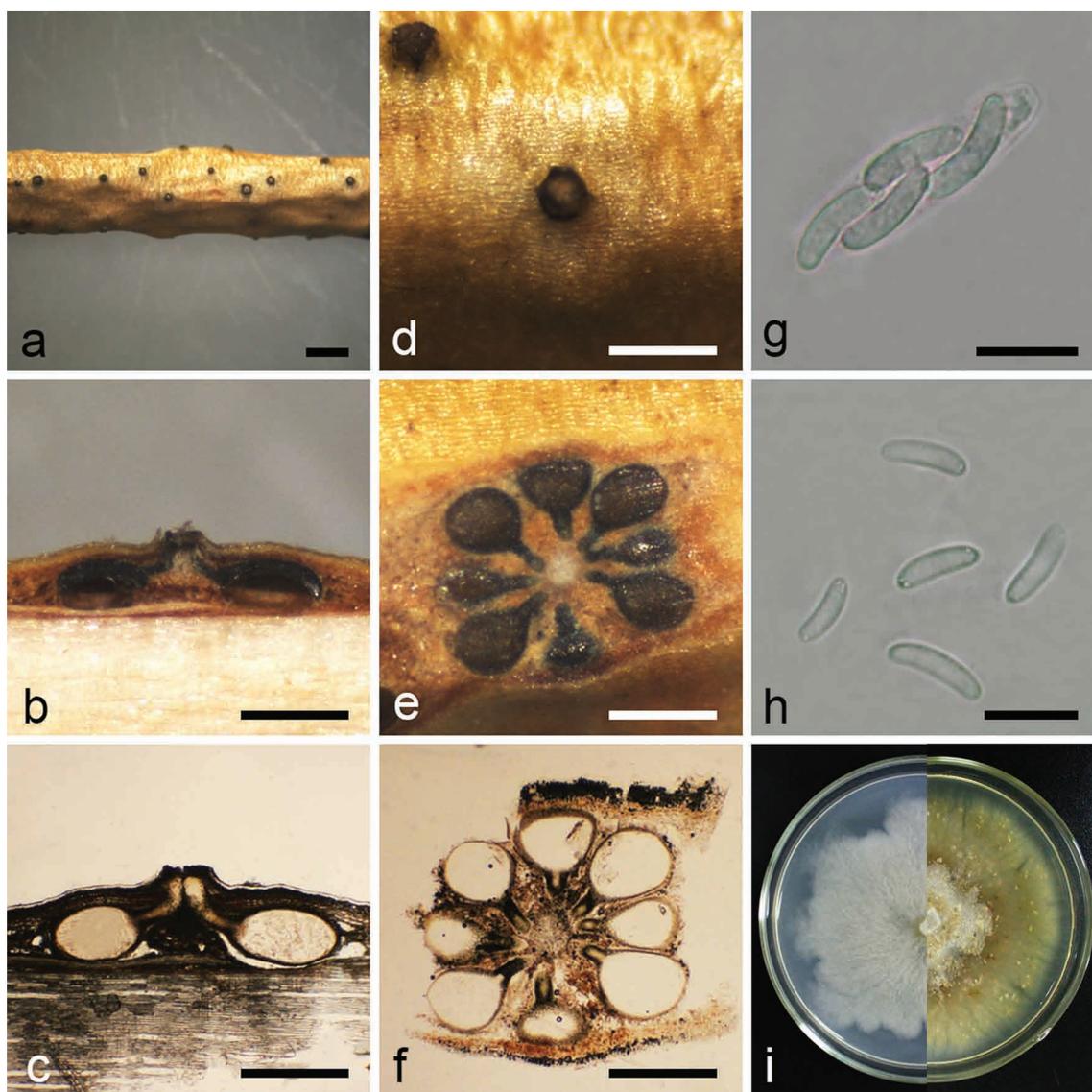
*Cytospora populina* (Fuckel) C.M. Tian, X.L. Fan & K.D. Hyde, *comb. nov.* (Fig. 4)

Mycobank MB 809224, Facesoffungi number: FOF00308.

Basionym:—*Valsa populina* Fuckel, Jb. nassau. Ver. Naturk. 25–26:314 (1871)

Host and Distribution:—Known from *Alnus inokumai* and *Pseudotsuga taxifolia* in Japan; and Salicaceae in China, Europe and Japan.

Pathogen on twigs and branches of *Salix psammophila*. Sexual state:—Stromata immersed in bark. Ascstromata, erumpent through the surface of bark, lenticular, extending to a large circular area, (900–)950–1130(–1210)  $\mu\text{m}$  (average = 1010  $\mu\text{m}$ , n = 20) in diameter. Conceptacle absent. Disc light grey to black, nearly flat, circular to ovoid, (100–)110–160(–180)  $\mu\text{m}$  (average = 140  $\mu\text{m}$ , n = 20) in diameter. Ostioles numerous, dark brown to black, at the same level as the disc, occasionally area below disc a lighter entostroma, (33.1–)33.8–47.6(–48.3)  $\mu\text{m}$  (average = 42.4  $\mu\text{m}$ , n = 20) in diameter. Locules dark brown, arranged circularly, flask-shaped to spherical, (220–)240–330(–370)  $\mu\text{m}$  (average = 280  $\mu\text{m}$ , n = 20) in diameter. Asci free, clavate to elongate obovoid, (51.5–)54.7–64.4(–66.9)  $\times$  (7.5–)8.1–13(–14.2)  $\mu\text{m}$  (average = 61.5  $\times$  11.1  $\mu\text{m}$ , n = 20), 4-spored. Ascospores biserial, elongate-allantoid, thin-walled, hyaline, lacking guttules, aseptate, (17.5–)18.9–24.3(–25.1)  $\times$  (4–)4.4–5.8(–6.4)  $\mu\text{m}$  (average = 21  $\times$  5.2  $\mu\text{m}$ , n = 50).



