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An Emerging Pathogen from Rotted Chestnut in China: *Gnomoniopsis daii* sp. nov.

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Abstract: Nut quality is fundamental to the economic viability of the Chinese sweet chestnut industry, but fruit rot disease significantly reduces this quality. In this study, we investigated chestnut rot in Anhui and Hubei provinces in China. Typical brown rot symptoms were observed, affecting nuts from different plantations. Isolates were obtained from symptomatic tissues of rotted fruits that were identified based on morphological comparison and phylogenetic analyses of partial internal transcribed spacer (ITS), and *tef1* and *tub2* gene sequences. The inoculation results showed that the tested fungal species is pathogenic to chestnut fruits. Hence, a new and severe pathogen that causes Chinese sweet chestnut brown rot, *Gnomoniopsis daii* sp. nov., is introduced herein.

Keywords: *Castanea mollissima*; fruit disease; plant pathology; taxonomy

1. Introduction

China has the largest chestnut industry in the world, producing more than 2×10^6 tons of chestnuts annually since 2013 [1]. The Chinese sweet chestnut (*Castanea mollissima* Bl.) is widely cultivated in most provinces in China, providing gluten-free, low fat, and cholesterol-free crop nuts for human consumption [2]. Chestnut orchards and stands are also important to the economy as sources of timber [3].

Traditionally, several pathogens were considered the causal agents of chestnut rot in China, including *Alternaria* Nees, *Botryosphaeria* Ces. & De Not., *Colletotrichum* Corda, *Diaporthe* Nitschke, *Fusarium* Link, and *Penicillium* Link species [4–7]. However, no detailed studies on these pathogens have been conducted in China in the past decade. European sweet chestnut (*Castanea sativa* Mill.), known as one of the four major chestnut species in the world, has been well studied in nut rot by several phytopathologists and taxonomists [8–15]. Several important fungal species, *Cryphonectria parasitica* M.E. Barr, *Gnomoniopsis smithogilvyi* L.A. Shuttlew., E.C.Y. Liew & D.I. Guest (syn. *G. castaneae* Tamietti), *Phytophthora cinnamomi* Rands, and *Sirococcus castanea* J.B. Mey., Senn-Irlet & T.N. Sieber, have been reported on *Castanea sativa* from Australia and Europe [16–20].

The genus *Gnomoniopsis* Berl. (Gnomoniaceae G. Winter, Diaporthales Nannf.) was first described as a subgenus within *Gnomonia* Ces. & De Not. for species having ascospores that develop additional septa [21]. However, the development of additional septa was thought to be an occasional occurrence; *Gnomoniopsis* was subsequently proposed as a synonym of *Gnomonia* [22]. Sogonov et al. reevaluated concepts of the leaf-inhabiting genera in Gnomoniaceae based on the DNA sequence data of these genera, and restricted the genus *Gnomoniopsis* to *G. chamaemori* Berl. (type), *G. comari* Sogonov, *G. fructicola* Sogonov, *G. macounii* Sogonov, *G. paraclavulata* Sogonov, *G. racemula* Sogonov, and *G. toementillae* Sogonov [21]. Subsequently, nine additional species were added to this genus [23]. *Gnomoniopsis castaneae* and *G. smithogilvyi* were described independently from Europe and Australia, but Shuttleworth

et al. proved that both names refer to a single species based on a comparative morphological analysis and five-marker phylogenetic analysis [18].

During the surveys of chestnut rot conducted in Anhui and Hubei provinces in China, typical brown rot symptoms were observed (Figure 1). Our aim in this study was to identify pathogens associated with chestnut brown rot in China. We conducted pathogenicity tests on healthy nuts to assess their pathogenicity.

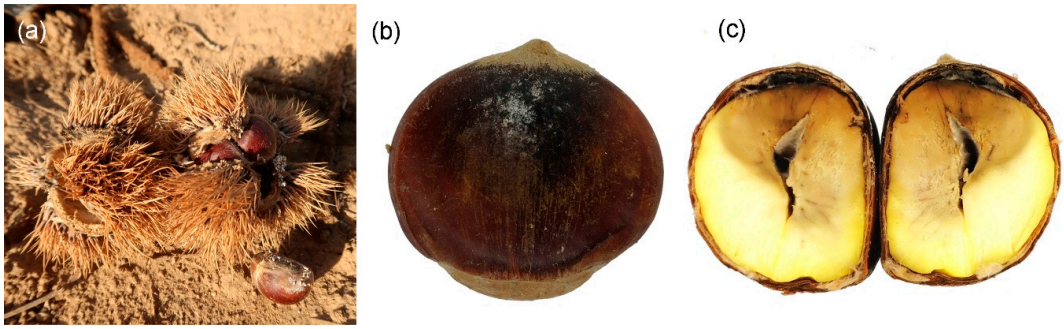

Figure 1. Symptoms of chestnut brown rot: (**a**,**b**) diseased nuts and (**c**) discolored kernels.

