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Posted Date: 21 November 2023

doi: 10.20944/preprints202311.1304.v1

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Article

Alternaria alternata and *A. koreana*, the Causal Agent of a New Leaf Spot in *Celtis sinensis* and Their Sensitivity to Fungicides

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Abstract: *Celtis sinensis* is a highly versatile species that is commonly cultivated in the southern regions of China. In June 2022, leaf blotch disease was detected in *C. sinensis* in Nanjing, Jiangsu, China. Based on morphological characteristics, three isolates were determined to be of the *Alternaria* species. Phylogenetic analysis of combined ITS, *GAPDH*, *TEF1-α*, *RPB2* and *Alt a 1* sequences identified the three isolates we obtained as *Alternaria alternata* and *Alternaria koreana*. Determination of the pathogenicity of *A. alternata* and *A. koreana* in *C. sinensis* leaves by inoculation in vivo experiments. Symptoms of inoculation onset in indoor pots were in accordance with those witnessed the open country. *A. alternata* and *A. koreana* can grow at 15-35 °C, with the best growth occurring at 25 °C. The findings from fungicide susceptibility experiments demonstrated that *A. alternata* and *A. koreana* were the most sensitive to prochloraz, which could offer an effective approach for future prevention and control measures against *A. alternata* and *A. koreana*. This study provides the initial step towards further research on *A. alternata* and *A. koreana* as pathogens of *C. sinensis* and provides the stage for future control strategy development.

Keywords: *Celtis sinensis*; *Alternaria alternata*; *Alternaria koreana*; fungi; identification; fungicides

1. Introduction

Celtis sinensis is an important member of the Ulmaceae family [1] that is native to China, Japan, and Korea [2]. Mainly distributed in the central and eastern regions of China, with straight trunks and beautiful tree shapes, it is a common street and landscaping tree species and a valuable resistant tree species that can adsorb toxic gases such as sulfur dioxide and chlorine and has a certain degree of adsorption capacity for dust [3]. *C. sinensis* demonstrates remarkable resilience, as it is able to withstand both dry conditions and moist, infertile soils. These trees possess a strong adaptability to diverse environmental factors. Moreover, the branches, leaves, roots, and bark of *C. sinensis* hold important medicinal value and can be utilized in the treatment of burns and urticaria [4]. In addition, it has a wide range of industrial uses. The roots and branches can be used to make artificial cotton and furniture, and the fruits can also be used as raw materials for lubricant production [5]. However, in Xuanwu District, Nanjing, Jiangsu Province, a large number of leaf spot and leaf curling occurred in *C. sinensis*, which seriously affected the ornamental value of *C. sinensis* and damaged the ecological environment.

Alternaria is a widely distributed group of fungi in the natural environment and is also economically important as a dematiaceous fungus [6]. More than 95% of the species are plant parthenogenetic parasites. They can grow and multiply in low-temperature and humid environments, causing a variety of plant diseases, as well as fruit rot, which severely jeopardizes the growth of crops and plants and causes enormous economic losses while destroying natural ecosystems globally [7]. In addition, the spores of *Alternaria* can also release allergens [8], triggering diseases such as asthma [9] and posing a major threat to human health. The pathogenic fungi of

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Alternaria establish their population by colonizing the surface of seeds with mycelium or conidia [10] on diseased plant residues and in the soil. These serve as primary infection sources for the following year. The conidia of the fungi are spread and dispersed by air currents, leading to secondary infections, thus perpetuating the cycle of transmission and spread of disease.

At present, the most direct and effective method for the control of diseases caused by *Alternaria* is chemical control [11]. Fungicides such as dithiocarbamates, triazoles, strobilurins, iprodione, or copper fungicides are used in most areas for disease control [12]. Copper-based fungicides are widely used due to their long residual effectiveness and resistance to washing [13]. Methoxyacrylate, dimethomorph, and mancozeb fungicides have also been registered for the control of brown spot disease in countries such as the United States, Israel, and Spain [14]. Due to the difficulty of mixing copper-based formulations with other pesticides and the single-site action of methoxyacrylate and dimethomorph fungicides, resistant populations can quickly emerge in the field, leading to decreased or ineffective efficacy of these fungicides. Recently, nicotinamide fungicides such as cyazofamid have been registered in the United States for the control of this disease [15]. These fungicides can be used for preseed disinfection, as well as direct application to the soil, effectively providing preventive protection [16].