**FIGURE 4.** Morphology of *Cytospora populina* from *Salix psammophila* (BJFC-S978). a, d: Habit of ascromata on a twig. b, c: Longitudinal sections through ascromata. e, f: Transverse sections through ascromata. g: Ascus. h: ascospores. i: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: a=1 mm; b–f=0.5 mm; g–h=20  $\mu\text{m}$ .

Cultures/Asexual state:—Colony white, flat, felty, texture uniform, conidiomata sparse, irregularly distributed.

Material examined:—**CHINA Shaanxi Province**: Yulin, Hongshi Gorge, 38°19'40.83" N, 109°42'37.98" E, 1092 m asl., on twigs and branches of *Salix psammophila*, collected by X.L. Fan, 29 July 2013 (BJFC-S978); living culture, CFCC 89644.

Notes: *Cytospora populina* (= *Valsa populina*) is a species responsible for poplar canker. It differs from other *Cytospora* species as asci have four ascospores; the asexual state is unknown. The definition of this species has always been confused. Kobayashi (1969) regarded it as the synonym of *V. salicina* Fr. and described it with "4 ascospores". However, the original description from Saccardo (1882) distinguished *Valsa salicina* and *Cytospora populina* with eight ascospores and four ascospores, respectively. Species Fungorum (<http://www.speciesfungorum.org/>) list *Naemaspora populina* Pers., as the possible asexual state of *Valsa populina* cited by Fuckel (1871), as a synonym of *Cytospora ambiens*, although the latter species also had eight ascospores. Sogonov *et al.* (2008) regarded *Valsa populina* as a synonym of *Plagiostoma salicellum* Sogonov, and described it as "ascospores one-septate, ellipsoid to fusiform". This is inconsistent with the original Latin description of *V. populina*. In the current study, isolate CFCC 89644 was shown to be *Valsa populina* based on previous described morphological characters, host, and distribution (Saccardo 1882, Tai 1979). The species was first recorded on *Salix psammophila* worldwide and treated as a novel combination.

*Cytospora populina* has not been epitypified and therefore we treat BJFC-S978 as an authentic specimen. This has been proved as consistent results from several other authors without epitypification (Saccardo 1882; Kobayashi 1969; Tai 1979). It makes sense to epitypify this here for further study. Thus fresh collections of *Cytospora populina* are needed for epitypification.

### *Cytospora elaeagni* Allesch., Hedwigia 36 (Beibl.): 162 (1897) (Fig. 5)

Host and Distribution:—Known from *Elaeagnus angustifolia* in, China, German and USA.

Pathogen on twigs and branches of *Elaeagnus angustifolia*. Sexual state:—Unknown.

