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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF CYTOSPORA SPP. OF ECONOMIC FORESTS AND SHELTERBELT IN XINJIANG

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Abstract

Cytospora species are found in Xinjiang with stem canker disease. *Cytospora* species were identified based on morphological characteristics and molecular biology from economic forests and shelter belt in Xinjiang. A total of 281 isolates were obtained from woods canker of 17 hosts in Xinjiang. The strains identified were *Valsa mali* (anamorph: *Cytospora sacculus*), *Valsa sordida* (anamorph: *Cytospora chrysosperma*), *Valsa macolica* (anamorph: *Cytospora schulzeri*), *Leucostoma niveum* (anamorph: *Cytospora nivea*) and *Cryptosphaeria pullmanensis* (anamorph: *Cytosporina pullmanensis*). The *V. mali* and *V. sordida* wwere the main pathogens of economic forest and shelter forest, respectively. It is also hoped that this will lead to improve management strategies for diseases associated with these fungi.

Introduction

Xinjiang region is the largest and driest area in China, it spans over 1.6 million km2, but the forest area accounts only for 2.94% (Pan *et al.* 2013). The afforestation area of Xinjiang is about 1.49×105 hm^2, including economic forest area 3.86 $\times 10^4$ hm2, timber forest area 0.8 $\times 10^4$ hm2, shelter forest 1.06 $\times 10^5$ hm^2 and fuelwood area 612 hm^2 (Statistic Bureau of Xinjiang Uygur Municipality 2015, Niu *et al.* 2015). Therefore, to maintain the biodiversity, ecological balance and national ecological security have great significance to protect the healthy development of agriculture and forestry in Xinjiang.

Species of *Cytospora* Ehrenb cause cankers and dieback on many genera of hardwoods and coniferous trees, but rarely on herbaceous plants (Sinclair *et al*. 1987, Farr *et al*. 1989). *Cytospora* cankers are especially destructive on *Prunus* spp. and *Malus* spp. in commercial orchards (Biggs 1989, Kepley *et al*. 2000). *Cytospora* are the anamorphs of the ascomycete genus *Valsa* Fr. Fruit bodies consist of stromata that usually contain either labyrinthine chambers or clusters of pycnidia, having filamentous conidiophores and allantoid hyaline conidia. An investigation in 2013 and 2015 in Xinjiang Province showed 30% diseased trees; whereas, in some commercial orchards, the incidence of diseased trees was 100%. The infection always leads to expanding cankers, resulting in the death of twigs, branches, trunks, and eventually the entire tree (Abe *et al.* 2007). The commercial orchards destroyed by this disease are prevalent in many apple-producing regions in China. The objective of this study was to identify species of *Cytospora* of shelter belt, economic forests in Xinjiang based on DNA-based characterization and morphology. Thus, the present study aimed was to increase the understanding of geographical and host range relationships, and to discuss possible origins of the pathogens in Xinjiang.

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Materials and Methods

In 2013-2017, 170 samples from economic forests and 111 samples from shelter belt in Xinjiang were collected with the developed symptoms of death of twig tips, branch dieback, and canker formation. Fragments (5 × 5 mm^2) from the junction of diseased and healthy tissues were surface sterilized with 1% NaClO for 60 s and then rinsed twice in sterile distilled water. Then, the fragments were incubated at 25°C for three days, and mycelial plugs were cut with a cork borer from the margins of the colonies and transferred to water agar plates. After incubation at 25°C for five days, single hyphal tips were cut with a sterile scalpel under the microscope and transferred to PDA to obtain a single hyphal colony. Stock cultures of all single hyphal isolates were stored in 15% glycerol at –80°C. Horizontal and longitudinal section was observed in the microscope.