This study reveals the characteristics of the pathogen related to leaf spot in *C. sinensis* in Xuanwu District, Nanjing, Jiangsu Province, China. This disease has caused significant damage to the local ecosystem. This study was conducted through the following methodologies: to determine the causative agent of brown spot disease in *C. sinensis* using Koch's postulates; to identify the pathogen through molecular biological and morphological identification methods; and to screen fungicides with significant inhibitory effects on the mycelial growth of the pathogen through culture-based plate phenotype experiments.

2. Materials and Methods

2.1. Sampling and fungal isolations

In June 2022, the disease was observed on the leaves of *C. sinensis* on the campus of Nanjing Forestry University, Nanjing City, Jiangsu Province (119°46'43"E, 32°02'38"N), and 20 diseased leaves were collected as samples from across 0.75-1.5 meters of *C. sinensis*. The samples were collected and returned to the laboratory, and the diseased leaves were wiped with absorbent paper with sterile water to remove surface dust and impurities. One hundred small tissues (3×3 mm^2) were cut from lesion margins and surface-sterilized in 75% ethanol for 30 s, followed by 5% NaClO for 90 s, rinsed in sterile water 3 times, dried on sterilized filter paper, plated onto potato dextrose agar (PDA) for 4 days at 25 °C [17]. On the third day, fungal hyphae emerging from leaf tissue were shifted to fresh PDA [18]. The isolated strains were preliminarily classified according to morphological characteristics and ITS sequence alignment for subsequent experiments.

2.2. Pathogenicity tests

Healthy *C. sinensis* and seedlings were obtained from the laboratory of Nanjing Forestry University, and the seedlings had heights of approximately 80 cm. Before the pathogenicity experiment, the leaf surface was wiped with 75% alcohol twice, and sprayed with sterile water three times. The water stains on the surface of the leaves were wiped dry with absorbent paper. The colony morphology of 100 fungal samples was assessed, and they were divided into different groups. Three isolates (11, 12 and 13) from the group with the highest frequency of occurrence were selected for pathogenicity testing and inoculation of leaf blades and seedlings, respectively [19]. Five isolated leaves were inoculated with each isolate, wounds were created on both sides of the leaf veins with a sterile needle, and a 6 mm mycelium block was placed face down on each puncture wound and removed after 24 hours [20]. In addition, five leaves were inoculated with sterilized PDA agar blocks as a control. The samples were cultured for four days under environmental conditions of 25 °C temperature, 70%-80% humidity, and 24 hours of light exposure per day. Nine healthy *C. sinensis* seedlings were selected on each plant, 3 leaves were inoculated with spores of one of the 3 isolates by

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puncturing the leaf with a sterile needle as a wound suspension (10^6 conidia/mL), and the other three plantlets were inoculated with sterile water as a control group. The plantlets were covered with plastic bags and sprayed with water to maintain a high level of humidity.

2.3. Morphological identification and biological characteristics

Three isolates (11, 12 and 13) were cultured on PDA medium at 25 °C for 7 days in a constant temperature incubator. Morphological identification relied on colony appearance and conidia characteristics. Morphology of conidia using Zeiss Axio Imager A2m microscope (Carl Zeiss, Germany) (n=30).

Three isolates (11, 12, and 13) were chosen for the purpose of conducting morphological observations and biological characterization. Isolates were cultured on potato dextrose agar medium (PDA), Czapek dox agar medium (CDA), Richard medium (Richard), corn meal agar medium (CMA), oatmeal agar medium (OA), and potato saccharose agar medium (PSA) for 7 days at 25 °C, and the appearance and coloration of the colonies were documented.

In order to ascertain the isolates' optimal growth temperature, mycelial plugs with a diameter of 6 mm were positioned on freshly prepared PDA medium, which had a diameter of 90 mm, and incubated at 5 °C, 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. The colony growth diameter was measured and recorded daily.

2.4. DNA extraction, PCR amplification, and sequencing

The isolated fungi were cultured on PDA medium for 7 days, and mature mycelium was scraped off using a sterilized surgical blade. DNA extraction was carried out utilizing the CTAB method [21]. All DNA extracts were preserved at a temperature of -20 °C for future utilization.

Polymerase chain reaction was performed on ITS [22], *TEF1-α* [23], *GAPDH* [24], *RPB2* [25], and *Alt a 1* [26] five genes, which were sequenced using primers ITS1/4, EF1-728F/EF1-986R, GPD1/GPD2, RPB2-5F2/RPB2-7C and Alt-for/Alt-rev, respectively. PCR was carried out in a 25 μL reaction mixture containing 21 μL of Tmix, 1 μL of DNA template, 1 μL of Taq DNA polymerase, 1 μL of forward primer and 1 μL of reverse primer (Table 1). The PCR products were sequenced at Sangon Biotech Co. Ltd. (Nanjing, China).