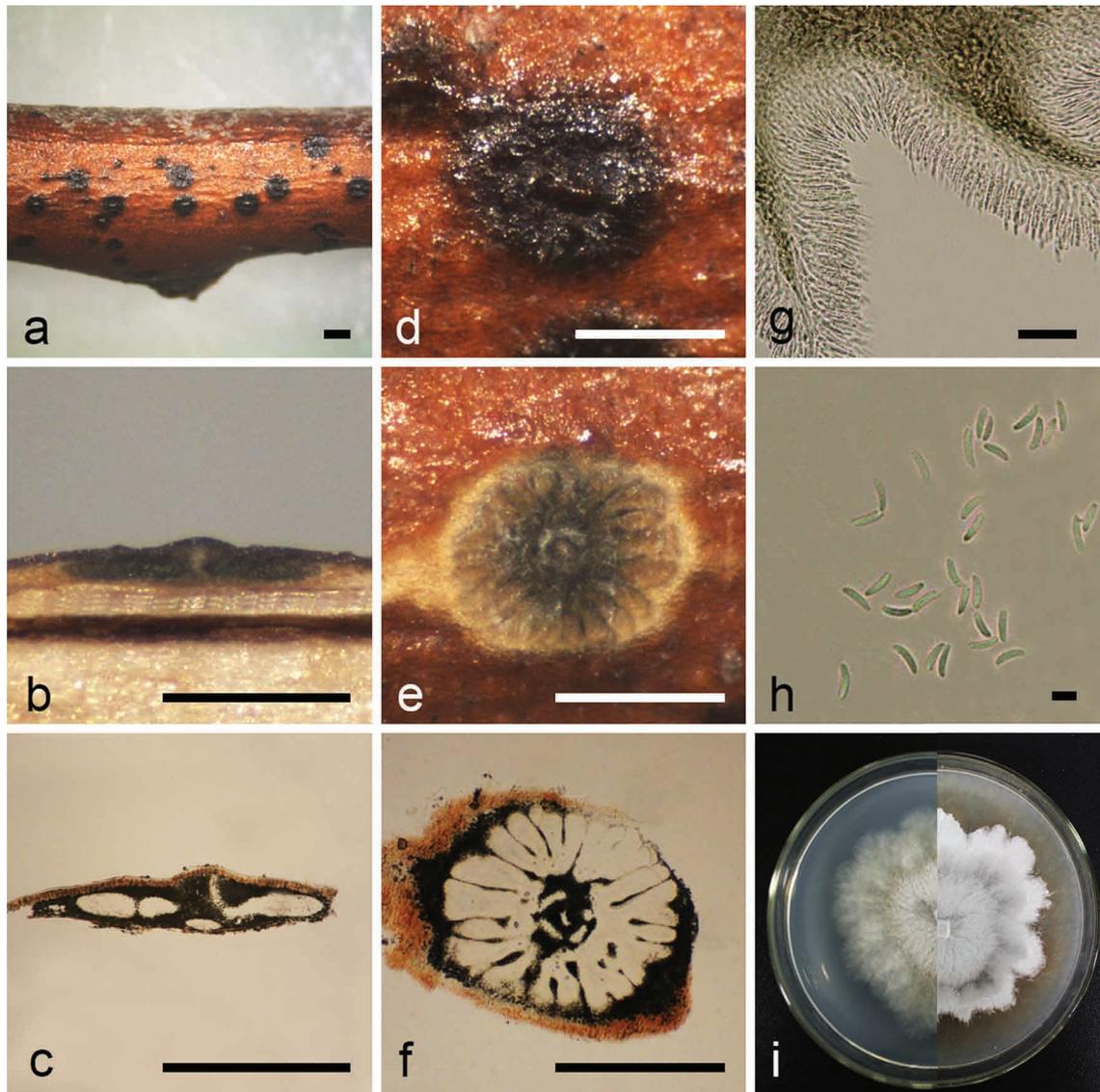
Asexual state:—Stromata immersed in bark. Conidiomata, slightly erumpent through the surface of bark, discoid, nearly flat, with small multiple locules. Conceptacle absent. Disc dark, unobscured, circular to ovoid, (70–) 90–140(–150)  $\mu\text{m}$  (average = 110  $\mu\text{m}$ , n = 20) in diameter, with one ostiole per disc. Ostiole in the center of the disc, unobscured, at the same level as the disc surface, (49.8–)51.8–62.8(–63.4)  $\mu\text{m}$  (average = 57.8  $\mu\text{m}$ , n = 20) in diameter. Multi-locules arranged in circles or ellipse with common walls, (580–)630–920(–940)  $\mu\text{m}$  (average = 790  $\mu\text{m}$ , n = 20) in diameter. Conidiophores hyaline, unbranched or branched at the bases, (14.2–)16.8–27.4(–28.1)  $\mu\text{m}$  (average = 22.5  $\mu\text{m}$ , n = 20). Conidia hyaline, elongate-allantoid, eguttulate, aseptate, (6.1–)6.3–9.3(–9.6)  $\times$  (1.7–)2–2.9(–3)  $\mu\text{m}$  (average = 7.7  $\times$  2.6  $\mu\text{m}$ , n = 50).

Cultures:—Colony originally white, producing light brown pigment after 7–10 days, flat, with a thick texture at the center with thin surrounding texture, conidiomata irregular on medium surface.

Material examined:—**CHINA Qinghai Province**: Haidong District, Pingan County, Bazang Gou, 36°27'32.44" N, 102°09'16.90" E, 1348 m asl., on twigs and branches of *Elaeagnus angustifolia*, collected by X.L. Fan, 15 Aug 2012 (BJFC-S642); living culture, CFCC 89631; **Ningxia Province**, Guyuan, Changchengliang, 36°03'01.77" N, 106°16'18.63" E, 1767 m asl., on twigs and branches of *Elaeagnus angustifolia*, collected by X.L. Fan, 24 Jul 2013 (BJFC-S965); living culture, CFCC 89632; *ibid.*, living culture, CFCC 89633.

Notes: *Cytospora elaeagni* has been recorded from *Elaeagnus angustifolia* in China, Germany and North America, but these records lacked any detailed descriptions, illustrations, and molecular data (Saccardo 1889; Chen 2002; Zhuang 2005). In the current study, the isolates CFCC 89631 and CFCC 89632 were shown to be *C. elaeagni* based on previous described morphological characters, host, and distribution (Saccardo 1889, Zhuang 2005). Therefore, this species from *E. angustifolia* was redescribed firstly based on morphology and phylogeny.

*Cytospora elaeagni* has not been epitypified and therefore we treat BJFC-S965 as an authentic specimen. This has been proved as consistent results from several other authors without epitypification (Saccardo 1889, Zhuang 2005). It makes sense to epitypify this here for further study. Thus fresh collections of *Cytospora elaeagni* are needed for epitypification.