Total genomic DNA was extracted from the hyphae harvested using a standard hexadecyltrimethyl-ammonium bromide method (Edwards *et al.* 1991). The ribosomal DNA internal transcribed spacer (ITS) region was amplified by PCR using the primer pair ITS1 and ITS4 (White *et al*. 1990). PCR products were purified with the QIAquickH PCR Purification Kit (QIAGEN, Valencia, CA) and directly sequenced with the ABI PRISM BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequences were resolved on an ABI 3130XL automated sequencer. Phylogenetic analyses were performed with MEGA 5.0 with Neighbor-Joining (NJ) using a heuristic search and 1000 random addition sequence replicates.

To confirm pathogenicity, mycelial PDA plugs of a culture of each fungi were inoculated onto five twigs and wrapped by plastic wrap, inoculated branches at both the ends with moist cotton wool wrapped by plastic wrap. Five disinfected twigs were inoculated with non-colonized PDA plugs as the control. Pathogenicity tests maintained in a plant growth chamber (28 °C, RH 80%, light 12 h per day) and 4 days later, incidence and lesion diameter of inoculated shoots was measured.

Results and Discussion

1. *Cytospora sacculus* (teleomorph *Valsa mali*, also called *V. ceratosperma*) (Abe *et al.* 2007, Chen *et al.* 1987, Spielman 1985, Wei *et al.* 2010).

Teleomorph not seen. Conidiomatal stromata immersed in bark, erumpent, discoid, convex to conical, 2-1.4 mm diam. Discs dark brown to grey, nearly flat to convex, circular, up to 0.5 mm diameter with one ostiole. Ostioles brown to grey, level with disc surfaces, surrounded by brown stroma. Conidioma of multiple locules united at the shared ostiole. Locules tear-shaped to elongate ovoid, not sharing common walls, surrounded by entostromata, each locule with an ostiole converging towards the disc to one shared ostiole per disc. Conidia, hyaline, eguttulate, allantoid, aseptate, (3.0~4.0) × (0.75~1.0) μm (Fig. 1).

Cultures: Conidia colony growth on PDA is greyish to brownish grey with an edge of dark and brownish on the surface. Colour of the reverse is white and gray. Colony texture is felty, slightly raised with faint growth zones. Conidiomata have white to grey surfaces during development (Fig.1).

Host: Malus, Pyrus, Zizyphus jujube, Prunus armeniaca, Populus nigra, Sophora japonica, Morus alba, Salix matsudana, Populus euphratica

2. ***Cytospora chrysosperma* (teleomorph *Valsa sordida*)** (Adams *et al*. 2005, Adams *et al*. 2006, Abbasi *et al*. 2015, Yang *et al*. 2015)

Teleomorph not seen, anamorph interspersed amongst ascostromata, discrete. Conidiomatal stromata immersed in bark, erumpent, labyrinthine cytosporoid, 1–2 mm diam. Discs white to

light grey, nearly flat, circular to ovoid, 0.4–0.5 mm diam. Locules multi-chambered, subdivided by invaginations into irregular chambers sharing common walls. Conidia hyaline, eguttulate, elongate-allantoid, aseptate, (3.5~5.0) µm × (0.9~1.4) µm (Fig. 2).

Cultures: Colony growth on PDA is pale white to faint yellow and had regular margins on the surface. Colour of the reverse is dark orange-yellow to brown. Colony texture is felty, slightly raised with no growth. Conidiomata have white to yellowish white surfaces during development (Fig. 2).

Host: Juglans, Malus, Populus alba, Salix matsudana, Elaeagnus angustifolia, Populus euphratica.

