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Lasiodiplodia pseudotheobromae, a new record of pathogenic fungus from some subtropical and tropical trees in southern China

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Abstract – *Lasiodiplodia pseudotheobromae*, a species recently segregated from *L. theobromae*, is reported from southern China for the first time. The identification was based on morphological features and comparison of DNA sequence data from nuc-ITS and partial *tef-1α* gene. *Acacia confusa*, *Albizia falcataria*, *Eucalyptus* sp., *Mangifera sylvatica*, and *Paulownia fortunei* are reported as new hosts for this species. A key to the *Lasiodiplodia* spp. mainly based on the characteristics of conidia and paraphyses is provided.

Forest pathology / tree canker

Résumé – *Lasiodiplodia pseudotheobromae*, une espèce récemment différentiée du complexe *L. theobromae*, est reportée pour la première fois de Chine méridionale. L'identification est basée sur l'analyse morphologique et la comparaison de séquences d'ADN des ITS ribosomaux et du gène *tef-1α*. *Acacia confusa*, *Albizia falcataria*, *Eucalyptus* sp., *Mangifera sylvatica* et *Paulownia fortunei* sont reportées comme nouvelles plantes hôtes pour cette espèce. Une clé d'identification des espèces de *Lasiodiplodia* est proposée.

Pathologie forestière / chancre

INTRODUCTION

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., the anamorph of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx, was a common, pantropical species, reported from about 500 host plants (Punithalingam, 1976). The species was recorded from both healthy, symptomless plant tissues as endophyte (Mohali *et al.*, 2005; Rubini *et al.*, 2005) or as a pathogen causing or associated with damping-off, wilt, die-back, root rot, collar rot, witches' brooms, or fruit rots (Punithalingam, 1976).

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However, as it is the case for other taxa reported from a wide geographical and host range, recent morphological and molecular studies evidenced that, in its common circumscription, *L. theobromae* is a complex of so-called cryptic taxonomic entities. Various cultural (colony morphology, pigment production), physiological (temperature relationships), and morphological features (size, shape, septation of the conidia, or septation of paraphyses) were found to be critical to distinguish closely related species within this complex (Pavlic *et al.*, 2004; Slippers *et al.*, 2004a, b; Phillips *et al.*, 2005; Alves *et al.*, 2006; Burgess *et al.*, 2006).

For instance, Alves *et al.* (2008) distinguished *L. pseudotheobromae* and *L. parva* from *L. theobromae*, on the basis of the conidial size and shape: conidia are larger and distinctly more ellipsoid in *L. pseudotheobromae*, and do not taper towards the base as strongly as in *L. theobromae*, while they are distinctly smaller in *L. parva.* Pavlic *et al.* (2004) and Burgess *et al.* (2006) also used the conidial features to distinguish *L. gonubiensis*, *L. crassispora*, *L. venezuelensis*, and *L. rubropurpurea*.

Some authors emphasized also the importance of paraphyses septation in *Lasiodiplodia* spp. For instance, paraphyses were described as septate in *L. crassispora*, *L. venezuelensis* (Burgess *et al.*, 2006), and *L. parva* (Alves *et al.*, 2008) while they were mostly aseptate in *L. pseudotheobromae* (Alves *et al.*, 2008), and variably described as aseptate (Burgess *et al.*, 2006) or septate (Alves *et al.*, 2008) in *L. theobromae*.

During a survey of *Lasiodiplodia* species in Southern China, several strains isolated from trees apparently healthy or showing canker and dieback symptoms were analyzed. Using both morphological and molecular data, several of these isolates were identified as *L. pseudotheobromae*. This represents so far the first record of this species for China. The Chinese isolates are then described and illustrated in present study.

