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# *Cytospora palm* sp. nov. (Diaporthales, Ascomycota), a canker agent on *Cotinus coggygria* (Anacardiaceae) in Northern China

*Qing-Tao ZHANG[a,b,c], Quan LU[a*], Ming HE[b], Cony DECOCK[d] & Xing-Yao ZHANG[a]*

*[a]Key Laboratory of Forest Protection, State Forestry Administration; Research Institute of Forest Ecology, Environment and Protection, Chinese Academy of Forestry, Beijing, 100091, China*

*[b]School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, 200240, China*

*[c]College of Mechanics, Taiyuan University of Technology, Taiyuan, 030024, China*

*[d]Mycothèque de l'Université catholique de Louvain (BCCM / MUCL), Earth and Life Institute – Microbiology, Croix du Sud 2 bte L7.05.06, B-1348 Louvain-la-Neuve, Belgium*

**Abstract** – *Cytospora palm* sp. nov. is described on *Cotinus coggygria* (Anacardiaceae) in China, on the basis of both morphological and DNA sequence data (ITS and tef1-α). The species is involved in canker disease of *C. coggygria*. Its key diagnostic characters are gregarious, circular, erumpent conidiomata, locules of a rosette cytosporoid type, with invaginations and 7-10 irregularly disposed chambers sharing common walls, emerging beaks, and conidia 4-4.7 × 1-1.5 µm. The optimum temperature for growth and sporulation is approximately 32°C.

**Phylogeny / Sordariomycetes / taxonomy / tree canker / Valsaceae**

## INTRODUCTION

*Cytospora* Ehrenberg (1818) was previously associated with the telemorphic genera *Valsa* Fr., *Leucostoma* (Nitschke) Höhn., *Valseutypella* Höhn and *Valsella* Fuckel. Currently, however, only *Valsa* is considered (Adams *et al.*, 2005). *Cytospora* or its teleomorphic form *Valsa* (Adams *et al.*, 2005) are well-known fungal tree pathogens in family Valsaceae (Ascomycota, Diaporthales) causing mostly canker diseases. The *Index fungorum* (http://www.indexfungorum.org/names/names.asp) lists more than 540 and 550 names of *Cytospora* and *Valsa*, respectively; nevertheless, many names are believed to be synonyms (Adams *et al.*, 2006).

* Corresponding author: Luquan@caf.ac.cn

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As far as their taxonomic and phylogenetic diversity is concerned, *Cytospora*/*Valsa* have received much attention in the last decade. The most recent taxonomic works, including phylogenetic approaches and detailed morphological descriptions of species clades shown by DNA sequence data analysis, include those of Adams *et al.* (2005, 2006) for species growing on *Eucalyptus* (Myrtaceae) in South Africa, Wang *et al.* (2011) for species on *Malus* (Rosaceae) in China, Fotouhifar *et al.* (2010) for species on various angiosperms of which several Salicaceae (*Populus*, *Salix*), Betulaceae (*Alnus*) or Rosaceae (*Malus*) in Iran, and Fan *et al.* (2013) for species on *Sophora* (Fabaceae) in China.

During a continuing survey of *Cytospora*/*Valsa* in China, two specimens of a *Cytospora* species were associated with canker development on *Cotinus coggygria* (Anacardiaceae) in Beijing Xiangshan. Based on disease symptoms, morphological features, and phylogenetic inferences from ITS and tef1-α DNA sequence data, these two specimens were determined to represent an undescribed species.

## MATERIAL AND METHODS

*Cultural and morphological studies.* – The isolates used in this study were obtained from diseased plants originating from Beijing Xiangshan in 2006. Diseased barks and twigs with symptoms of *Cytospora* cankers were removed and brought to the laboratory for further studies. Strains were isolated from the wood tissue, purified, and routinely cultured on PDA at 25°C under alternating 12 h light/dark. Cultures are deposited in the culture collection of Chinese Academy of Forestry (CXY).