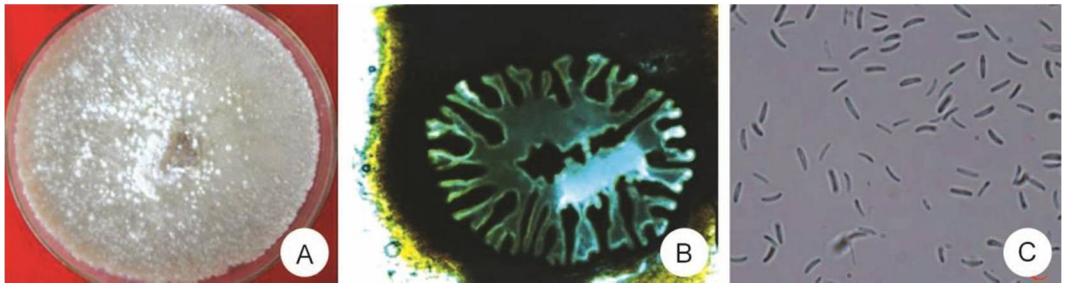

Fig. 1. Colony and Morphologic Characteristics of *Cytospora sacculus* (teleomorph: *Valsa mali*) A. Colony on PDA, B. Horizontal and Longitudinal section through conidioamata, C. Conidiospore

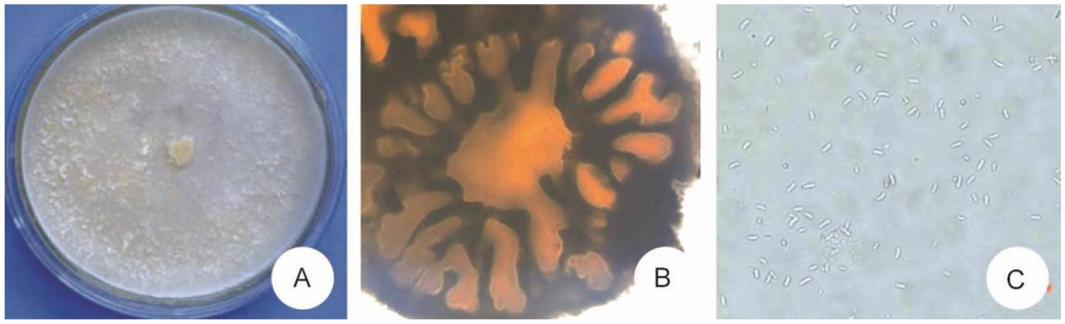

Fig. 2. Colony and Morphologic characteristics of *Cytospora chrysosperma* (teleomorph: *Valsa sordida*) A. Colony on PDA, B. Horizontal and Longitudinal section through conidioamata, C. Conidiospore

3. ***Cytospora schulzeri* (teleomorph *Valsa malicola*)** (Adams *et al.* 2005, Adams *et al.* 2006)

Teleomorph not seen. Conidiomatal stromata immersed in bark, erumpent, rosette to labyrinthine cytosporoid with regular radially arranged chambers, circular to ovoid 0.9–1.5 mm diam. Discs light to medium brown, nearly flat, circular, up to 0.4 mm diam., with one ostioles per disc. Ostioles dark brown, circinately arranged, at the same level as the disc surface, about 100 µm diam. Locules multi-chambered, subdivided by entire invaginations into regular radially arranged chambers sharing common walls. Conidia hyaline, eguttulate, elongate-allantoid, aseptate, (5.5-6.0) × (0.9-1.0)µm (Fig. 3).

Cultures: Colony growth on PDA is pale white to olive-grey and had no regular margins on the surface. Colour of the reverse is pale white to olive-grey. Colony texture is slightly raised with no growth. Conidiomata have white to yellowish white surfaces during development (Fig. 3).