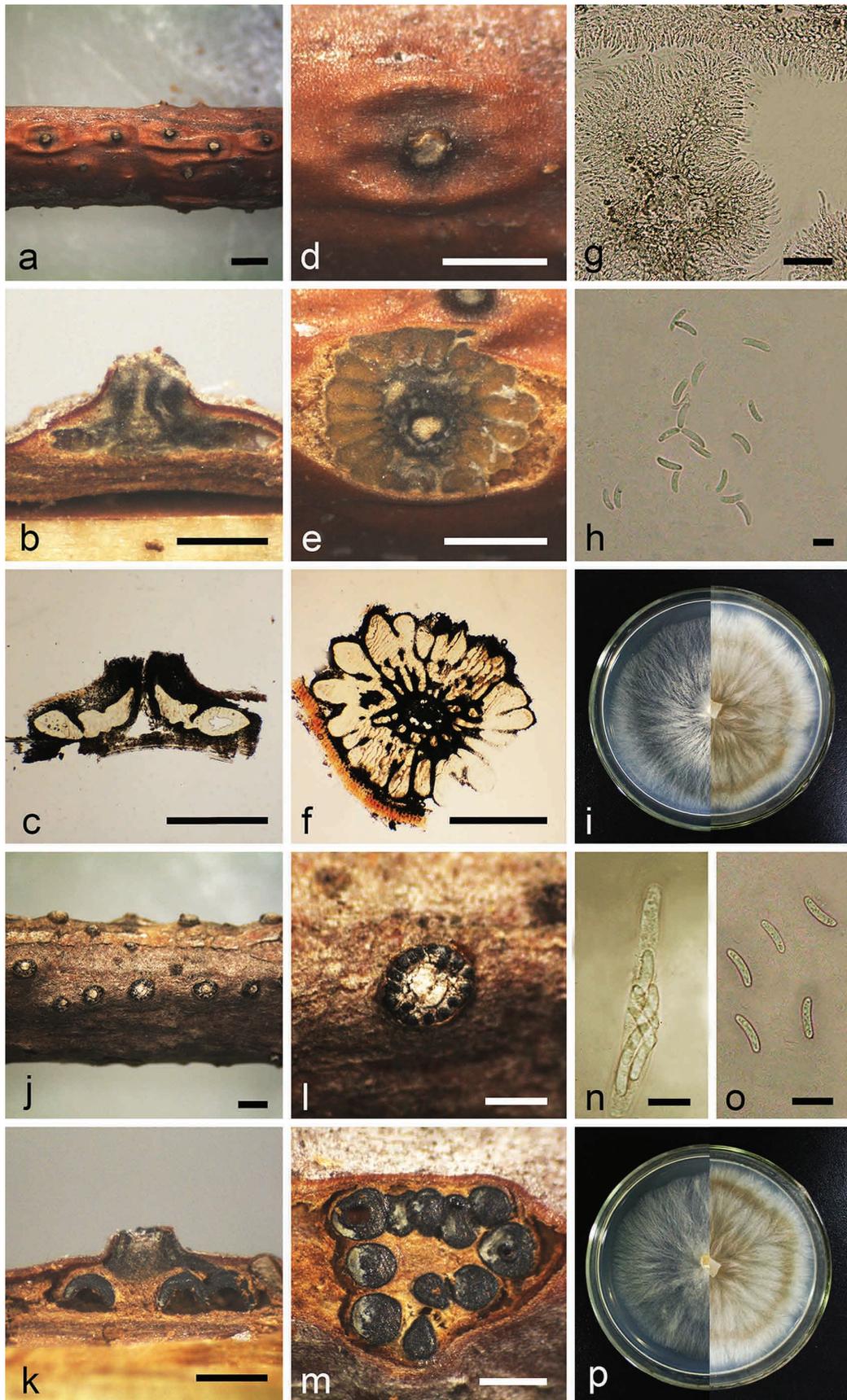


**FIGURE 5.** Morphology of *Cytospora elaeagni* from *Elaeagnus angustifolia* (BJFC-S642). a, d: Habit of conidiomata on a twig. b, c: Longitudinal sections through conidiomata. e, f: Transverse sections through conidiomata. g: Conidiophores. h: Conidia. i: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: a=1 mm; b–f=0.5 mm; g=20  $\mu\text{m}$ ; h=5  $\mu\text{m}$ .

***Cytospora hippophaes*** Thum., *Fungi austr.*: 282 (1872) (Fig. 6)

Host and Distribution:—Known from *Hippophae rhamnoides* in Australia, China, and Spain.

Pathogen on twigs and branches of *Hippophae rhamnoides*. Sexual state:—Stromata immersed in bark. Ascstromata, erumpent through the surface of bark, lenticular, extending to a large circular area, (1100–)1170–1600(–1710)  $\mu\text{m}$  (average = 1370  $\mu\text{m}$ , n = 20) in diameter. Conceptacle absent. Disc white to light brown, usually surrounded by tightly ostiolar necks, nearly flat, circular to ovoid, (390–)420–770(–820)  $\mu\text{m}$  (average = 560  $\mu\text{m}$ , n = 20) in diameter. Ostioles numerous, at the same level as the disc or slightly above, concentrated, dark brown to black, arranged circularly in a disc, (55.7–)58.5–88.1(–89)  $\mu\text{m}$  (average = 73.3  $\mu\text{m}$ , n = 20) in diameter. Locules dark brown, arranged circularly to triangularly, flask-shaped to spherical, (260–)280–320(–360)  $\mu\text{m}$  (average = 300  $\mu\text{m}$ , n = 20) in diameter. Asci free, clavate to elongate obovoid, (33.8–)38.6–47.4(–49.5)  $\times$  (5.1–)5.9–7.8(–8.2)  $\mu\text{m}$  (average = 44.5  $\times$  7.1  $\mu\text{m}$ , n = 20), 8-spored. *Ascospores* biseriolate, elongate-allantoid, thin-walled, hyaline, eguttulate, aseptate, (10.4–)11.8–15(–16.1)  $\times$  (2.6–)3–4.1(–4.4)  $\mu\text{m}$  (average = 13.6  $\times$  3.4  $\mu\text{m}$ , n = 50).



**FIGURE 6.** Morphology of *Cytospora hippophaes* from *Hippophae rhamnoides* (BJFC-S779). a, d: Habit of conidiomata on twig. b, c: Longitudinal sections through conidiomata. e, f: Transverse sections through conidiomata. g: Conidiophores. h: Conidia. i: Colonies on PDA at 3 days (left) and 30 days (right). j: Habit of ascomata on twig. k: Longitudinal sections through ascomata. l: Disc of ascomata on twig. m: Transverse sections through ascomata. n: Asci. o: ascospores. p: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: a, j=1 mm; b–f, k–m=0.5 mm; g=20  $\mu$ m; h=5  $\mu$ m; n–o=10  $\mu$ m.