Morphology *in vivo*: diagnostic characteristics of the species, whether teleomorphic or anamorphic, are mostly formed on living plants in nature (Adams *et al.*, 2005). Sporulation was induced on the host plant for morphological studies following the methodology of Adams *et al.* (2005). *Cotinus coggygria* leaves were autoclaved for 20 min submerged in water. Two autoclaved leaves were placed on the surface of 2% MEA agar. The isolates were inoculated near the leaves and incubated at room temperature. Leaves colonized by the mycelium were transferred onto water agar, incubated at 24°C in cool white fluorescent light 12 h and 12 h dark (Adams *et al.*, 2005).

Microscopical features: conidiomatal details including their arrangement (single or aggregated), number and size were examined under a LEICA S6D stereomicroscope following the method of Adams *et al.* (2005) after 30 d. Locules, conidiophores, and conidia were examined from sections of 20 µm thick of the conidiomatal stromata. Sections were obtained with a Sectioning Cryostat LEICA CM1900. Photographs were taken by a Ziness Axio Imager A2 m microscope.

Cultural features and growth rates were recorded from cultures grown on PDA in 9 cm glass Petri plates, in dark at the temperatures of 4, 25, 32 and 37°C (Adams *et al.*, 2005). The diameter of the colony was measured daily for 7 d, in $2 \times 3$ replicates.

*Molecular protocols.* – Genomic DNA was extracted from mycelium grown in 100 ml potato-dextrose broth incubated in a shaking incubator at 100 rpm at room temperature for 6-7 days. Mycelia were washed by vacuum

filtration through sterile filter paper and freeze-dried. The freeze-dried mycelium hyphae were ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was extracted following the cetyl trimethyl ammonium bromide (CTAB) method (Hallen *et al.*, 2003). ITS1-5.8S-ITS2 rDNA and elongation factor 1 alpha (tef1-α) sequences were amplified by the primer pairs ITS1/ITS4 (White *et al.*, 1990) and EF1-728F/EF1-986R (Carbone & Kohn 1999), respectively. The PCR products were purified with the TIANquick Midi Purification Kit (TIANGEN BIOTECH, Beijing, China) and directly sequenced with the ABI 3730XL (TIANGEN BIOTECH, Beijing, China). Sequence data for the isolates of the unknown species were deposited in GenBank. Other reference rDNA-ITS and tef1-α sequences included in this study were obtained from GenBank (Table 1).

DNA sequences were aligned with Clustal X 1.83 (Thompson *et al.*, 1997). The alignments are deposited at TreeBASE under the accession number http://purl.org/phylo/treebase/phylows/study/TB2:S15777?x-access-code=7b6fc4860ac0b6903799fa07eef56ca4&format=html.

*Phylogenetic analysis.* – Phylogenetic analysis was performed with Maximum parsimony (MP) and Bayesian approaches. MP analysis was conducted with PAUP 4.0b10 (Swofford 2003). Gaps were treated as missing data. The equally weighted most parsimonious searches were carried out with the heuristic search algorithm with TBR branch swapping and random sequence addition. Topological robustness was assessed through bootstrapping with 1000 replicates (Felsenstein *et al.*, 1985). The species *Phomopsis vaccinii* shear *et al.* was selected as outgroup for both (ITS and tef1-α) analysis, following the results of Adams *et al.* (2005).

Bayesian analysis was carried out with MrBayes 3.1.2 (Huelsenbeck *et al.*, 2001). The best-fit models of nucleotide substitution (GTR+I+G) and (SYM+G) were selected by AIC in MrModeltest 2.3. Two independent runs of Markov chain Monte Carlo (MCMC) with four chains were run 1.000.000 generations. Trees were sampled every 100 generations and 200.000 trees were discarded as burn-in.

*Pathogenicity test.* – Pathogenicity was confirmed by inoculating 20 *Cotinus coggygria* twigs. Twigs were superficially disinfected with 70% ethanol. The bark was superficially scalped so that a rectangular flap was formed that remains attached at one side, exposing inner bark, cambial tissues, and xylem. An agar disc from an actively growing culture was placed beneath the bark flap, the flap pressed and wrapped with tape. Inoculated twigs were incubated at 25°C for 10 days (Scorza & Pusey 1984). The maximum discoloration length was measured after 10 d of incubation. Another two cuttings were treated with water agar as controls. The maximum canker length was considered as an index of pathogenicity. The tests were repeated two times.