Host: Malus, Pyrus, Crataegus pinnatifida

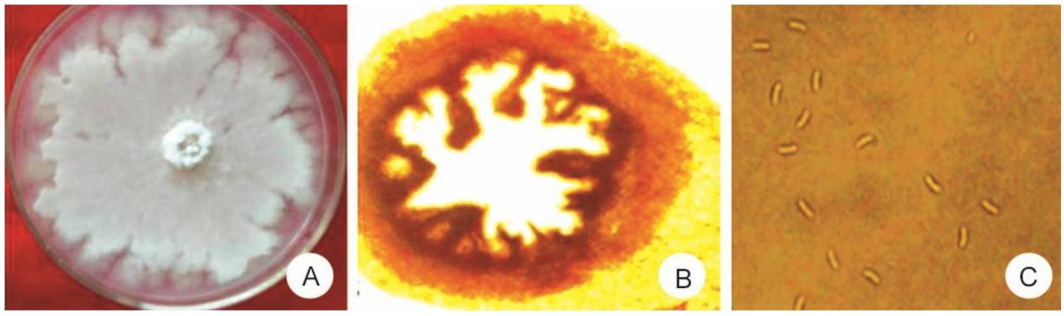

Fig. 3. Colony and Morphologic characteristics of *Cytospora schulzeri* (teleomorph: *Valsa malicola*) A. Colony on PDA, B. Horizontal and Longitudinal section through conidioamata, C. Conidiospore

4. ***Cytospora nivea*（teleomorph *Leucostoma niveum*）**(Adams *et al.* 2005, Adams *et al.* 2006).

Teleomorph not seen. Conidiomatal stromata embedded in bark, erumpent, labyrinthine and leucocytosporoid, 1.4~2.0 mm diam. Discs white, nearly flat to convex, circular, up to 0.2 mm diam. one ostiole per disc. Locules multi-chambered, subdivided by invaginations into irregular chambers sharing common walls. Conidia hyaline, eguttulate, elongate-allantoid, aseptate, (6.0~7.5) × (1.1~1.2) μm (Fig. 4).

Cultures: Colony growth on PDA is faint yellow to dark brown and had regular margins on the surface. Color of the reverse is orange-yellow to dark brown. Colony texture is felty, slightly raised with no growth. Conidiomata have white to brown surfaces during development (Fig.4).

Host: Juglans, Populus alba L., Salix matsudana.

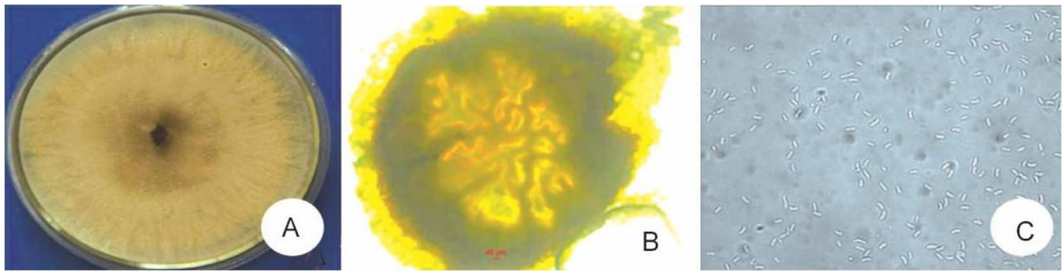

Fig. 4. Colony and Morphologic characteristics of *Cytospora nivea* (teleomorph:*Leucostoma niveum*) A. Colony on PDA, B. Horizontal and Longitudinal section through conidioamata, C. Conidiospore

5. *Cytosporina pullmanensis* (teleomorph *Cryptosphaeria pullmanensis*) (Glawe 1984, Ma *et al.* 2016, Mehrabi *et al.* 2016)

Teleomorph not seen. Conidiomata immersed in bark stromatic, multi-chambered, emptying into common ostiole. Conidia produced on colonies were allantoid, with flattened bases, hyaline, (6.8-8.4) × (1.2-1.8) μm (Fig. 5).

Cultures: Fungal colonies on PDA were light orange to white-gray masses and irregular margins; reverse coloration bright yellow, fading to white at margin. Colony texture is felty, slightly raised with no growth. Conidiomata have white to brown surfaces during development (Fig. 5).