Table 1. Primers used for PCR amplification in the molecular identification of three isolates (11–13).

Gene	Primer	Sequence (5′-3′)	PCR amplification cycle parameters:
Internal transcribed spacer (ITS)	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	95 °C: 3 min, (95 °C: 30 s, 55 °C: 30 s,72 °C: 45 s) × 30 cycles, 72 °C: 10 min
Elongation factor 1-alpha (TEF1-α)	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	95 °C: 3 min, (95 °C: 30 s, 59 °C: 30 s, 72 °C: 20 s) × 30 cycles, 72 °C: 10 min
Glycerol-3-phosphate dehydrogenase (GAPDH)	GPD1 GPD2	CAACGGCTTCGGTCGCATTG GCCAAGCAGTTG GTTGT	95 °C: 3 min, (95 °C: 30 s, 59.5 °C: 30 s, 72 °C: 45 s) × 30 cycles, 72 °C: 10 min
RNA polymerase second largest subunit (RPB2)	RPB2-5F2 RPB2-7cR	GGGGWGAYCAGAAGA AGGC CCCATRGCTTGTYYRCCCAT	95 °C: 3 min, (95 °C: 30 s, 53 °C: 30 s, 72 °C: 50 s) × 30 cycles, 72 °C: 10 min
Alternaria major allergen gene (Alt a 1)	Alt-al-for Alt-al-rev	ATGCAGTTCACCACCATCGC ACGAGGGTGAYGTAGGCGTC	95 °C: 3 min, (95 °C: 30 s, 60.5 °C: 30 s, 72 °C: 30 s) × 30 cycles, 72 °C: 10 min

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2.5. Phylogenetic analyses

The phylogenetic tree was constructed using sequences from the fungal cultures in this study and sequences related to *Alternaria* sp. in GenBank (Table 2); *Alternaria alternantherae* was used as the outgroup. Sequence alignment analysis using MAFFT version 7 (https://mafft.cbrc.jp/alignment/software/) [27], to trim sequences to ensure a high degree of sequence alignment [28]. Phylogenetic analysis through concatenation of five loci (ITS, *TEF1-α*, *GAPDH*, *RPB2*, and *Alt a 1*). Phylogenetic trees based on combined genes were constructed using two independent optimality search criteria: maximum likelihood (ML) and Bayesian inference (BI) criteria. The ML analysis used the GTR + F + I + G4 model, and branch stability was determined by 1,000 bootstrap replicates. BI analysis used the GTR + I + G + F model, including two parallel runs of 2,000,000 generations. The resulting trees were plotted using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 2. The isolates of *Alternaria* species used in this study for phylogenetic analysis.

Species	Isolate	Accession Numbers				
		ITS	*GAPDH*	*TEF1-α*	*RPB2*	*Alt a 1*
Alternaria alstroemeriae	CBS 118809^T	KP124297	KP124154	KP125072	KP124765	KP123845
Alternaria sp.	CBS 108.27	KC584236	KC584162	KC584727	KC584468	-
A. alternantherae	CBS 124392	KC584179	KC584096	KC584633	KC584374	KP123846
A. alternata	CBS 916.96^T	AF347031	AY278808	KC584634	KC584375	AY563301
A. alternata	**11**	**OP476716**	**OP609771**	**OP609768**	**OP604538**	**OP609775**
A. alternata	**13**	**OP476718**	**OP609773**	**OP609770**	**OP604540**	**OP609776**
A. arctoseptata	MFLUCC 21-0139^T	-	OK236702	0K236608	OK236655	OK236755
A. baoshanensis	MFLUCC 21-0124^T	MZ622003	OK236706	OK236613	OK236659	OK236760
A. betae-kenyensis	CBS 118810^T	KP124419	KP124270	KP125197	KP124888	KP123966
A. breviconidiophora	MFLUCC 22-0075^T	MZ621997	OK236698	OK236604	OK236651	-
A. burnsii	CBS 107.38^T	KP124420	JQ646305	KP125198	KP124889	KP123967
A. doliconidium	KUN-HKAS 100840^T	NR158361	-	-	-	-
A. ellipsoidialis	MFLUCC 21-0132^T	MZ621989	OK236690	OK236596	OK236643	OK236743
A. eupatoriicola	MFLUCC 21-0122^T	MZ621982	OK236683	OK236589	OK236636	OK236736
A. eichhorniae	CBS 489.92^T	KC146356	KP124276	KP125204	KP124895	KP123973
A. eichhorniae	CBS 119778	KP124426	KP124277	KP125205	KP124896	KP123973
A. falcata	MFLUCC 21-0123^T	MZ621992	OK236693	OK236599	OK236646	OK236746
A. gaisen	CBS 632.93	KC584197	KC584116	KC584658	KC584399	KP123974
A. gaisen	CBS 118488	KP124427	KP124278	KP125206	KP124897	KP123975
A. gossypina	CBS 104.32^T	KP124430	JQ646312	KP125209	KP124900	JQ646395
A. gossypina	CBS 107.36^T	KP124431	JQ646310	KP125210	KP124901	JQ646393
A. iridiaustralis	CBS 118404	KP124434	KP124283	KP125213	KP124904	KP123980
A. iridiaustralis	CBS 118486^T	KP124435	KP124284	KP125214	KP124905	KP123981