## RESULTS

*Phylogeny.* – The length of PCR product for ITS and tef1-α were 566 and 271 bp, respectively. The rDNA-ITS sequences alignment included 649 characters, with 477, 46, and 126, constant, parsimony uninformative, and parsimony informative positions, respectively. The maximum parsimony analysis resulted in a single most parsimonious tree (Fig. 1) with a length (TL) of 377 steps

Table 1. China taxa and reference taxa in this study

| *Taxon* | *Culture* | *Geographic origin* | *Host* | *GenBank* | |
|---|---|---|---|---|---|
| | | | | *ITS* | *EF1-α* |
| *Cytospora palm* sp. nov. | CXY 1276[1] | China | *Cotinus coggygria* | JN402990 | KJ781296 |
| | CXY 1280 | China | *C. coggygria* | JN411939 | KJ781297 |
| *C. abyssinica* | CBS 116189[2] | Ethiopia | *Eucalyptus globulus* | AY347353 | JX438558 |
| | CBS 117605 | Ethiopia | *E. globulus* | AY347352 | – [6] |
| | CBS 117004 | Ethiopia | *E. globulus* | – | JX438559 |
| *C. berkeleyi* | CBS 116823 | USA | *E. globulus* | AY347350 | – |
| | CBS 116825 | USA | *E. globulus* | AY347349 | JX438562 |
| | CBS 116824 | USA | *E. globulus* | – | JX438561 |
| *Valsa fabianae*/ *C. eucalyticola* | CBS 116840 | Australia | *E. nitens* | AY347358 | – |
| | Dunnii | South Africa | *E. dunnii* | AY347360 | – |
| | CBS 116853 | South Africa | *E. saligna* | – | JX438590 |
| | CBS116851 | South Africa | *E. dunnii* | – | JX438591 |
| *C. nitschkii* | CBS 117606 | Ethiopia | *E. globulus* | AY347355 | JX438567 |
| | CBS 116854 | Ethiopia | *E. globulus* | AY347356 | – |
| *C. rhizophorae* | MUCC 302[3] | Australia | *E. grandis* | EU301057 | – |
| | HAB16R14 | Malaysian | | JN083837 | – |
| | ATCC 38475 [4] | USA | *Rhizophora mangle* | – | JX438609 |
| *Leucostoma cinctum* | 292 | Iran | *Armeniaca vulgaris* | EF447407 | – |
| | 299 | Iran | *A. vulgaris* | EF447408 | – |
| | A48 | USA | *Malus domestica* | – | JX438579 |
| | CBS 148.42 | Switzerland | *Larix* sp. | – | JX438580 |
| *L. niveum* | CMW 5274[5] | South Africa | *Populus canescens* | DQ243794 | – |
| | CBS 118561 | South Africa | *Populus simonii* | DQ243795 | – |
| | CBS 109489 | Russia | *Populus* sp. | – | JX438533 |
| | CBS 259.34 | Switzerland | *Populus nigra* | – | JX438532 |
| *L. persoonii* | 209-2 | Iran | *Vitis vinifera* | EF447373 | – |
| | 261 | Iran | *V. vinifera* | EF447375 | – |
| | SXYLt | China | *Prunus persica* | – | JQ900339 |
| | 32-2w | China | *M. domestica* | – | JQ900340 |
| *V. ambiens* | ATCC 52279 | USA | *Acer rubrum* | AY347339 | – |
| | CBS191.42 | Switzerland | *Taxus baccata* | – | JX438576 |
| | CBS 118089 | USA | *Acer* sp. | AY347346 | – |
| *V. cypri* | CBS 201.42 | Switzerland | *Syringa* sp. | DQ243801 | JX438582 |
| | 163 | Iran | *Morus alba* | EF447411 | – |
| | Sterkfontein | South Africa | *Olea europaea* var. *africana* | – | JX463522 |
| *V. malicola* | CBS 118570 | USA | *M. domestica* | DQ243802 | – |
| | CBS 118559 | South Africa | *M. domestica* | DQ243792 | – |
| | 03-7-1 | China | *M. domestica* | – | JQ900337 |
| | SXQS1 | China | *M. domestica* | – | JQ900336 |
| *V. sordida* | CBS 197.50 | UK | *Populus tremula* | AY347322 | – |
| | CMW 5269 | South Africa | *Salix* sp. | AY347324 | – |
| | KepTFR3w_1 | USA | *Populus tremuloides* | – | JX438549 |
| | KepTFR3w_2 | USA | *P. tremuloides* | – | JX438550 |
| *Phomopsis vaccinii* | CBS 160.32 | USA | *Vaccinium macrocarpon* | AF317578 | GQ250326 |