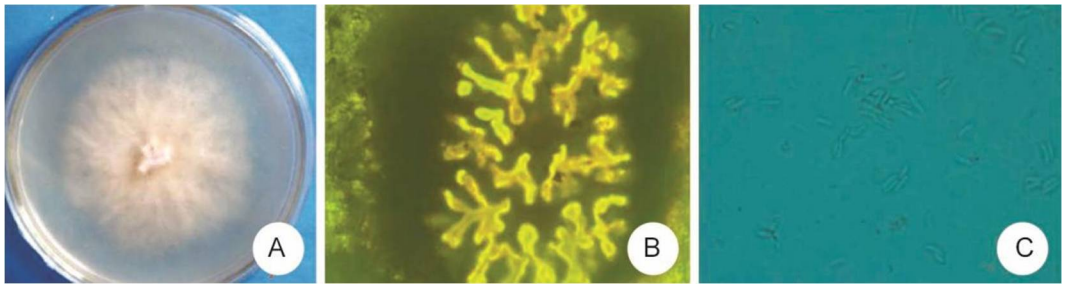

Fig. 5. Colony and Morphologic characteristics of *Cryptosphaeria pullmanensis* (teleomorph: *Cytosporina pullmanensis*). A.Colony on PDA, B. Horizontal and Longitudinal section through conidioamata, C. Conidiospore

Host: Populus alba, Salix matsudana.

Comparisons with sequences from the international GenBank database (http://www.ncbi.nlm.nih.gov/) were conducted using BLASTN search. In total, 228 strains from economic forests and shelterbelt in Xinjiang were identified by morphological features of culture, 18S rDNA-ITS and β-tubulin gene sequences analysis. In which 28 representative strains were assigned to the genus level based on 16S rRNA genes and β-Tubulin sequencing results.

The sequences of related species retrieved from GenBank *Valsa sordida* (HM156067, KC787361), *Valsa malicola* (JN545839, KF293799), *Leucostoma niveum* (KF293930, KJ739499), *Valsa mali* (GU174587, HM156066), *Cryptosphaeria pullmanensis* (GQ293966, KM588263), and *Alternaria* sp. (KC584210)] were initially aligned using the multiple alignment program Clustal X 1.81, which was included in the DNAman software package version 5.2.2 (Lynnon BioSoft). The phylogenetic tree was constructed from the evolutionary distance data calculated from Kimura's two-parameter model using the neighbor–joining method with 1000 bootstrap replicates. The result of Xingjiang wood canker pathogen identification based on morphological characteristics, rDNA-ITS sequences and β-Tubulin sequences analysis are consistent (Figs 6-7).

The isolated and identified 281 strains were inoculated with branches of 2 year old healthy economic trees such as *Malus, Pyrus, Juglans,* and shelter forest such as *Populus alba, Populus euphratica and Salix matsudana,* which were inoculated in the corresponding of the host plant show water soaked, and emits a foul odor, necrosis, inner bark discoloration, similar to those observed in branches and lateral discrete ostiolar beaks of conidiomata were visible on bark of branches. The strains were inoculate was round and has obvious junction of diseased and healthy. The lesion was brown, shrinkage, and emits a foul odor, at the same time the color of xylem was brown in the inoculation site, after 20 days, black pycnidia were appeared *in vitro* branches. Hence, Koch's postulates were supported by pathogenicity tests conducted on branches after inoculation *in vitro* (Fig. 8).

Identification of *Cytospora* species using morphological characteristics, rDNA-ITS sequences and β-Tubulin sequences has made it possible to recognize many species of *Cytospora* in Xinjiang. A total of 281 isolates were obtained from woods canker come from 17 kinds of hosts in Xinjiang, all strains are anamorph, and pathogenicity of these isolated were tested according to