MATERIALS AND METHODS

Isolates and materials – Six strains of *Lasiodiplodia* sp., originating from the Guangxi, Guangdong, and Chongqing province, in southern China, were used in this study (Table 1). Original strains were isolated from bark tissue during fieldwork on 2% PDA medium (Shuangxuan Bio. Co., Beijing), incubated at 25°C, and later purified in the case of persistent bacterial contamination. Single-conidia strains of all isolates were later obtained in the laboratory and maintained on PDA medium at 25°C. The single-conidia strains as well as the mother isolates are deposited at the Research Institute of Forest Ecology, Environment and Protection, Chinese Academy of Forestry (CAF), Beijing, China (Table 1).

Morphology – Colony morphology, color, and growth rates at t° ranging from between 5-35°C with 10°C interval were determined on 2% PDA medium. Pigment production was also tested at 38°C and 40°C. Pycnidial morphology and color were determined on sterilized pine needles on 2% water agar (WA) medium, at 25°C under near-UV light.

Preliminary identification of the isolates was achieved based on conidial morphology. *In vitro* pycnidia on sterilized pine needles were cut with an American Optical Freezing Microtome. Microscopical observations were carried

Table 1. Isolates included in this study

Culture no.[1]	*Identity*	*Host*	*Location*	*GenBank Accession no.* *rDNA-ITS*	tef-1α
CMW18420	*Lasiodiplodia theobromae*	*Casuarina cunninghamii*	Uganda	DQ103534	DQ103564
CMW18422	*L. theobromae*	*Pinus patula*	Mpumalanga, S. Africa	DQ103544	DQ103562
CMW18423	*L. theobromae*	*P. patula*	Mpumalanga, S. Africa	DQ103545	DQ103563
CMW14077	*L. gonubiensis*	*Syzygium cordatum*	Eastern Cape, S. Africa	AY639595	DQ103566
CMW14078	*L. gonubiensis*	*S. cordatum*	Eastern Cape, S. Africa	AY639594	DQ103567
CMW7773	*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	AY236936	DQ235142
CMW7772	*B. ribis*	*Ribes* sp.	New York, USA	AY236935	AY236877
CMW7774	*B. obtusa*	*Ribes* sp.	New York, USA	AY236953	AY236902
CBS494.78	*L. parva*	Cassava-field soil	Colombia	EF622084	EF622064
CBS456.78	*L. parva*	Cassava-field soil	Colombia	EF622083	EF622063
CBS495.78	*L. parva*	Cassava-field soil	Colombia	EF622085	EF622065
CBS120832	*L. plurivora*	*P. salicina*	Western Cape, S.Africa	EF445362	EF445395
CBS304.79	*L. pseudotheobromae*	*Rosa* sp.	Netherlands	EF622079	EF622061
CBS116459	*L. pseudotheobromae*	*Acacia mangium*	Costa Rica	EF622077	EF622057
CBS447.62	*L. pseudotheobromae*	*Citrus aurantium*	Suriname	EF622081	EF622060
WAC12538	*L. rubropurpurea*	*Eucalyptus grandis*	Tully, Queensland, Australia	DQ103556	DQ103574
WAC12537	*L. rubropurpurea*	*E. grandis*	Tully, Queensland, Australia	DQ103555	DQ103573
CMW13513	*L. venezuelensis*	*A. mangium*	Acarigua, Venezuela	DQ103549	DQ103570
CMW 13512	*L. venezuelensis*	*A. mangium*	Acarigua, Venezuela	DQ103548	DQ103569
CMW7060	*B. stevensii*	*Fraxinus excelsior*	Netherlands	AY236955	AY236904
CBS115038	*B. sarmentorum*	*Malus pumila*	Netherlands	AY573206	AY573223
CMW7780	*B. dothidea*	*F. excelsior*	Switzerland	AY236947	AY236896
CMW8000	*B. dothidea*	*Prunus* sp.	Switzerland	AY236949	AY236898
CBS110492	*L. crassispora*	Unknown	Unknown	EF622086	EF622066
CMW13488	*L. crassispora*	*E. urophylla*	Acarigua, Venezuela	DQ103552	DQ103559
CBS115035	*B. iberica*	*Quercus ilex*	Spain	AY573213	AY573228
CMW26162	*L. margaritacea*	*Adansonia gibbosa*	Washington, USA	EU144050	EU144065
CMW26163	*L. margaritacea*	*A. gibbosa*	Washington, USA	EU144051	EU144066
CXY580	*L. pseudotheobromae*	*Acacia confusa*	Nanning, Guangxi, China	FN645639	FN645643
CXY585	*L. pseudotheobromae*	*Mangifera svlvatica*	Nanning, Guangxi, China	FN645637	FN645642
CXY611	*L. pseudotheobromae*	*Eucalyptus* sp.	Zengcheng, Guangdong, China	FN645638	[2]
CXY616	*L. pseudotheobromae*	*Albizia falcataria*	Zengcheng, Guangdong, China	–	–
CXY617	*L. pseudotheobromae*	*A. falcataria*	Zengcheng, Guangdong, China	FN645641	FN645645
CXY828	*L. pseudotheobromae*	*Paulownia fortunei*	Jiangbei, Chongqing, China	FN645640	FN645644