[1] Accession numbers with the prefix CXY are the culture collection of Chinese Academy of Forestry.
[2] Accession numbers with the prefix CBS are Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands.
[3] Accession numbers with the prefix MUCC are Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie Prefecture, Japan.
[4] Accession numbers with the prefix ATCC are from the America Type Culture Collection, Manassas, Virginia, USA.
[5] Accession numbers with the prefix C.M.W. are of the culture collection of M.J. Wingfield at the Tree Protection.
[6] The sequence is not available in GenBank.

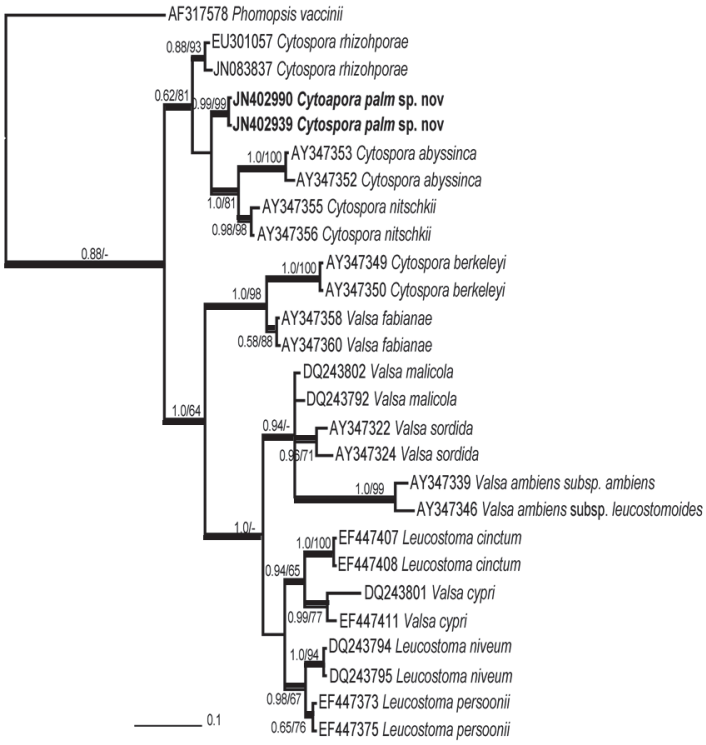


Fig. 1. Phylogenetic tree based on an alignment of the sequences of the ITS regions of *Cytospora*, *Valsa* and *Leucostoma* species, which was generated using the MP method in PAUP and the Bayesian method in MrBayes. Numbers separated by a slash (below or above branches) represent bootstrap values > 50% and posterior probability distributions. The strains obtained in this study are shown in bold.

(consistency index (CI) = 0.6207, retention index (RI) = 0.7951, homoplasy index (HI) = 0.3793, rescaled consistency index (RC) = 0.4935). The topologies of the trees obtained using MP or Bayesian analyses were similar (Fig. 1). Bootstrap and posterior probability values were also mostly congruent in their support for major relationships. The resulting rDNA-ITS tree topology is similar to those of previous studies (Adams *et al.*, 2005, 2006).