Asexual state:—Stromata immersed in bark. Conidiomata, erumpent through the surface of bark, flat to discoid, with multi-locules. Conceptacle absent. Disc light brown, nearly flat, ovoid to ellipsoid, (230–) 270–460(–480)  $\mu\text{m}$  (average = 360  $\mu\text{m}$ ,  $n=20$ ) in diameter, with 3–5 ostioles per disc. Ostioles at the same level as the disc, dark grey to black, surrounded below disc by lighter entostroma, (72.3–)75.1–107.9(–109.5)  $\mu\text{m}$  (average = 98.2  $\mu\text{m}$ ,  $n = 20$ ) in diameter. multi-locules arranged regularly with common walls, (910–)950–1190(–1220)  $\mu\text{m}$  (average = 1070  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Conidiophores hyaline, unbranched, or occasionally branched at the bases, (15.1–)15.6–21.9(–22.2)  $\mu\text{m}$  (average = 18.7  $\mu\text{m}$ ,  $n = 20$ ). Conidia hyaline, elongate-allantoid, aseptate, eguttulate, (7.2–)7.7–10(–10.8)  $\times$  (1.4–)1.8–2.3(–2.5)  $\mu\text{m}$  (average =  $9.2 \times 2.1$   $\mu\text{m}$ ,  $n = 50$ ).

Cultures:—Colony originally white, and producing dark brown pigment after 7–10 days, flat, felt-like, with concentric circular texture, conidiomata irregular on medium surface.

Material examined:—**CHINA Ningxia Province**: Guyuan, Jingyuan, Baimian No.2 Middle School, 35°25'08.02" N, 106°24'00.64" E, 1760 m asl., on twigs and branches of *Hippophae rhamnoides*, collected by X.L. Fan, 22 Jul 2013 (BJFC-S966); living culture, CFCC 89636; Guyuan, Liupan Mountain, 36°03'01.77" N, 106°14'20.83" E, 2094 m asl., on twigs and branches of *Hippophae rhamnoides*, collected by X.L. Fan, 15 Jul 2013 (BJFC-S970); living culture, CFCC 89637; **Shaanxi Province**, Yulin, Yulin Middle School, 38°14'03.73" N, 109°44'22.92" E, 1055 m asl., on twigs and branches of *Hippophae rhamnoides*, collected by X.L. Fan, 30 Jul 2013 (BJFC-S974); living culture, CFCC 89638; **Gansu Province**, Gannan, Diebu, Dianga, Jiangba, 34°02'06.70" N, 103°12'06.25" E, 2770 m asl., on twigs and branches of *Hippophae rhamnoides*, collected by X.L. Fan, 9 Aug 2012 (BJFC-S779); living culture (from conidia), CFCC 89639; *ibid.*, living culture (from ascospore), CFCC 89640.

Notes: *Cytospora hippophaes* was recorded from *Hippophae rhamnoides* and has also been reported from Australia, China, and Spain. However, these records lacked any detailed description, illustrations, and molecular data (Gonzalez 1916; Saccardo 1884; Zhuang 2005). Isolates CFCC 89637, CFCC 89638, and CFCC 89639 were shown to be *C. hippophaes* based on morphology and host (Saccardo 1884, Zhuang 2005). In the current study, this species from *Hippophae rhamnoides* is redescribed firstly with both states with illustrations and phylogeny.

*Cytospora hippophaes* has not been epitypified and therefore we treat BJFC-S779 as an authentic specimen. This has proved as consistent results from several other authors without epitypification (Saccardo 1884, Zhuang 2005). It makes sense to epitypify this here for further study. Thus fresh collections of *Cytospora hippophaes* are needed for epitypification.

### *Cytospora nivea* (Hoffm.) Sacc., *Michelia* 2: 264 (1881) (Fig. 7)

= *Sphaeria nivea* Hoffm., *Veg. Crypt.* 1: 28 (1787)