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A. jacinthicola	CBS 878.95	KP124437	KP124286	KP125216	KP124907	KP123983
A. jacinthicola	CBS 133751^{T}	KP124438	KP124287	KP125217	KP124908	KP123984
A. koreana	SPL2-1 (KACC49833)^{T}	LC621613	LC621647	LC621715	LC621681	LC631831
A. koreana	SPL2-4	LC621615	LC621649	LC621717	LC621683	LC631832
A. koreana	**12**	**OP476717**	**OP609772**	**OP609769**	**OP604539**	**OP609774**
A. lathyri	MFLUCC 21-0140^{T}	MZ621974	OK236675	OK236581	OK236628	OK236728
A. longipes	CBS 539.94	KP124441	KP124290	KP125220	KP124911	KP123987
A. longipes	CBS 540.94	AY278835	AY278811	KC584667	KC584409	AY563304
A. macroconidia	MFLUCC 21-0134^{T}	MZ622001	OK236704	OK236610	OK236657	OK236757
A. minimispora	MFLUCC 21-0127^{T}	MZ621980	OK236681	OK236587	OK236634	OK236734
A. muriformispora	MFLUCC 22-0073^{T}	MZ621976	OK236677	OK236583	OK236630	OK236730
A. ovoidea	MFLUCC 14-0427^{T}	MZ622005	OK236708	OK236614	OK236661	OK236761
A. phragmiticola	MFLUCC 21-0125^{T}	MZ621994	OK236696	OK236602	OK236649	OK236749
A. pseudoinfectoria	MFLUCC 21-0126^{T}	MZ621984	OK236685	OK236591	OK236638	OK236738
A. rostroconidia	MFLUCC 21-0136^{T}	MZ621969	OK236670	OK236576	OK236623	OK236723
A. salicicola	MFLUCC 22-0072^{T}	MZ621999	OK236700	OK236606	OK236653	OK236753
A. setosa	YZU 191101^{T}	OP341770	OP352306	OP374459	OP352294	OP293717
A. tectorum	YZU 161050^{T}	OP341728	OP352303	OP374456	OP352291	OP293714
A. tectorum	YZU 161052	OP341817	OP352304	OP374457	OP352292	OP293715
A. tomato	CBS 103.30	KP124445	KP124294	KP125224	KP124915	KP123991
A. tomato	CBS 114.35	KP124446	KP124295	KP125225	KP124916	KP123992
A. torilis	MFLUCC 14-0433^{T}	MZ621988	OK236688	OK236594	OK236641	OK236741
A. vitis	MFLUCC 17-1109^{T}	MG764007	-	-	-	-

^{T} ex-type isolate; bolded are the isolates for this study.

2.6. Evaluation of fungicides against A. alternata and A. koreana

Fungicides were kindly provided by Mrs. Tingting Dai of Nanjing Forestry University as 96% prochloraz [29], 97% myclobutanil [30], 97% tebuconazole [31], and 98% pyraclostrobin [32]. Each fungicide was prepared as a stock solution, which was then diluted to different concentrations. The solutions were added to sterilized PDA medium at a rate of 2% to create agar plates containing the respective concentrations of the fungicide. PDA plates without the use of any fungicide were used as a control. A fungal plug was extracted from the colony's edge using a 6mm punch and relocated to the center of the medium. After incubation at 25 °C for 7 days, the diameter of the fungal mycelium was measured. The EC50 value was calculated using GraphPad Prism 8.