The tef1-α alignment contains 370 characters, of which were 79, 31, and 260 constant, parsimony-uninformative and parsimony-informative characters, respectively. The parsimony analysis the tef1-α dataset was performed with 24 taxa and resulted in a single most parsimonious trees (TL = 892, CI = 0.6211, RI = 0.7361, HI = 0.3789, RC = 0.4572). The MP and Bayesian analyses resulted in similar topology (Fig. 2).

The phylogenetic relationships of the species *C. nitschkii*, *C. abyssinica*, *L. cinctum*, *L. niveum*, *L. persoonii*, *V. ambiens*, *V. sordida* and *V. malicola* were similar to previous analyses (Adams *et al.*, 2005, 2006). The sequences of our unidentified species from *C. coggygria* form a distinct, strongly supported clade (BP 99, PP 0.99).

## TAXONOMY

***Cytospora palm*** Q.T. Zhang et X.Y. Zhang **sp. nov.** **Fig. 3a-h**

*Mycobank*: 563349

*Etymology*: the species epithet refers to the conidiomatal shape of the fungus when fused in small groups with beaks.

*Stromata* not observed on natural material.

*Teleomorph* and *anamorph* not observed in nature.

*Colony* on PDA white to grey, mycelium clings to the surface of PDA.

*Temperatures relationships:* colonies mean radial growth on PDA at 4°C of 3 mm 7 days, conidiomata absent; at 25°C of 90 mm 7 days, *conidiomata* present, sporulation medium; at 32°C of 88 mm 7 days, *conidiomata* present, good sporulation; at 37°C of 33 mm 7 days conidiomata formed; poor sporulation.

*Pycnidial conidiomata* formed on the agar, usually single, dark-colored; in *in vitro* culture on sterilized leaves, *pycnidia* single, gregarious, or fused in small groups, dark-colored, circular, erumpent, 1-5 mm diam., with *beaks* emerging up to 1-3 mm above the disc surfaces; *locules* of the rosette cytosporoid type,

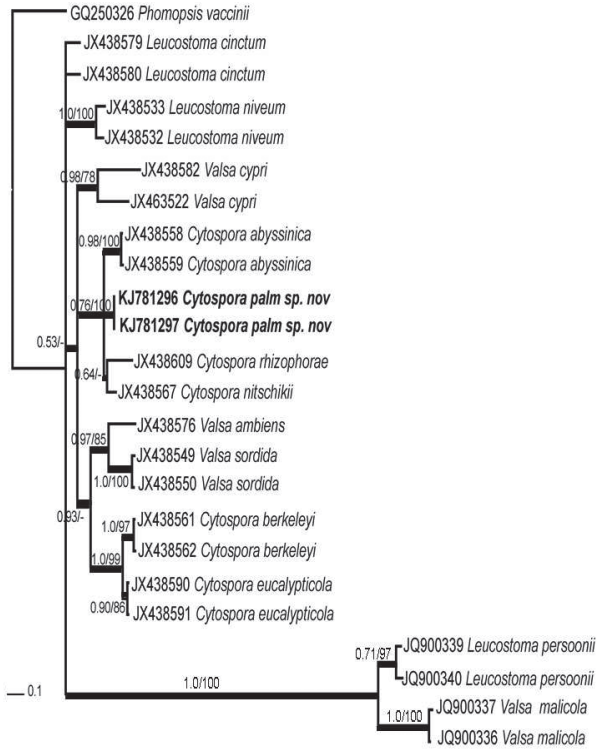


Fig. 2. Phylogenetic tree based on an alignment of the sequences of the tef1-α of *Cytospora*, *Valsa* and *Leucostoma* species, which was generated using the MP method in PAUP and the Bayesian method in MrBayes. Numbers separated by a slash (below or above branches) represent bootstrap values > 50% and posterior probability distributions. The strains obtained in this study are shown in bold.

subdivided by invaginations, with up to 7-10 irregularly arranged chambers sharing common walls.