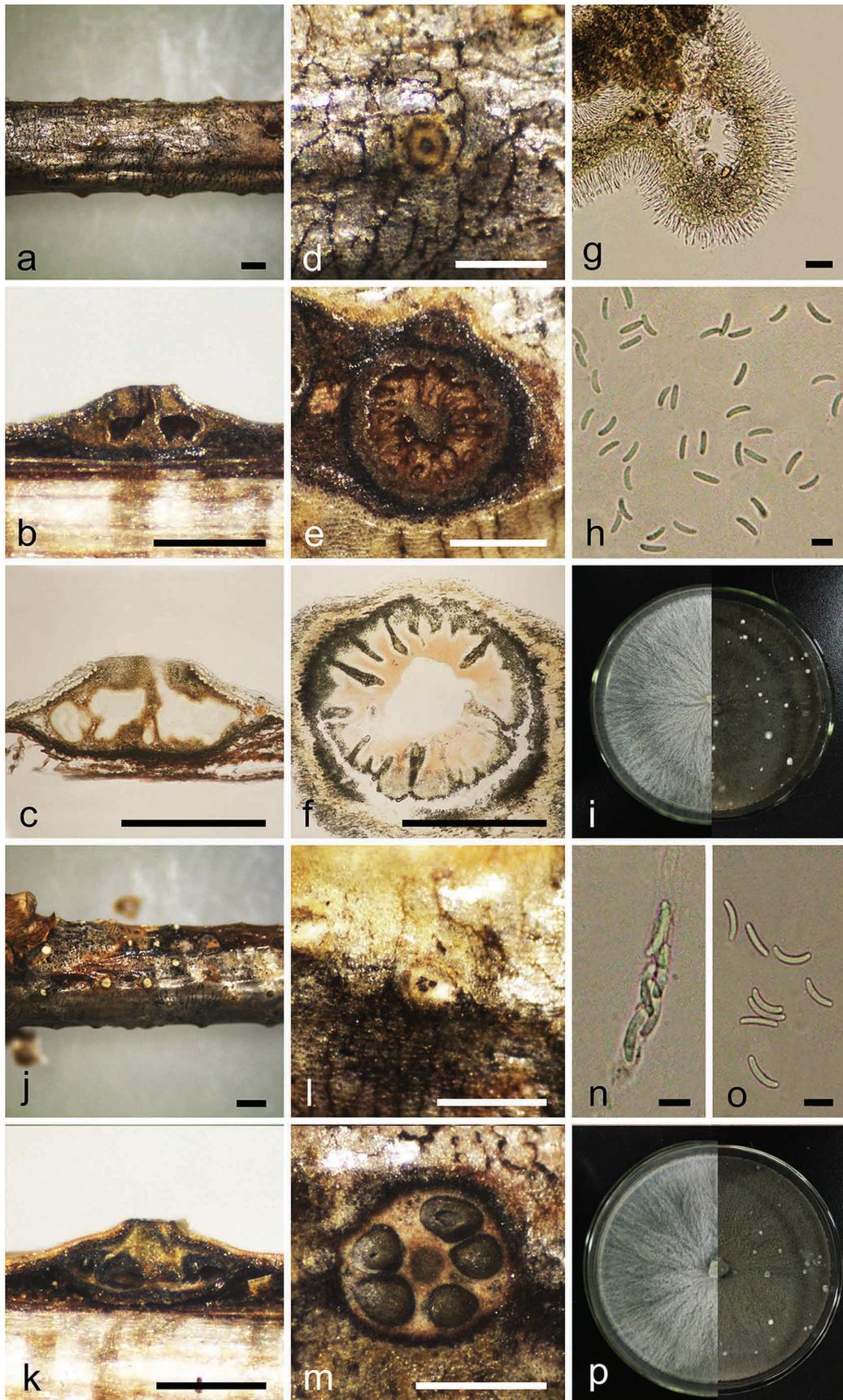
= *Valsa nivea* (Hoffm.) Fr., *Summa veg. Scand.*, Section Post. (Stockholm): 411 (1849)

= *Leucostoma niveum* (Hoffm.) Hohn., *Mitt. bot. Inst. tech. Hochsch. Wien* 5: 58 (1928)

Host and Distribution:—Known from *Elaeagnus angustifolia* in China; *Juniperus* and *Rosa* in Bulgaria; *Malus* in Iran and South Africa; and Salicaceae in Asia, Europe, North America and South Africa.

Pathogen on twigs and branches of *Elaeagnus angustifolia* and *Salix psammophila*. Sexual state:—Stromata immersed in bark. Ascstromata, erumpent through the surface of bark, lenticular, extending to a large circular area, (570–)620–1080(–1120)  $\mu\text{m}$  (average = 930  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Conceptacle dark, surround the ascstromata. Disc white to light brown, nearly flat, circular to ovoid, (170–)200–280(–300)  $\mu\text{m}$  (average = 250  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Ostioles numerous, dark brown to black, at the same level as the disc, occasionally area below disc a lighter entostroma,, (40.6–)42.5–86.1(–89.5)  $\mu\text{m}$  (average = 64.8  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Locules dark brown, arranged circularly, flask-shaped to spherical, (160–)210–260(–280)  $\mu\text{m}$  (average = 240  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Asci free, clavate to elongate obovoid, (30.1–)31.7–43.5(–44.4)  $\times$  (5.2–)5.5–7.4(–7.9)  $\mu\text{m}$  (average =  $39.8 \times 6$   $\mu\text{m}$ ,  $n = 20$ ), 8-spored. Ascospores biseriolate, elongate-allantoid, thin-walled, hyaline, eguttulate, aseptate, (9.7–)10.8–15.3(–16.3)  $\times$  (1.8–)2.1–3.2(–3.6)  $\mu\text{m}$  (average =  $13.2 \times 2.7$   $\mu\text{m}$ ,  $n = 50$ ).

Asexual state:—Stromata immersed in bark. Conidiomata, erumpent through the surface of bark, discoid to conicoid, with multiple locules. Conceptacle dark, surround the stromata. Disc brown to dark grey, nearly flat, ovoid to ellipsoid, (110–) 140–210(–270)  $\mu\text{m}$  (average = 190  $\mu\text{m}$ ,  $n=20$ ) in diameter, with one ostiole per disc. Ostiole at the same level as the disc, dark grey to black, (52.5–)54.3–90.9(–93.2)  $\mu\text{m}$  (average = 74.8  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Multi-locules arranged irregularly with common walls, (410–)470–650(–770)  $\mu\text{m}$  (average = 550  $\mu\text{m}$ ,  $n = 20$ ) in diameter. Conidiophores hyaline, unbranched, or occasionally branched at the bases, (16.9–)18.3–28.5(–29.1)  $\mu\text{m}$  (average = 23  $\mu\text{m}$ ,  $n = 20$ ). Conidia hyaline, elongate-allantoid, aseptate, eguttulate, (6–)6.2–9.2(–9.8)  $\times$  (1.5–)1.7–2.4(–2.9)  $\mu\text{m}$  (average =  $7.6 \times 1.9$   $\mu\text{m}$ ,  $n = 50$ ).