2. Materials and Methods

2.1. Sample Collection and Isolation

Anhui and Hubei provinces are two important chestnut production bases in China. Samples were randomly collected in local storehouses from different chestnut plantations after harvest, then packed in paper bags, and posted to the laboratory for further study. Rotted chestnuts were surface-sterilized for 1 min in 75% ethanol, 3 min in 1.25% sodium hypochlorite, and 1 min in 75% ethanol, then rinsed for 2 min in sterile water and blotted on dry sterile filter paper. Infected nut tissues were cut into small pieces (0.2 cm × 0.2 cm) using a sterile scalpel and transferred onto the surface of malt extract agar (MEA; 30 g malt extract, 5 g peptone, 15 g agar/L; Aobox Company Limited, Beijing, China). After inoculation, agar plates were left at 25 °C in the dark for 2 days. Then, single hyphal strands were transferred to fresh medium plates under a dissecting stereomicroscope with a sterile needle. Specimen of the new species was deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC). The ex-type culture was maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

2.2. DNA Extraction and Phylogenetic Analysis

Genomic DNA was extracted from 15-day-old mycelium grown on MEA using the CTAB (cetyltrimethylammonium bromide) method [24]. DNA sequences were generated for the internal transcribed spacer (ITS) regions including the *5.8S* gene of the ribosomal RNA operon amplified with primers ITS1/ITS4 [25], the translation elongation factor 1a (*tef1*) amplified with primers EF1-728F/EF1-1567R [26], and the b-tubulin gene 2 (*tub2*) amplified with primers T1/Bt2b [27]. The PCR conditions were: initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 50 s at 48 °C (ITS) or 54 °C (*tef1*) or 52 °C (*tub2*), and 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. The PCR amplification products were scored visually by electrophoresis in 2% agarose gel. The DNA sequencing was performed using an ABI Prism 3730xl DNA Analyzer (ABI, Foster City, CA, USA) with Big-Dye Terminator kit v.3.1 (Invitrogen, Beijing, China) at Shanghai Invitrogen Biological Technology Co. Ltd. (Beijing, China).

Sequences of the three individual loci (ITS, *tef1* and *tub2*) were aligned and edited manually using MEGA6 (Table 1). Maximum likelihood (ML) analysis was used for phylogenetic inferences of the concatenated alignments. ML analysis was implemented on the CIPRES Science Gateway portal using RAxML-HPC BlackBox v. 8.2.10 based on single ITS and combined sequences of ITS, *tef1*, and *tub2* [28]. The resulting trees were plotted using FigTree v. 1.4.2.

Table 1. Isolates and GenBank accession numbers used in this study.