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3. Results

3.1. Field observations and fungal isolation

The survey results indicated that nearly 50% of the *C. sinensis* in Xuanwu District, Nanjing, have shown signs of disease. Most of the spots were produced on the apex part of the leaves (Figure 1A), and the spots were brown with darker margins and round to irregular in shape (Figure 1B), on which dark brown chains of conidia were visible. Over time, the spots gradually expand, and the leaves become dark brown, dry, curled, and necrotic and eventually fall off. In total, 90 fungal colonies developed from the tissue pieces and were grouped into three types according to their colony characteristics, with frequencies of 84.4% (76 of 90), 10% (9 of 90), and 5.5% (5 of 90). Three isolates from the highest frequency were selected for purification. According to ITS sequences, 3 fungi belonged to *Alternaria* and were used for further research.

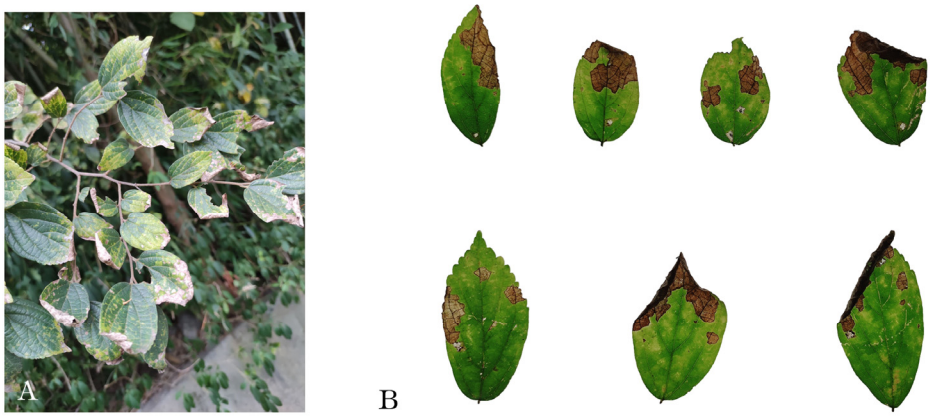

Figure 1. Symptoms of infected leaves in *C. sinensis* in the wild. **A.** Diseased leaves naturally. **B.** Close-up view of diseased leaves.

3.2. Pathogenicity test

Three representative isolates (11, 12 and 13) were inoculated on healthy leaves, and black spots were observed on the leaves after 4 days (Figure 2). The induced symptoms matched those observed in the wild. Conversely, the control maintained healthy with no symptoms of disease. Observing the size of the lesions, those caused by isolates 11 and 13 had smaller affected areas. In contrast, the lesions caused by isolate 12 exhibited a larger affected area. The isolates obtained from the inoculated diseased leaves displayed similar morphological characteristics and shape to the originally isolated strains. DNA was extracted and subjected to PCR using the primer ITS1/4, and the obtained sequences were similar to the original ones. The symptoms observed on seedlings inoculated in a controlled environment closely resembled those seen in the natural field conditions, thus meeting the criteria of Koch's postulates. Therefore, *A. alternata* and *A. koreana* are the pathogens of leaf spot disease of *C. sinensis*.

3.3. Morphological characteristics

Three representative isolates were grown on PDA medium and hatched for 7 days at 25 °C, and morphological characteristics were recorded based on visual observations (Figure 3). The colonies of isolates 11 and 13 on PDA were initially gray and became grayish brown over time. Conidiophores produced numerous conidia in long chains, mostly unbranched. Conidia were obclavate, dark brown, with two to five transverse and zero to two longitudinal or oblique septa, and measured 10.1 to 35.9 × 7.6 to 24.3 μm (n=30). The colony of isolate 12 on PDA was grayish-green. Hyaline (young) hyphae or brown (old) hyphae, with septa and smooth walls, were slightly constricted at the septa and branched. Conidiophores produced numerous conidia in long chains. These conidia were globose to ellipsoidal and 13.5 to 34.5 × 7.0 to 14.1 μm (n = 30). The morphological characteristics of

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the 3 isolates matched those of *Alternaria* spp. The three isolates showed different morphological characteristics on different media.