**FIGURE 7.** Morphology of *Cytospora nivea* from *Salix psammophila* (BJFU-S979). a, d: Habit of conidiomata on twig. b, c: Longitudinal sections through conidiomata. e, f: Transverse sections through conidiomata. g: Conidiophores. h: Conidia. i: Colonies on PDA at 3 days (left) and 30 days (right). j: Habit of ascomata on twig. k: Longitudinal sections through ascomata. l: Disc of ascomata on twig. m: Transverse sections through ascomata. n: Asci. o: ascospores. p: Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: a, j=1 mm; b–f, k–m=0.5 mm; g=20  $\mu$ m; h=5  $\mu$ m; n–o=10  $\mu$ m.

Cultures:—Colony originally white, and producing dark green to black pigment after 7–10 days, flat, felty, with concentric circles, and conidiomata irregular on the medium surface.

Material examined:—**CHINA Ningxia Province**: Guyuan, Changchengliang, 36°03'01.78" N, 106°16'18.09" E, 1777 m asl., on twigs and branches of *Elaeagnus angustifolia*, collected by X.L. Fan, 24 Jul 2013 (BJFC-S964); living culture, CFCC 89641; **Shaanxi Province**, Yulin, Hongshi Gorge, 38°19'32.43" N, 109°42'00.69" E, 1105 m asl., on twigs and branches of *Salix psammophila*, collected by X.L. Fan, 29 Jul 2013 (BJFC-S979); living culture (from conidia), CFCC 89642; *ibid.*, living culture (from ascospore), CFCC 89643.

Notes: *Cytospora nivea* is a commonly recorded species associated with Salicaceae hosts, whereas it has only been collected on *Populus* in China (Saccardo 1881, 1884; Chen 2002; Zhuang 2005). In the current study, the isolates CFCC 89641, CFCC 89642, and CFCC 89643 were shown to be *C. nivea* based on phylogenetic analysis and morphological characters (Adams *et al.* 2006). This is the first record of this species on *Salix psammophila* worldwide.

*Cytospora nivea* has not been epitypified and therefore we treat BJFC-S979 as an authentic specimen. This has proved as consistent results from several other authors without epitypification (Saccardo 1881, 1884; Zhuang 2005; Adams *et al.* 2006). It makes sense to epitypify this here for further study. Thus fresh collections of *Cytospora nivea* are needed for epitypification.

*Cytospora chrysosperma* (Pers.) Fr., *Sylv. mycol. berol.* (Berlin): 28 (1818).

= *Naemaspora chrysosperma* Pers., *Syn. meth. fung.* (Göttingen) 1: 108 (1801).

= *Valsa sordida* Nitschke, *Pyrenomyces Germanici* 2: 203 (1870).

Host and Distribution: 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 Iran; Salicaceae in China, Iran, Netherland, South Africa, Switzerland, UK and the USA; *Sophora japonica* in China; *Triticum* in Germany; and *Ulmus* in USA.

Pathogen on twigs and branches of *Salix psammophila*.

Material examined:—**CHINA Shaanxi Province**: Yulin, Jichang East Road, 38°19'21.16" N, 109°39'54.73" E, 1124 m asl., on stems of *Salix psammophila*, collected by X.L. Fan, 1 Aug 2013 (BJFC-S975); living culture, CFCC 89629; Yulin, Hongshi Gorge, 38°19'40.83" N, 109°42'37.98" E, 1092 m asl., on stems of *Salix psammophila*, collected by X.L. Fan, 29 Jul 2013 (BJFC-S978); living culture, CFCC 89630.

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). In the current study, the isolates CFCC 89629 and CFCC 89630 were shown to be *C. chrysosperma* based on phylogenetic analysis and morphological characters. This is the first record of this species on *Salix psammophila* worldwide.

*Cytospora chrysosperma* has not been epitypified and therefore we treat BJFC CGHs 10 as an authentic specimen (Fan *et al.* 2014). This has proved as consistent results from several other authors without epitypification (Adams *et al.* 2005, 2006; Fotouhifar *et al.* 2010). It makes sense to epitypify this here for further study. Thus fresh collections of *Cytospora chrysosperma* are needed for epitypification.

## Discussion

The naming of *Cytospora* species is complicated because unclear host ranges, barely distinguishable morphology in the asexual and sexual states and lack of ex-type sequence data in GenBank. Therefore when a strain of *Cytospora* is isolated from almost any host, the strains have to be compared with putatively named taxa in GenBank. In the present study we bring together all ex-type data and where ex-type strains are not available we assigned authentic strains to represent the species identified in this study.