*Conidiophores* hyaline, unbranched or occasionally branched at base, 8-15 × 1-1.5 µm; *conidiogenous cells* phialidic; *conidia* hyaline, aguttulate, allantoid, aseptate, 4-4.7 × 1-1.5 µm; occasionally exuded in yellow *cirrihi* (Fig. 3h).

*Teleomorph* not observed.

*Known host*: *Cotinus coggygria*

*Known distribution*: Beijing, China

*Material examined*: on twigs of *Cotinus coggygria* (Anacardiaceae), Xiangshan, Beijing, China, 40° 0' 2'' N 116° 11' 42'' E., 50 m asl., 1 May 2006,

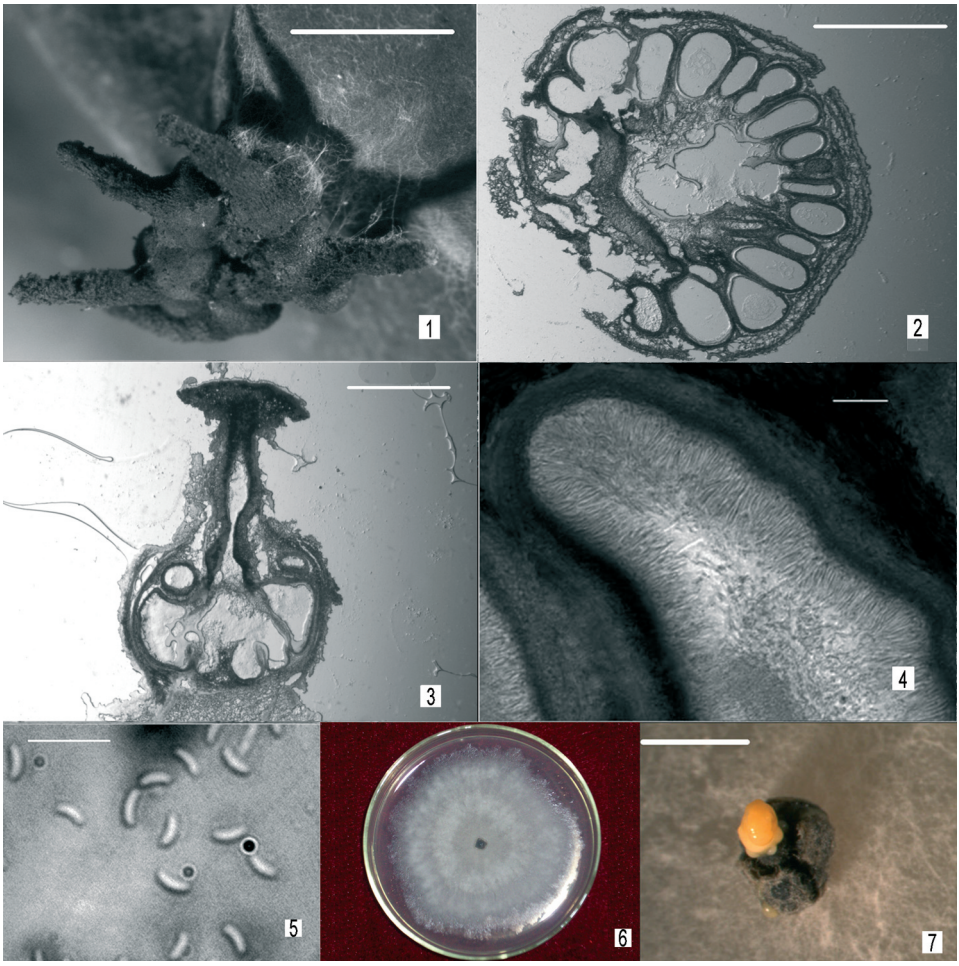


Fig. 3. *Cytospora palm* on *Cotinus coggygria* (from holotype). **1.** Conidiomata produced on PDA; **2.** Horizontal cross section through conidiomatal stroma. **3.** Longitudinal section through conidiomatal stroma. **4.** Sections through locules showing conidiophores in the hymenia. **5.** Conidia. **6.** Colonies on PDA at three days. **7.** Yellow tendril of conidia exuding from conidiomata. Scale bars: 1 = 1 mm, 2-3 = 0.5 mm, 4-5 = 10 µm, 7 = 1 mm.

