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 $2 \times 10^6$ 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 cinamomi* 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.](image)

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 1α (tef1) amplified with primers EF1-728F/EF1-1567R [26], and the β-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.
Isolates were allowed to grow on MEA for one week 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 inoculations, viz., CMF002A (ex-type from Hubei province), CMF002B (from Anhui province), CMF095 (from Hubei province), and CMF098 (from Hubei province). Isolates were selected for measurement. Cultural characteristics of isolates incubated on MEA were observed and recorded, including colony color and texture.

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 dainii* 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

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

| Species                     | Country    | Host                        | Strain           | Internal Transcribed Spacer (ITS) | tub2       | tef1       |
|-----------------------------|------------|-----------------------------|------------------|-----------------------------------|------------|------------|
| *Apiognomon veneta*         | France     | Platanus occidentalis       | CBS 342.86       | DQ313531                          | EU219225   | DQ318036   |
| *Gnomoniopsis alderdenensis*| USA        | *Rubus* pedatus             | CBS 125679       | GU320826                          | GU320788   | GU320813   |
| *Gnomoniopsis alderdenensis*| USA        | *Rubus* parviflorus         | CBS 125680       | GU320825                          | GU320787   | GU320801   |
| *Gnomoniopsis alderdenensis*| USA        | *Rubus* parviflorus         | CBS 125681       | GU320827                          | GU320789   | GU320802   |
| *Gnomoniopsis chaemorii*    | Finland    | *Rubus* chamaemorus         | CBS 804.79       | GU320817                          | GU320777   | GU320809   |
| *Gnomoniopsis cleserulata*  | 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                          | GU320799   | GU320814   |
| *Gnomoniopsis comari*       | Switzerland| *Comarum* palustre          | CBS 809.79       | EU254823                          | GU320778   | GU320794   |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF002A          | MN398671                          | MN605519   | MN605517   |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF002B          | MN398672                          | MN605520   | MN605518   |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF095           | MN398673                          | NA         | NA         |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF098           | MN398674                          | NA         | NA         |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF099           | MN398675                          | NA         | NA         |
| *Gnomoniopsis daii*         | China      | *Castanea* mollissimana     | CMF116           | MN398676                          | NA         | NA         |
| *Gnomoniopsis frankicola*   | Bulgaria   | *Agrimonia* eupatoria       | NA               | EU254812                          | NA         | NA         |
| *Gnomoniopsis frankicola*   | France     | *Frangaria* vesca           | CBS 121226       | EU254824                          | EU219144   | GU320792   |
| *Gnomoniopsis frankicola*   | USA        | *Frangaria* sp.             | CBS 208.34       | EU254826                          | EU219149   | GU320808   |
| *Gnomoniopsis garstulata*   | Bulgaria   | *Agrimonia* eupatoria       | NA               | EU254812                          | NA         | NA         |
| *Gnomoniopsis garstulata*   | USA        | *Rubus* sp.                 | CBS 125671       | GU320816                          | GU320776   | GU320793   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* sp.                 | CBS 125672       | GU320823                          | GU320781   | GU320797   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* sp.                 | CBS 125673       | GU320824                          | GU320782   | GU320798   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* sp.                 | CBS 125674       | GU320820                          | GU320780   | GU320796   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* sp.                 | CBS 125675       | GU320822                          | GU320763   | GU320799   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* procerus            | CBS 125676       | GU320821                          | GU320784   | GU320811   |
| *Gnomoniopsis idaeicola*    | USA        | *Rubus* procerus            | CBS 125677       | GU320821                          | GU320784   | GU320811   |
| *Gnomoniopsis idaeicola*    | USA        | *Spinosa* sp.               | CBS 121468       | EU254762                          | EU219126   | GU320804   |
| *Gnomoniopsis occulta*      | USA        | *Potentilla* sp.            | CBS 125677       | GU320828                          | GU320765   | GU320812   |
| *Gnomoniopsis occulta*      | Russia     | *Potentilla* anserina       | NA               | EU254811                          | NA         | NA         |
| *Gnomoniopsis paracerasia*  | USA        | *Cembrion* angustifolium    | CBS 121469       | EU254841                          | EU219125   | GU320803   |
| *Gnomoniopsis racemula*     | Switzerland| *Sangioisia minor*          | CBS 858.79       | GU320818                          | GU320790   | GU320805   |
| *Gnomoniopsis sanguiacinar* | Switzerland| *Sangioisia 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.
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.

*Gnomoniopsis daii* sp. nov.
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

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**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.
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](image_url) **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](image_url) **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.

| 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. 8.0–8.8 × 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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