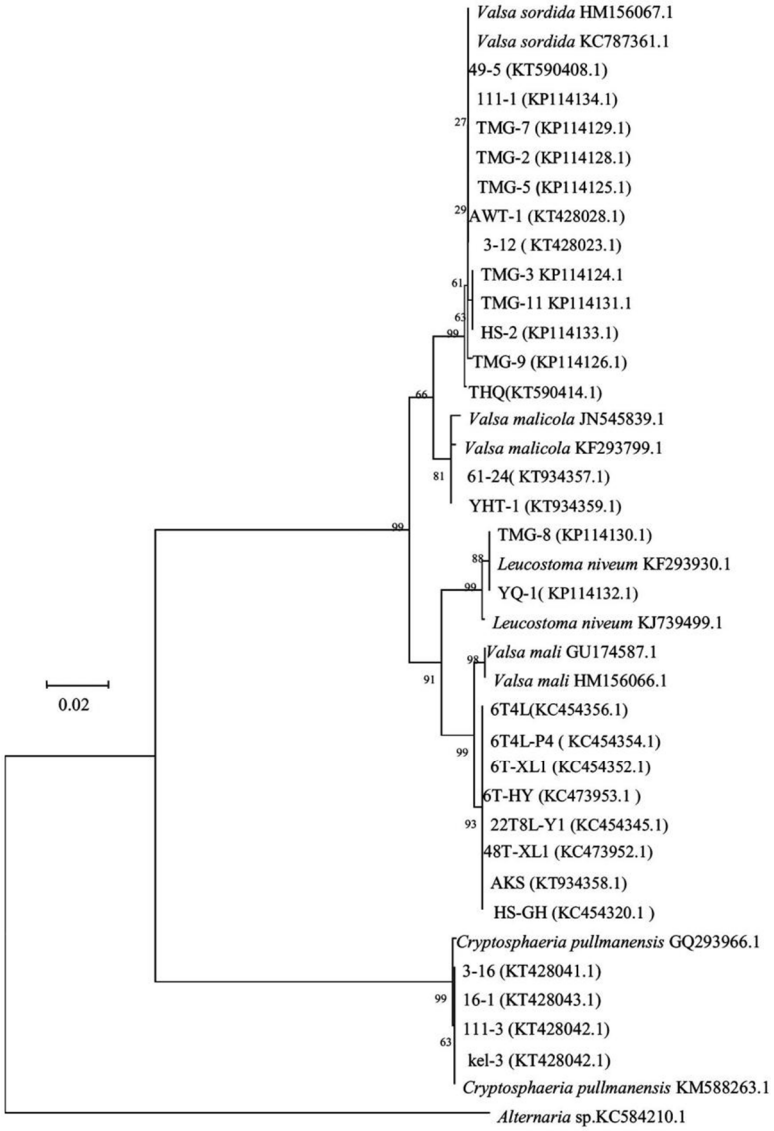

Fig. 6. Phylogram using ITS sequences data of *Valsa* strains.

Koch's rule. These isolated strains were identified as *Valsa mali* (anamorph: *Cytospora sacculus*), *Valsa sordida* (anamorph: *Cytospora chrysosperma*), *Valsa macolica* (anamorph: *Cytospora schulzeri*), *Leucostoma niveum* (anamorph: *Cytospora nivea*) and *Cryptosphaeria pullmanensis* (anamorph: *Cytosporina pullmanensis*), respectively. The *V. mali* and *V. sordida* are main pathogens of economic forest and shelter forest, respectively.

Valsa mali is the most serious and widespread pathogens associated with canker disease on multiple plants. Specially pear (*Pyrus*) and apple (*Malus*) (Wang *et al*. 2011, Suzaki 2008). It was considered a synonym of *Valsa ceratosperma* by Kobayashi and the species concept of

V. ceratosperma was broad from a wide array of host plants (e.g. *Eucalyptus*, *Fagus*, *Malus*, *Quercus*, *Rosa* and *Populus* spp.) distributed worldwide (Kobayashi 1970, Old *et al.* 1991, Adams *et al*. 2005). *Valsa sordida* (anamorph: *Cytospora chrysosperma*) is the causal agent of canker on different species of plant genera, *Populus* and *Salix*, and more rarely on other angiosperms all over the world (Abbasi *et al.* 2015, Kaistn *et al.* 2015). Because of *V. mali* were isolated and identified from *Salix matsudana*, *Populus* spp., it may become a common pathogenic of canker disease transmission between shelter forest and economic forest. At the same time our inoculation pathogenicity tests show that *V. mali* can infect branches of *Salix matsudana*, *Populus* spp., but the pathogenicity is weak.