Species	Country	Host	Strain	GenBank Accession Number		
				Internal Transcribed Spacer (ITS)	*tub2*	*tef1*
Apiognomonia veneta	France	*Platanus occidentalis*	CBS 342.86	DQ313531	EU219235	DQ318036
Gnomoniopsis alderdunensis	USA	*Rubus pedatus*	CBS 125679	GU320826	GU320788	GU320813
Gnomoniopsis alderdunensis	USA	*Rubus parviflorus*	CBS 125680	GU320825	GU320787	GU320801
Gnomoniopsis alderdunensis	USA	*Rubus parviflorus*	CBS 125681	GU320827	GU320789	GU320802
Gnomoniopsis chamaemori	Finland	*Rubus chamaemorus*	CBS 804.79	GU320817	GU320777	GU320809
Gnomoniopsis clavulata	USA	*Quercus falcata*	CBS 121255	EU254818	EU219211	GU320807
Gnomoniopsis comari	Finland	*Comarum palustre*	CBS 806.79	EU254821	EU219156	GU320810
Gnomoniopsis comari	Finland	*Comarum palustre*	CBS 807.79	EU254822	GU320779	GU320814
Gnomoniopsis comari	Switzerland	*Comarum palustre*	CBS 809.79	EU254823	GU320778	GU320794
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF002A**	**MN598671**	**MN605519**	**MN605517**
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF002B**	**MN598672**	**MN605520**	**MN605518**
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF095**	**MN598673**	**NA**	**NA**
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF098**	**MN598674**	**NA**	**NA**
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF099**	**MN598675**	**NA**	**NA**
Gnomoniopsis daii	**China**	***Castanea mollissima***	**CMF116**	**MN598676**	**NA**	**NA**
Gnomoniopsis fructicola	USA	*Fragaria vesca*	CBS 121226	EU254824	EU219144	GU320792
Gnomoniopsis fructicola	France	*Fragaria* sp.	CBS 208.34	EU254826	EU219149	GU320808
Gnomoniopsis fructicola	USA	*Fragaria* sp.	CBS 125671	GU320816	GU320776	GU320793
Gnomoniopsis guttulata	Bulgaria	*Agrimonia eupatoria*	NA	EU254812	NA	NA
Gnomoniopsis idaeicola	USA	*Rubus* sp.	CBS 125672	GU320823	GU320781	GU320797
Gnomoniopsis idaeicola	USA	*Rubus pedatus*	CBS 125673	GU320824	GU320782	GU320798
Gnomoniopsis idaeicola	France	*Rubus* sp.	CBS 125674	GU320820	GU320780	GU320796
Gnomoniopsis idaeicola	USA	*Rubus procerus*	CBS 125675	GU320822	GU320783	GU320799
Gnomoniopsis idaeicola	USA	*Rubus procerus*	CBS 125676	GU320821	GU320784	GU320811
Gnomoniopsis macounii	USA	*Spiraea* sp.	CBS 121468	EU254762	EU219126	GU320804
Gnomoniopsis occulta	USA	*Potentilla* sp.	CBS 125677	GU320828	GU320785	GU320812
Gnomoniopsis occulta	USA	*Potentilla* sp.	CBS 125678	GU320829	GU320786	GU320800
Gnomoniopsis occulta	Russia	*Potentilla anserina*	NA	EU254811	NA	NA
Gnomoniopsis paraclavulata	USA	*Quercus alba*	CBS 123202	GU320830	GU320775	GU320815
Gnomoniopsis racemula	USA	*Chamerion angustifolium*	CBS 121469	EU254841	EU219125	GU320803
Gnomoniopsis sanguisorbae	Switzerland	*Sanguisorba minor*	CBS 858.79	GU320818	GU320790	GU320805
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130190	JQ910642	JQ910639	KR072534
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130189	JQ910644	JQ910641	KR072535
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130188	JQ910643	JQ910640	KR072536
Gnomoniopsis smithogilvyi	Italy	*Castanea sativa*	MUT 401	HM142946	KR072532	KR072537
Gnomoniopsis smithogilvyi	New Zealand	*Castanea sativa*	MUT 411	HM142948	KR072533	KR072538
Gnomoniopsis tormentillae	Switzerland	*Potentilla* sp.	CBS 904.79	EU254856	EU219165	GU320795
Sirococcus castaneae	Switzerland	*Castanea sativa*	CBS 142041	KX929744	KX958443	KX929710

Note: NA, not applicable. Strains in this study are identified in bold.

2.3. Morphological Identification and Characterization

Morphological descriptions of the new *Gnomoniopsis* species were based on cultures sporulating on MEA in the dark at 25 °C after a month. Micromorphological features were observed under a Leica compound microscope (DM 2500, Leica, Wetzlar, Germany). More than 50 conidia were randomly selected for measurement. Cultural characteristics of isolates incubated on MEA were observed and recorded, including colony color and texture.

2.4. Pathogenicity Trials

Four isolates representing *Gnomoniopsis daii* were selected for inoculations, viz., CMF002A (ex-type from Anhui province), CMF002B (from Anhui province), CMF095 (from Hubei province), and CMF098 (from Hubei province). Isolates were allowed to grow on MEA for one week at 25 °C before the tests. We collected 160 asymptomatic nuts from a chestnut orchard in Anhui province, and 10 of them were randomly chosen and dissected to confirm healthy status. The remaining 150 chestnuts

were surface-sterilized for 1 min in 75% ethanol, 3 min in 1.25% sodium hypochlorite, and 1 min in 75% ethanol, then rinsed for 2 min in sterile water and blotted on dry sterile filter paper. Using a cork borer (7 mm diameter), we wounded the nuts by removing the seed coat to expose the seed. Same-sized gar discs were removed from the actively growing margins of cultures and placed into the wounds with the mycelium facing the exposed seed. Sterile MEA discs were used for the negative controls. Wounds with the inoculated mycelium or sterile MEA were covered with masking tape to prevent contamination and desiccation. We ran 30 replicates for each strain and negative control. These inoculated nuts were maintained in a greenhouse at 25 °C. After 15 days, all the replicates were examined for disease, and re-isolations were conducted for all the symptomatic nuts.