1. Culture collections: CMW = Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; WAC = Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia; CXY = Culture Collection of Research Group leading by Xing-Yao Zhang, Chinese Academy of Forestry, Beijing, China. 2. The sequence missed because of unsuccessful gene amplification.

out in lactic acid Cotton blue using a Leica DM 6000B light microscope (Leica, Germany). Fifty conidia were measured with SPOT4.0.8 software (Leica, Germany). The standard deviation (95%) was determined.

DNA isolation and amplification – DNA extraction and amplification of the ribosomal ITS regions (including 5.8S) and a fragment of the *tef-1α* were as described in Zhao (2007). The primer pairs ITS1 / LR5 (Crous *et al.*, 2006) and EF1-728F / EF1-986R (Carbone & Kohn, 1999) were used to amplify the ITS region and *tef-1α* gene respectively. The resulting amplificons were cloned into T plasmid vector (Promega, USA) after purification, and then sequenced in ABI Prism 377 autosequencer (PE, USA). The sequences were then deposited in GenBank (Table 1).

Phylogenetic analysis – To compare our *Lasiodiplodia* strains with other *Lasiodiplodia* species, 20 sequences (rDNA-ITS and *tef-1α*) of *Lasiodiplodia* spp. and 8 of *Botryosphaeria* spp. were retrieved from GenBank, and included in a phylogenetic analysis (Table 1). Sequence data were analyzed with MEGA v4.0 (Tamura *et al.*, 2007) using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Some ambiguous sites were manually checked and then adjusted where necessary. The ITS and *tef-1α* alignments combined as a whole dataset were used for phylogenetic analysis. The ITS and *tef-1α* datasets consisted of 535 and 325 characters, respectively, including alignment gaps (data not shown). In the analysis all characters were unweighted and unordered; gaps were treated as missing data.

Phylogenetic analysis were performed using maximum parsimony (MP) method as implemented in PAUP* v4.0b10 (Swofford, 2003) and Bayesian inference (BI) method as implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). The following parameters were used for the MP analyses: Hsearch addseq, Bootstrap, nreps=1000. Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI). Gaps were treated as a fifth character, all ambiguous characters and parsimony uninformative characters were excluded before analysis.

Bayesian analysis was conducted on the same combined datasets as that used in the parsimony analysis. Firstly, Modeltest v.3.7 (Posada & Crandall, 1998) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes applying K80 substitution model with gamma (G) parameters to accommodate variable rates across sites. The following parameters were used: lset nst = 6, rates = invgamma, mcmcp, ngen =10 000 000, nchains = 4, burnin = 10 000. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 90 000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis.

RESULTS

DNA analysis – Preliminary indications of the relationships of our *Lasiodiplodia* isolates were obtained using a BLAST search at GenBank (Altschul *et al.*, 1997). The search using the ITS and the fragment of the *tef-1α* demonstrated homology

with members of *Lasiodiplodia*, *L. pseudotheobromae* having the highest similarity score (100%). Importantly, the Chinese isolates had 100% similitude with the type strain of *L. pseudotheobromae* for both the ITS and *tef-1α* sequences.