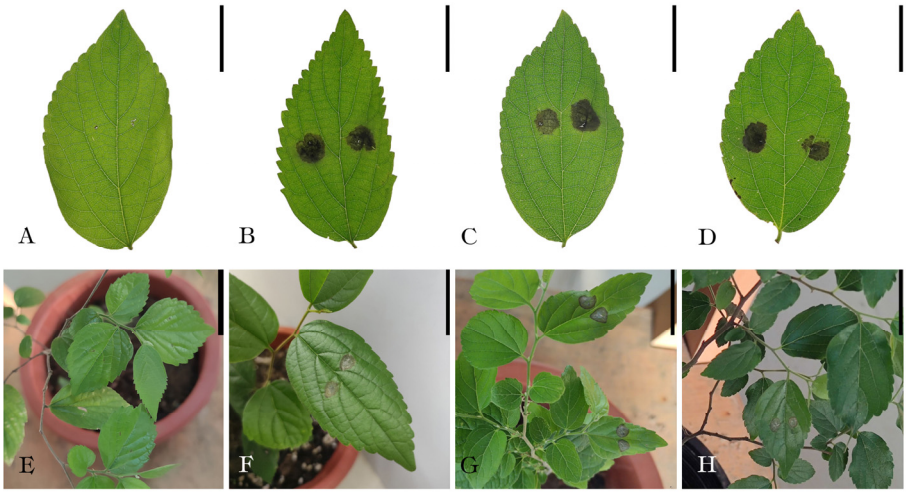

Figure 2. Pathogenicity of fungal isolates. **A.** Symptomless leaves of controls treated with PDA after 4 days. **B-D.** Symptoms on leaves inoculated with mycelium blocks of isolates 11, 12 and 13 after 4 days. **E.** Symptomless leaves of controls treated with sterile water after 5 days. **F-H.** Symptomless leaves of controls treated with sterile water after 5 days. Symptoms on leaves inoculated with 10 µl of conidial suspension (10^6 conidia/mL) of isolates 11, 12 and 13 after 5 days.

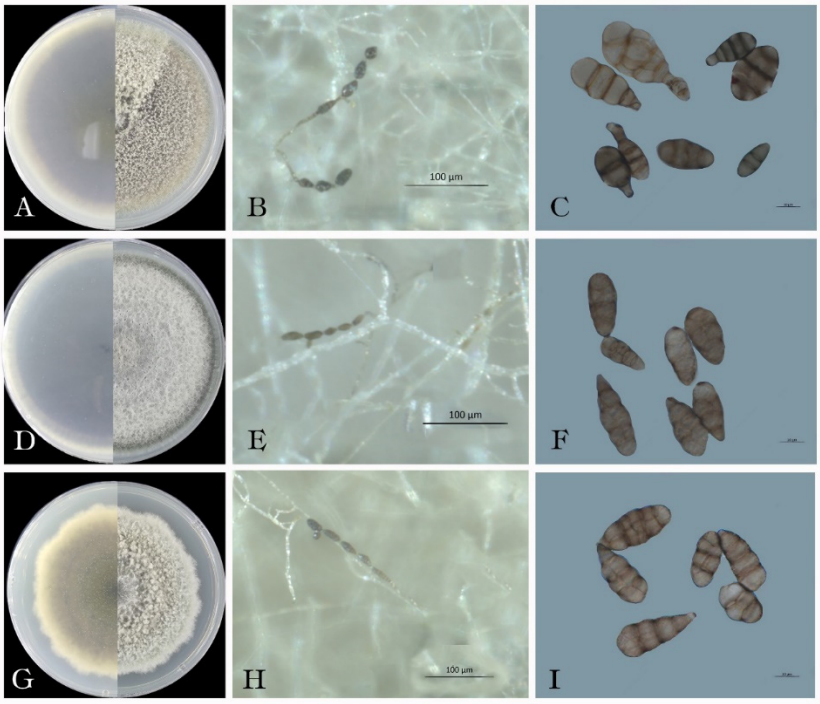

Figure 3. Morphological characteristics of fungal isolates. **A.** 7-day-old front and back view colony of 11 on PDA. **B-C.** Conidial chain and conidia of isolate 11, Bars: E = 100 µm, F =10 µm. **D.** 7-day-old front and back view colony of 12 on PDA. **E-F.** Conidial chain and conidia of isolate 12, Bars: I = 100 µm, J =10 µm. **G.** 7-day-old front and back view colony of 13 on PDA. **H-I.** Conidial chain and conidia of isolate 13, Bars: M = 100 µm, N =10 µm.

3.4. Multigene phylogenetic analysis

BLAST results showed that the ITS, *GAPDH*, *TEF1-α*, *RPB2* and *Alt a 1* sequences of isolate 12 were highly similar (≥98%) to those of *A. koreana* (culture ex-type SPL2-1), while sequences of isolates

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11 and 13 were highly similar (≥99%) to those of *A. alternata* (culture representative CBS 918.96). The sequences of genes/region ITS, *GAPDH*, *TEF1-α*, *RPB2* and *Alt a 1* from the three isolates (11, 12 and 13) were deposited in GenBank, and the accession numbers are shown in Table 2. Phylogenetic analyses using concatenated sequences placed isolate 12 in the clade of *A. koreana* and isolates 11 and 13 in the clade of *A. alternata*. Based on the phylogeny and morphology, isolate 12 was identified as *A. koreana*, and isolates 11 and 13 were identified as *A. alternata* (Figure 4).