3. Results

3.1. Fungal Isolation and Identification

Most of the pieces from infected nut tissue yielded a fungus, and 125 isolates were obtained. The isolates were primarily identified based on the morphology of conidia formed on the plates and ITS sequences. As a result, four isolates were *Alternaria*, six isolates were *Botryosphaeria*, 53 isolates were *Colletotrichum*, seven isolates were *Diaporthe*, 42 isolates were *Gnomoniopsis*, 11 isolates were *Fusarium*, and two isolates were *Penicillium*. Only one fungus was obtained from one rotted chestnut. For the first time in China, *Gnomoniopsis* isolates were obtained from rotted chestnut. Hence, detailed studies on them were conducted during the present study.

3.2. Phylogeny

To identify the phylogenetic position of our isolates within *Gnomoniopsis*, phylogenetic analyses were performed based on ITS and combined ITS, *tef1*, and *tub2* sequence data. The ITS alignment contained 38 sequences (including one outgroup) with 542 characters including alignment gaps. Of these, 428 characters were constant, 34 were variable and parsimony-uninformative, and 80 were parsimony-informative. The six *Gnomoniopsis* strains from this study form a well-supported clade distinguished from known species (Figure 2). The combined ITS, *tef1*, and *tub2* alignment contained 38 sequences (including one outgroup) and 1685 characters including alignment gaps; 960 of these were parsimony-informative, 174 were variable and parsimony-uninformative, and 551 were constant. A similar phylogram was obtained from multi-genes to single ITS (Figure 3), which indicated strains from this study as a new *Gnomoniopsis* species. All 42 *Gnomoniopsis* isolates were identical in our primary comparison; hence, the six are shown in Figures 2 and 3.