Subsequently, a combined ITS - *tef-1α* data set was built with all the *Lasiodiplodia* species available at GenBank, *viz. L. crassispora*, *L. gonubiensis*, *L. margaritacea*, *L. parva*, *L. plurivora*, *L. pseudotheobromae*, *L. rubropurpurea*, *L. theobromae* and *L. venezuelensis*.

The combined dataset contained 860 characters, of which 243 were parsimony informative, 21 were variable and parsimony uninformative, and 596 were constant. Maximum parsimony analysis of the combined dataset yielded two equally most parsimonious trees. The trees differed only in the terminal arrangement of taxa and showed a topology identical to the 50% majority-rule consensus bootstrap tree. In addition, the 50% majority-rule consensus tree of 90,000 trees sampled during the Bayesian analysis was identical in topology. One of the two most parsimonious trees is presented in Fig. 1 with bootstrap support and Bayesian posterior probabilities reported above the node.

Two main clades were resolved, distinguishing species with a *Diplodia* or *Lasiodiplodia* anamorph. Within the *Lasiodiplodia* clade, the analysis recovered the different clades corresponding to the 9 species included (Fig. 1).

All the isolates originating from southern China were placed within the *L. pseudotheobromae* clade together with the ex-type strain (CBS116459).

DESCRIPTION

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28: 1-13, 2008. Fig. 2-13

Growth rate – Colony diameter on PDA after 72 h in darkness, 25-36 mm at 15°C, ≥ 90 mm at between 25°C and 35°C, 46-60 mm at 40°C. No growth on PDA at 5°C. The optimal temperature for fungal isolates growth is 35°C.

Colony feature – Colonies on PDA grey to black with dense aerial cottony mycelium. All isolates produce a pink pigment in PDA cultures when incubated at temperature ≥ 35°C.

Conidiomata pycnidial, uni- to multilocular on PDA and sterile pines needles, grey or brown, thick-walled with a textura angularis, the wall formed of dark brown thick-walled cells; *ostiole* central, single, papillate; *paraphyses* hyaline, flexuous, cylindrical, aseptate, ends rounded, sometime branched, up to 60 µm long; *conidiophores* reduced to conidiogenous cells; *conidiogenous cells* hyaline, smooth, cylindrical to sub-obpyriform, holoblastic, discrete, determinate or indeterminate and proliferating percurrently with one or two distinct annelations, formed from cells lining the inner wall of the pycnidium; *conidia* obovoid, broad oblong, straight, thick wall, at first hyaline and aseptate, 18-30 × 8-15 µm; immature conidia could remain a long time, ultimately becoming dark brown and one-septate with irregular longitudinal striations, broadly rounded at apex, truncate at base, 22~30 × 13~17 µm, mean and standard deviation of 50 conidia = 26.2 ± 1.4 × 15 ± 0.5 µm, length/width ratio = 1.9 ± 0.1.

Teleomorph - unknown.

Hosts plants - On bark with canker symptom of the species *Acacia confusa* Merr., *Albizia falcataria* (L.) Fosberg, *Mangifera sylvatica* Roxb., and *Paulownia fortunei* (Seem.) Hemsl., and of healthy *Eucalyptus* spp.

Known distribution in China - Guangxi, Guangdong, and Chongqing Province.