Xingyao Zhang (CXY1280; holotype); idem, 39° 59' 54'' N 116° 11' 53'' E., 100 m asl., 1 May 2006, Xingyao Zhang (CXY1276).

*Pathogenicity*: After 10 days of incubation, 90% of the infected twigs showed significant brown discoloration of the cambium (n = 18 mean = 1.66 ± 0.39 cm). The cambium appeared water soaked, and released a foul odor. *Cytospora palm* was re-isolated from symptomatic tissues and confirmed by morphology and sequence comparisons. Inoculations were later repeated two times with similar results.

## DISCUSSION

Morphological and ecological features and phylogenetic inferences evidenced a new species of *Cytospora* in Northern China, *Cytospora palm*, causing canker diseases on *Cotinus coggygria*.

Growth temperature relationships are an important physiological characteristic to distinguish *Cytospora* species *in vitro* (Adams *et al.*, 2005). The critical temperatures are 37°C and 32°C (Adams *et al.*, 2005). In our case, our isolates of *C. palm* have their optimum growth temperature at 32°C.

Still, little is known about the biology and ecology of *C. palm*. So far no teleomorph is known. The species is only known from *Cotinus coggygria*; it has not been recorded on other plants co-inhabiting the same locality. The geographic distribution range of *C. palm* remains of course insufficiently documented. *Cotinus coggygria* is widely distributed in China (Zheng & Min 1980) and *C. palm* might follow its host throughout its range.

From a phylogenetic perspective, *C. palm* is genetically distinct from all other *Cytospora* species for which DNA sequence data are available. For the time being, its closest relatives are *C. abyssinica*, *C. nitschikii*, and *C. rhizophorae*. *Cytospora palm* is phylogenetically distantly related to some of the well-known species occurring in China including *V. malicola*, *V. sodida*, and *L. niveum*.

Morphologically, *C. palm* shares similarities with *V. myrtagena*, e.g. the rosette cytosporoid organization of the locules. However, these two species differ in several aspects, including emerging beaks and the conidiogenous cells and conidia. *Cytospora palm* is characterized by beaks 1-3 mm tall *versus* 0.25-0.4 mm in *V. myrtagena*. The conidiophores are occasionally branched and longer (8-15 × 1-1.5 µm) and the conidia are longer (4-4.7 × 1-1.5 µm) in *C. palm* compared to those of *V. myrtagena* (5-7 × 1 µm and 3-4 × 1 µm, respectively, Adams *et al.*, 2005).

Intensive surveys of more ecosystems in China harboring a large tree diversity will certainly lead to the discovery of more *Cytospora* species and perhaps, using phylogenetic methods, new endemic lineages within *Cytospora*.

In the last decades, *Cytospora* has received much attention because of their involvement in canker diseases of many economically important trees. *Cytospora* cankers were found especially damaging on *Malus* spp., *Pyrus* spp., *Prunus* spp. in commercial orchards and on *Picea* spp., *Acer* spp. and *Populus* spp. in forestry (Wang *et al.*, 2011; Adams *et al.*, 2005, 2006). Little is known about host specificity of *Cytospora* species mainly because of poorly defined species concepts (Adams *et al.*, 2006). Whereas the identification of *Cytospora* by the sole means of morphology proved challenging or almost impossible (Adams *et al.*, 2005), the combination of molecular, morphological, and ecological data (host relationships)

greatly helped to clarify the taxonomy of *Cytospora* (Adams *et al.*, 2006). As far as DNA sequence data are concerned, the ITS regions have proved to be reliable for identification in *Cytospora* (Adams *et al.*, 2005, 2006; Wang *et al.*, 2011; Fotouhifar *et al.*, 2010). Our study shows that partial DNA sequence of tef1-α can be an alternative, reliable marker to identify species and to reconstruct phylogenetic relationships.

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