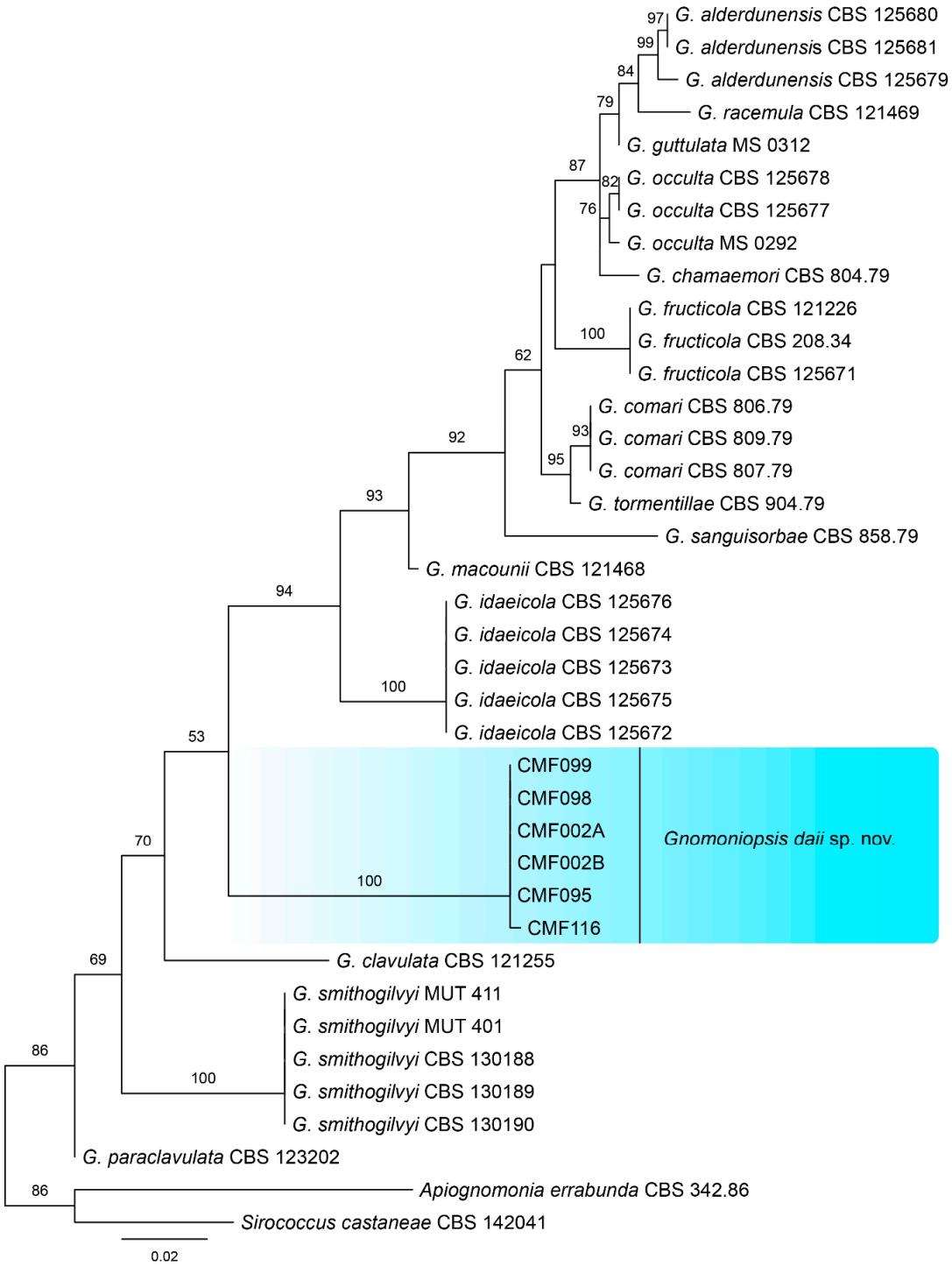

Figure 2. Consensus tree resulting from a RAxML analysis of ITS sequence alignment for species of *Gnomoniopsis*. The scale bar represents the expected number of changes per site.