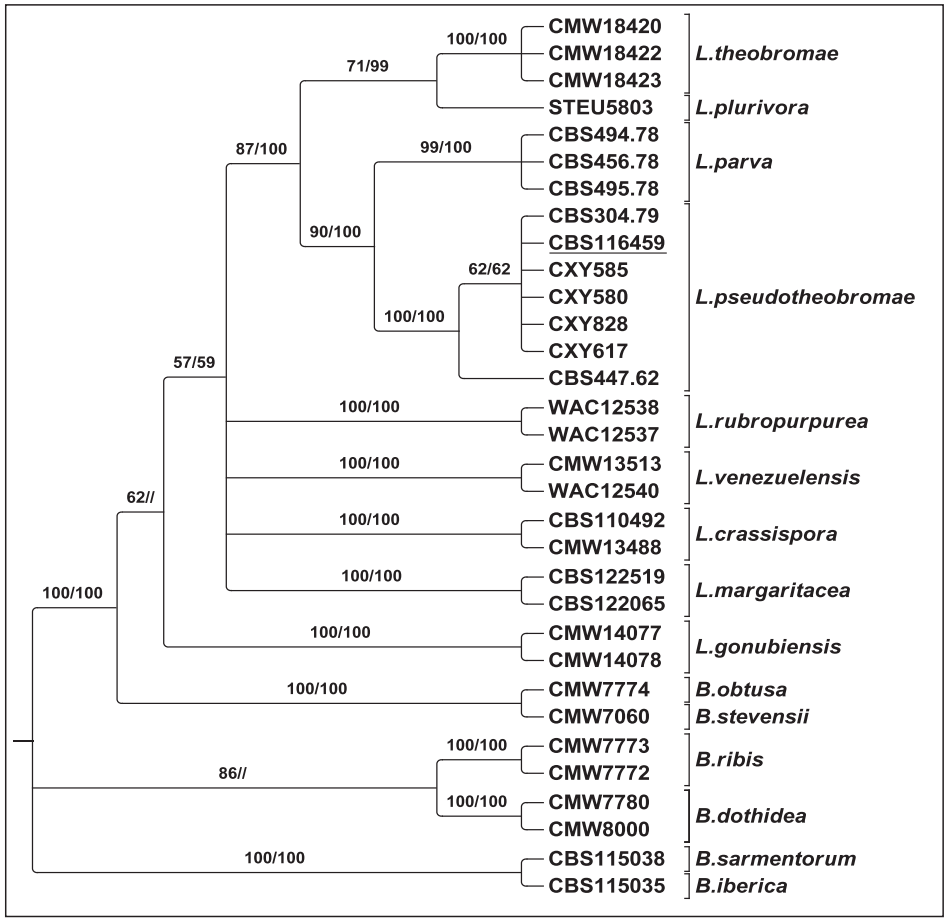

Fig. 1. Most parsimonious tree with 50% majority-rule consensus bootstrap obtained from the combined analysis of ITS and *tef-1α* DNA-sequence data (tree length = 449 steps; CI = 0.7817; RI = 0.8951; RC = 0.6997; HI = 0.2183). Numbers above the node represent the bootstrap/ Bayesian posterior probabilities. The isolate CBS116459 is the ex-type of *L. pseudotheobromae*.

DISCUSSION

Lasiodiplodia pseudotheobromae was recently segregated from the *L. theobromae* complex on the basis of several isolates obtained from various host plants (*Acacia mangium, Citrus aurantium*, *Coffea* sp., *Gmelina arborea*, and *Rosa* sp.) (Alves *et al.*, 2008). Its geographical distribution and host plant range are far to be completely known, however.

The analysis of several Chinese *Lasiodiplodia* isolates, using both morphological features and molecular data, confirmed for the first time the presence of this species in its southern, tropical to subtropical areas. The host plants *Acacia confusa*,