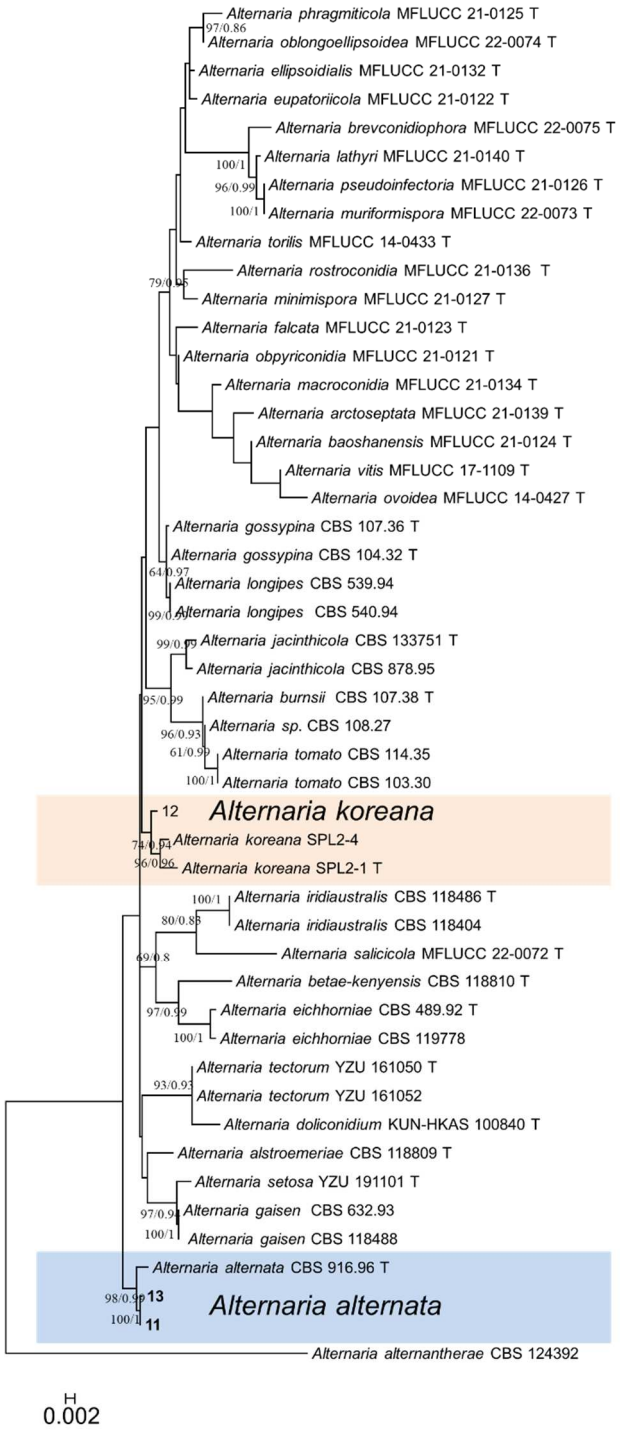

Figure 4. Phylogenetic relationship of 11, 12 and 13 and related taxa derived from concatenated sequences of the ITS, *GAPDH*, *TEF1-α*, *RPB2* and *Alt a 1* genes using the maximum likelihood algorithm and Bayesian analysis. Bootstrap values >75% (1,000 replications) and Bayesian posterior probability (PP ≥ 0.90) are shown at the nodes (ML/PP). *Alternaria alternantherae* (CBS 124392) was used as an outgroup. Bar = 0.002 substitutions per nucleotide position. T indicates ex-types.

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3.5. Biological characteristics

The colonies of isolates 11 and 13 on CDA appeared as yellowish colonies with flattened colonies (Figure 5B, N). On Richard's medium, the colony appeared grayish-white, with very few hyphae and weak growth (Figure 5C, O). The colony of isolate 11 was dark green with dense mycelium on CMA and OA media, and on PSA media, the colony was dark gray in the middle with a brown ring around it with abundant mycelium (Figure 5D, E). The colony of isolate 13 was light green and radial on CMA, OA and PSA media (Figure 5P, Q, R). The colony of isolate 12 on CDA was almost identical to the colony morphology on PDA and PSA, with grayish-white colonies with dense hyphae (Figure 5G, H, L), showing a brownish color on Richard, with reticulated colonies and fewer hyphae (Figure 4I), a whitish-green color on CMA, with loosely packed colonies (Figure 5J), and a dark green color on PSA medium, with fuzzy colonies (Figure 5K).