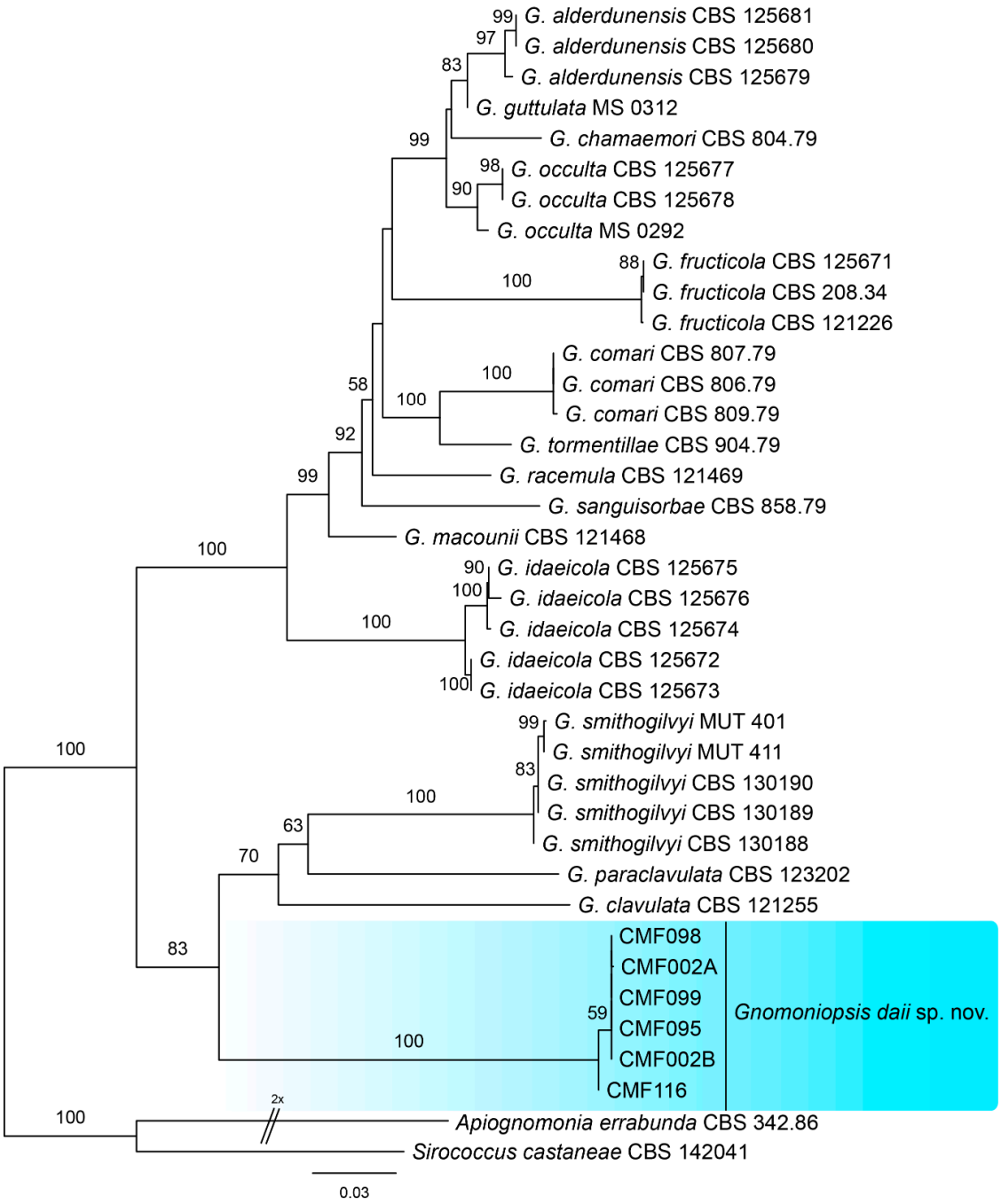

Figure 3. Consensus tree resulting from a RAxML analysis of combined ITS, *tef1*, and *tub2* sequence alignment for species of *Gnomoniopsis*. The scale bar represents the expected number of changes per site.

3.3. Morphology and Taxonomy

Gnomoniopsis daii C.M. Tian & N. Jiang, sp. nov. (Figures 4 and 5)

MycoBank MB 833088

Holotype: BJFC-C005

Etymology: in honor of Fanglan Dai, who is one of the most famous Chinese taxonomists.

Host/Distribution: on rotted *Castanea mollissima* fruits in China.

Original description: Colonies on MEA attaining 60 mm in one week at 25 °C, with undulate margin, whitish; after one month at 25 °C, light orange to white conidiomata distributed irregularly on the surface. Pycnidia globose to oval, solitary or confluent, light orange to white, 150–950 µm

diameter; conidiophores indistinct, often reduced to conidiogenous cells. Conidiogenous cells oval, hyaline, one-celled, 5–18 µm. Conidia oval, oblate, fusiform, straight to slightly curved, hyaline, finely guttulate or not, (5.0–)5.5–7.0(–8.0) × 2.0–3.5 µm.

Material examined: CHINA, Anhui province, Liuan city, on rotted fruits of *Castanea mollissima*, Ning Jiang and Chengming Tian, 7 October 2017 (BJFC-C005 holotype; ex-type culture, CMF002A = CFCC 54043); Liuan city, on rotted fruits of *Castanea mollissima*, Ning Jiang and Chengming Tian, 7 October 2017 (living culture, CMF002B). Hubei province, Huanggang city, on rotted fruits of *Castanea mollissima*, Ning Jiang and Chengming Tian, 20 September 2019 (living culture, CMF095, CMF098, CMF099, CMF116).

Notes: *Gnomoniopsis daii* has similar conidia to *G. smithogilvyi* (5.0–8.0 × 2.0–3.5 µm in *Gnomoniopsis daii* vs. 6.1–9.8 × 2.4–4.9 µm in *G. smithogilvyi*), but they are different in host species and distribution (*Gnomoniopsis daii* on *Castanea mollissima* in China vs. *G. smithogilvyi* on *Castanea sativa* in Europe) [18]. They are obviously separated in the phylogram base on ITS (Figure 2) and combined ITS, *tef1*, and *tub2* (Figure 3).

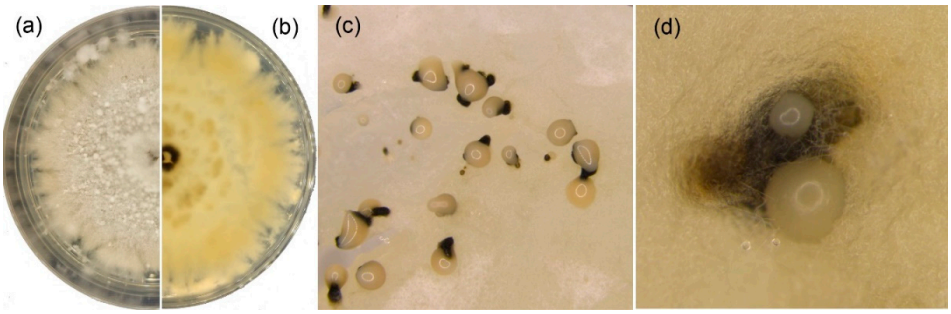

Figure 4. *Gnomoniopsis daii* (BJFC-C005 from CMF002A) cultures on MEA. (**a**,**b**) Colony on MEA after 15 days at 25 °C; (**c**,**d**) conidiomata formed on MEA.