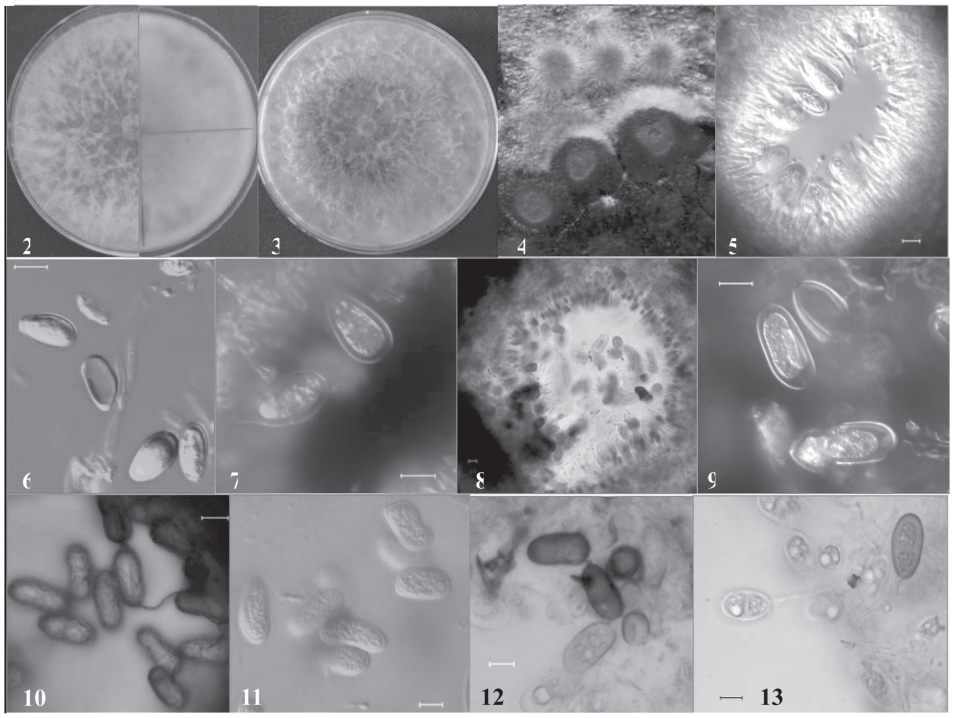

Fig. 2-13. Morphological characteristics of *Lasiodiplodia pseudotheobromae* CXY828. 2, colony on PDA at 25°C; 3, colony on PDA at 38°C, show pink pigment produced; 4, pycnidium; 5, paraphyses, immature conidia and conidiogenous cell; 6, branched paraphyses and conidia; 7, conidiogenous cell and conidia; 8-11, conidia; 12-13, matured dark brown conidia with 0 or 1 septate. Bars = 10 μm.

Albizia falcataria, *Mangifera sylvatica*, and *Paulownia fortunei*, all with bark canker, are reported as new hosts for the fungus. In addition, the species was also isolated from the bark of symptomless *Eucalyptus* sp.

Lasiodiplodia theobromae was widely reported in China (Zhao 2007). However, reports of that species should be carefully revised as they might represent in fact one of the other species recently described (Pavlic *et al.*, 2004; Alves *et al.*, 2006; Burgess *et al.*, 2006). In all probability, the host range *L. pseudotheobromae* in China (or elsewhere) will reveal to be more diverse still.

In order to facilitate the recognition of species within the complex, a key to the known *Lasiodiplodia* species is provided here. It is mainly based on the septation of conidia and paraphyses characteristics (Pavlic *et al.*, 2004; Burgess *et al.*, 2006; Damm *et al.*, 2007; Alves *et al.*, 2008; Pavlic *et al.*, 2008).

KEY TO *LASIODIPLODIA* SPECIES

1. Mature conidia 1-septate 2
1. Mature conidia 1-3-septate . . . . . *L. gonubiensis*

2. Conidia mostly globose, subglobose to obovoid, l/w < 1.5 . . . . . *L. margaritacea*
2. Conidia mostly ellipsoid to obovoid, l/w > 1.5 3

3. Paraphyses on average > 50 µm long. 4
3. Paraphyses on average < 50 µm long. 7

4. Paraphyses 130 µm long . . . . . *L. plurivora*
4. Paraphyses 50-130 µm long. 5

5. Paraphyses septate 6
5. Paraphyses mostly aseptate. . . . . *L. pseudotheobromae*

6. Pink pigment not produced on PDA at temperature higher than 35°C. . . . . *L. theobromae*
6. Pink pigment produced on PDA at temperature higher than 35°C . . . . *L. parva*

7. Paraphyses aseptate, pycnidia fluffy and reddish-purple . . . . . *L. rubropurpurea*
7. Paraphyses septate. 8

8. Immature conidia thin-walled (< 2 µm); mature conidia narrowly striated . . . . . *L. venezuelensis*
8. Immature conidia thick-walled (> 2 µm); mature conidia widely striated . . . . . *L. crassispora*

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