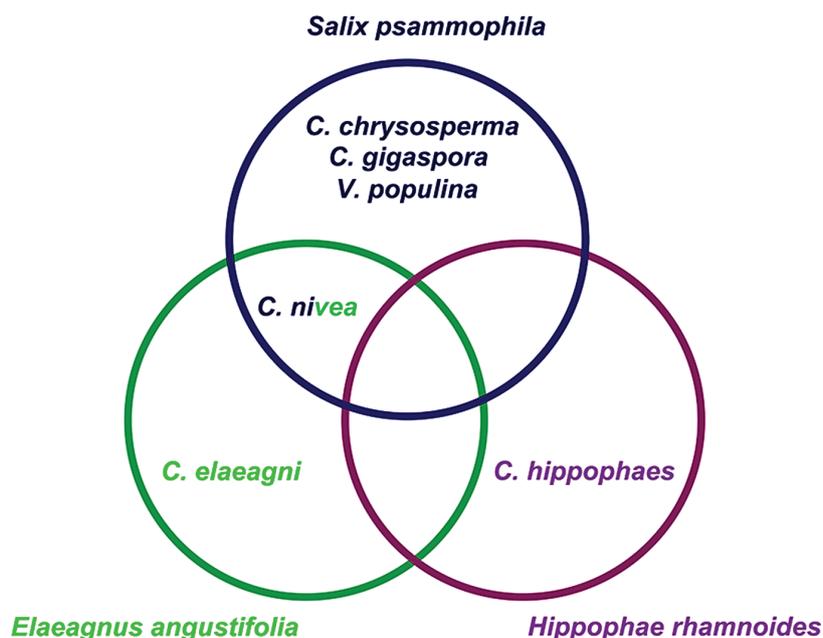
The current study characterizes six species (*Cytospora chrysosperma*, *C. elaeagni*, *C. gigaspora*, *C. hippophaes*, *C. nivea* and *C. populina*) associated with *Cytospora* canker disease of *Elaeagnus angustifolia*, *Hippophae rhamnoides* and *Salix psammophila* in northwest China. The study is based on a systematic taxonomic approach using morphology and analysis of ITS sequence data. The six species have distinct morphological characters in their fruiting bodies, spore size, and cultural characteristics. The multilocus phylogeny combining four gene region sequences (ITS, nrLSU, RPB2, and ACT) indicates that these species belong to six individual clades. Although *C. elaeagni* and *C. hippophaes*

have been recorded previously in China, there are no detailed descriptions, illustrations, or molecular data associated with these records. This has resulted in confusion amongst mycologists and plant pathologists, in the naming of species. Other species are reported for the first time from the hosts *Elaeagnus angustifolia* and *Salix psammophila*, with a novel species.

*Cytospora* pycnidia usually contain single or multiple irregular locules, filamentous conidiophores, and allantoid hyaline conidia. In moist conditions, conidia ooze from the pycnidia as yellow-orange gelatinous tendrils. The sexual state is characterized by clustered, erumpent ascomata, which are globose to ellipsoid, with beaks converging at the disc or surface, and usually with many ostioles per disc. Asci are ellipsoid to clavate and free, with 4–8 ascospores. Ascospores are hyaline, allantoid, aseptate, thin-walled, and smooth-walled (Adams *et al.* 2005, 2006). Formerly, *Cytospora* species were largely named based on morphology and host association. However, the overlap of morphological characters and lack of host-specificity has resulted in confusion in the identification of species (Fries 1823; Saccardo 1884; Deng 1963; Tai 1979; Wei 1979; Spielman 1983, 1985; Adams *et al.* 2005, 2006). More than one *Cytospora* species may generally be present on one host plant, and one species may occur on multiple host plants. Therefore, previous naming of *Cytospora* species may have been subjective.

*Cytospora gigaspora* has multi-locules but with a single ostiole, and black conceptacle and occurs on *Salix psammophila*. It is similar with *C. nivea* on *Populus*. However, *C. gigaspora* has unusually long conidia (10.4 µm on average) and flat locules compared with those of other *Cytospora* species (Figs 3b and 3h). In the phylogenetic analysis (Fig. 2) *C. gigaspora* from a distinct clade with high support values (MP-BS/ML-BS/BPP = 99/96/1.00). *Cytospora chrysosperma*, *C. nivea*, and *C. populina* have previously been reported from *Populus* spp. as the causative agent of poplar canker disease (Saccardo 1884; Deng 1963; Tai 1979; Wei 1979; Chen 2002; Zhuang 2005). These three *Cytospora* species represent the first records from *Salix psammophila* worldwide. *Cytospora elaeagni* and *C. hippophaes* were previously identified according to their hosts.

With the exception of *C. nivea*, all species found in this study were only associated with a single host *Elaeagnus angustifolia* L., *Hippophae rhamnoides* L. and *Salix psammophila* (Fig. 8). This species might be relatively host specific (Zhou *et al.* 2001), as *Cytospora* species, such as *C. australiae* Speg., *C. eucalyptina* Speg., *C. eucalypticola* Van der Westh., and *C. agarwalii* Soni, Dadwal & Jamaluddin have only been isolated from *Eucalyptus* sp. (Adams *et al.* 2005). Previous reports have indicated that many *Cytospora* species have broad host ranges; however, these reports lacked molecular data (Fries 1823; Deng 1963; Saccardo 1884; Tai 1979; Wei 1979). Some widespread species (e.g., *C. chrysosperma* and *C. nivea*) were identified and verified by molecular data, to occur in many host species (Adams *et al.* 2005, Fan *et al.* 2014). Species occurrence may therefore reflect factors influencing distribution, such as environmental factors and transmission, rather than the taxa actually being host-specific. In order to clarify certain host ranges and selective mechanisms of *Cytospora* species, advanced genetic studies, such as verifying host jumping and genome evolution, will need to be carried out using a more intensive and wider sampling of isolates.



**FIGURE 8.** Pattern of overlapping *Cytospora* species among *Elaeagnus angustifolia*, *Hippophae rhamnoides* and *Salix psammophila*.

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