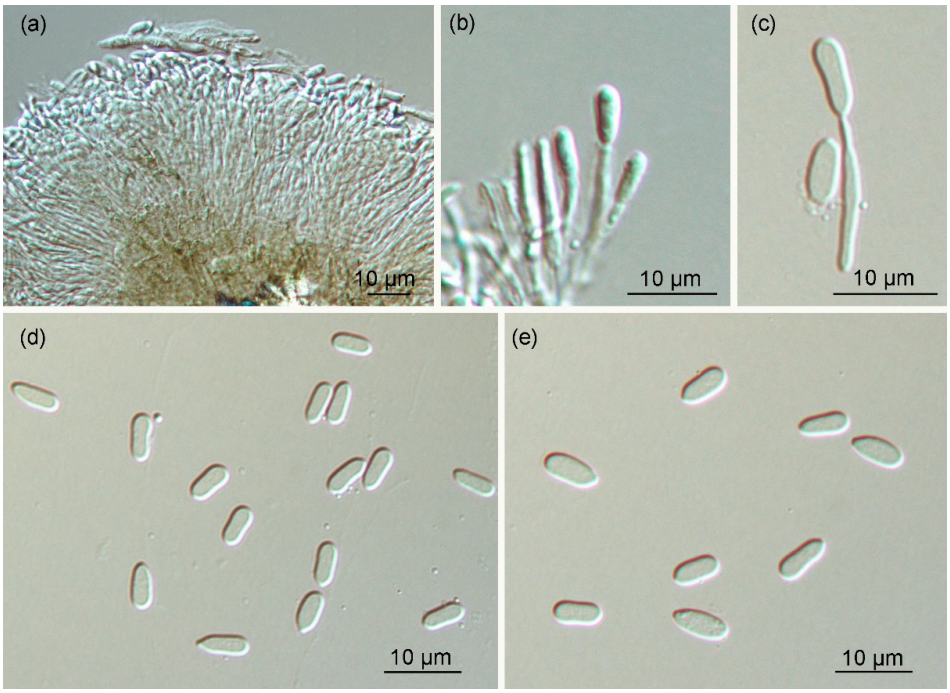

Figure 5. Morphology of *Gnomoniopsis daii* (BJFC-C005 from CMF002A): (**a**–**c**) conidiogenous cells with attached conidia and (**d**,**e**) conidia.

3.4. Pathogenicity Trials

All 10 nuts assayed to test their health were found to be intact. The four tested strains showed brown rot symptoms and were detected in 83% of the artificially infected nuts (Figure 6). No obvious differences were found among the four strains (Table 2). Re-isolates were obtained from affected nuts and identified based on ITS sequence, which were all *Gnomoniopsis daii*. The asymptomatic nuts and negative controls did not show any symptoms.

Table 2. Results of pathogenicity trials.

Strain	No. of Affected Nuts	No. of Asymptomatic Nuts
CMF002A	26	4
CMF002B	24	6
CMF095	22	8
CMF098	23	7