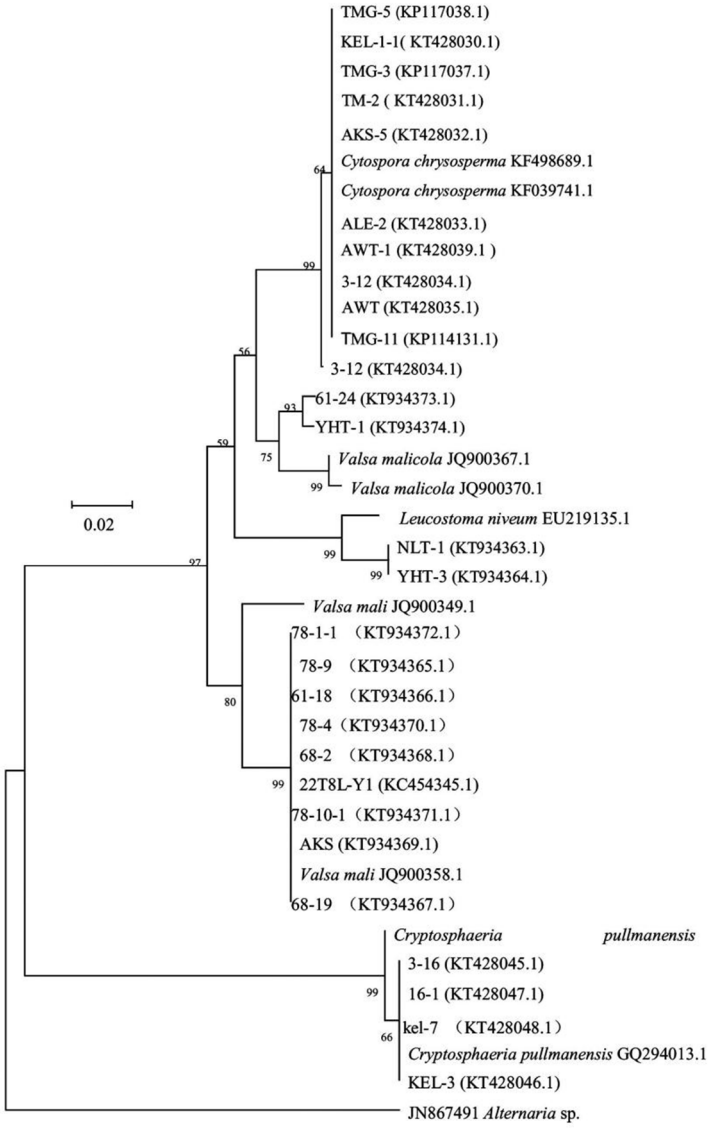

Fig. 7. Phylogram using β-Tubulin sequences data of *Valsa* strains

Fig. 8. Canker symptoms on stems after inoculation *in vitro*. A-H. represent *Malus*, *Pyrus*, *Juglans*, *Populus alba* L., *Salix matsudana*, *Populus euphratica*, *Elaeagnus angustifolia* and *Populus nigra* L., respectively

The present study about *Cytospora* species (Spielman 1985, Adams *et al.* 2005), based on sequences for some genes have substantially elevated identifications over past reports. It has also provided a reproducible method for recognizing distinct species or species aggregates in *Cytospora*. It is also hoped that this will lead to improve management strategies for diseases associated with these fungi.

Acknowledgments

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