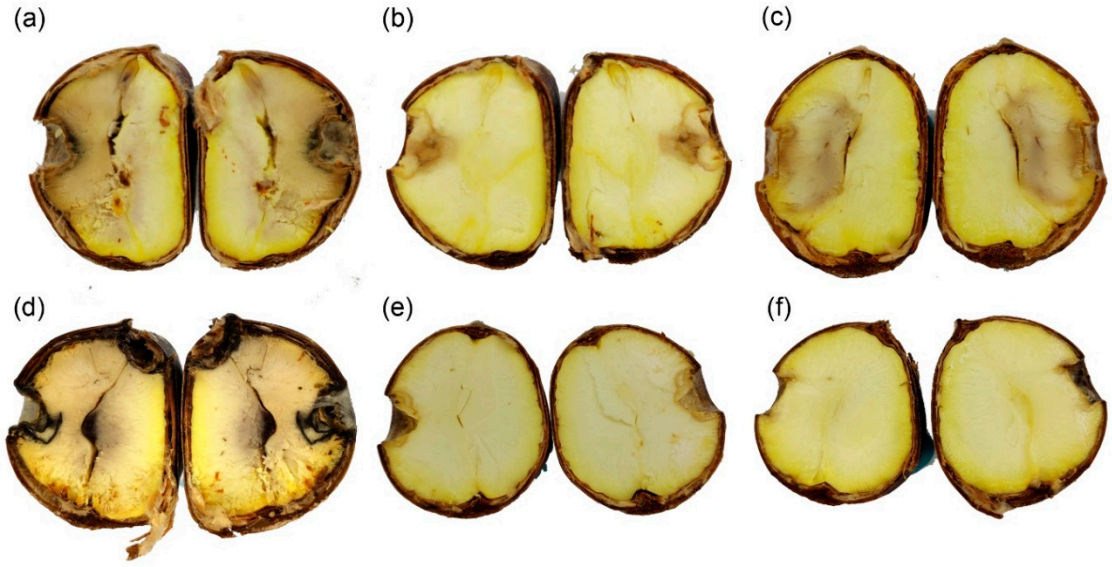

Figure 6. Results of pathogenicity trials after 15 days: (**a–d**) Mild to severe symptoms caused by CMF002A (ex-type), (**e**) asymptomatic chestnut after incubation, and (**f**) negative control.

4. Discussion

In this paper, *Gnomoniopsis daii* is introduced as a new species in the genus *Gnomoniopsis*. This species was found to be an emerging causal agent of chestnut brown rot in Anhui and Hubei provinces in China. Identification for describing the fungus as a new taxon was based on the results of phylogenetic analyses of sequence data for combined ITS and *tef1* and *tub2* genes, as well as the morphological characteristics. However, only the asexual state of *Gnomoniopsis daii* was discovered from rotted seeds of chestnut trees.

The related species of *Gnomoniopsis daii*, *G. smithogilvyi*, has been reported to cause serious disease on *Castanea sativa* and *C. crenata* × *C. sativa* hybrids in Europe and Oceania [13,19]. The infection process and cycle of chestnut disease has been demonstrated, and ascospores of *G. smithogilvyi* from chestnut buds are key to causing fruit rot [11,13]. The pathogen was later isolated from cankers on stems and branches [11,13]. However, we did not discover the sexual morph of *Gnomoniopsis daii* on the chestnut bud during this study.

Gnomoniopsis species inhabited three families of host, viz., Fagaceae, Rosaceae, and Onagraceae [18,20,23]. *Gnomoniopsis daii*, *G. smithogilvyi*, *G. clavulata*, and *G. paraclavulata* were discovered from Fagaceae trees and formed a close phylogenetic relationship differing from other species (Figures 2 and 3). *Gnomoniopsis smithogilvyi* was first reported as a nut rot pathogen [8].

Subsequently, the authors isolated this fungus from chestnut branches [13]. *Gnomoniopsis daii* was described as a novel pathogen of chestnut rot disease in China depending on its asexual state. *Gnomoniopsis clavulata* and *G. paraclavulata* were collected from overwintered leaves belonging to *Quercus* species in the form of sexual states [21]. These two species were only reported in the USA [21]. Conidial size can only barely separate these four close species (5.0–8.0 × 2.0–4.0 µm in *G. clavulata* vs. 5.0–8.0 × 2.0–3.5 µm in *G. daii* vs. 6.0–9.5 × 2.0–3.5 µm in *G. paraclavulata* vs. 4.9–9.8 × 2.9–4.9 µm in *G. smithogilvyi*) [8,20,23], but the combined evidence of host species, distribution, and molecular data (ITS, *tef1*, and *tub2*) clearly distinguishes these related species.

During our pathogenicity test, we confirmed that *Gnomoniopsis daii* also causes chestnut brown rot. Hence, this *Gnomoniopsis* species represents the second species in this genus infecting *Castanea* hosts. *Castanea* is an important plant genus worldwide, so it is necessary to further research the fundamental aspects of the relationship between the pathogen genus *Gnomoniopsis* and host genus *Castanea*.

Accurate identification and diagnostics of fungal pathogens are important for determining the disease cycle and route of transmission. As an emergent disease agent in chestnut orchards in China, chestnut tree loss appears to be closely associated with *Gnomoniopsis* nut rot. Further studies should focus on methods to prevent increased damage to this valuable crop tree.

5. Conclusions

A novel fungal species, *Gnomoniopsis daii*, is an emerging pathogen causing Chinese sweet chestnut brown rot in China.

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Conflicts of Interest: The authors declare no conflict of interest.

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