Isolates 11, 12, and 13 were cultured between 5°C-35°C for 7 days, and the colony diameter reached a maximum and grew most vigorously at 25°C, but at 5°C, the mycelium hardly grew, which shows that 25°C is the optimum culture temperature for isolates 11, 12, and 13 (Figure 6).

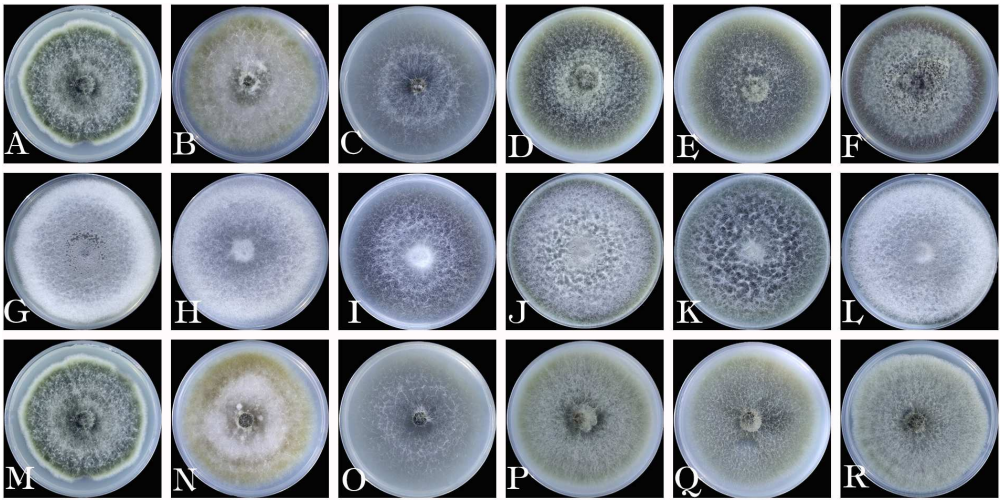

Figure 5. Colony formation of isolates 11, 12, and 13 isolated from *Celtis sinensis* hatched for 7 days on different media at 25°C. **A-F.** Colony morphology of isolate 11 cultured on PDA, CDA, Richard, CMA, OA, and PSA media for 7 days. **G-L.** Colony morphology of isolate 12 cultured on PDA, CDA, Richard, CMA, OA, and PSA media for 7 days. **M-R.** Colony morphology of isolate 13 cultured on PDA, CDA, Richard, CMA, OA, and PSA media for 7 days.

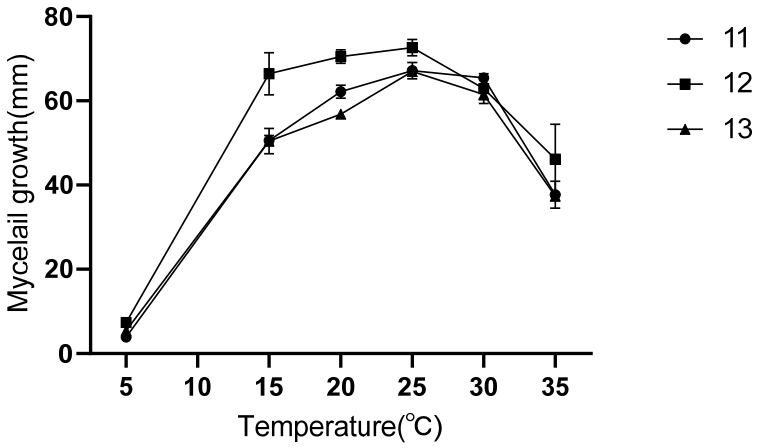

Figure 6. The impact of temperature on the growth of colony diameters in isolates 11, 12, and 13 after 7 days of culture on PDA media.

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3.6. Susceptibility of Alternaria isolates to fungicides

The three isolates showed similar biological responses to the four fungicides (Figure 7). All four fungicides showed significant growth inhibition of the isolates on PDA media. Prochloraz had a lower EC50 on mycelial growth than the other three fungicides and showed the best inhibitory effect on isolates 11, 12, and 13 (Table 3). Pyraclostrobin had the highest EC50 on mycelial growth of 13 isolates and the weakest inhibitory effect but still had a strong inhibitory effect on 11 and 12 isolates. Based on the EC50 values, myclobutanil inhibited isolates 11 and 13 almost equally but more strongly against isolate 12. Isolate 12 was the most sensitive to tebuconazole compared to 11 and 13, and isolate 13 was the least sensitive. The results indicated that prochloraz was the most effective fungicide against *Alternaria* spp. in this study.

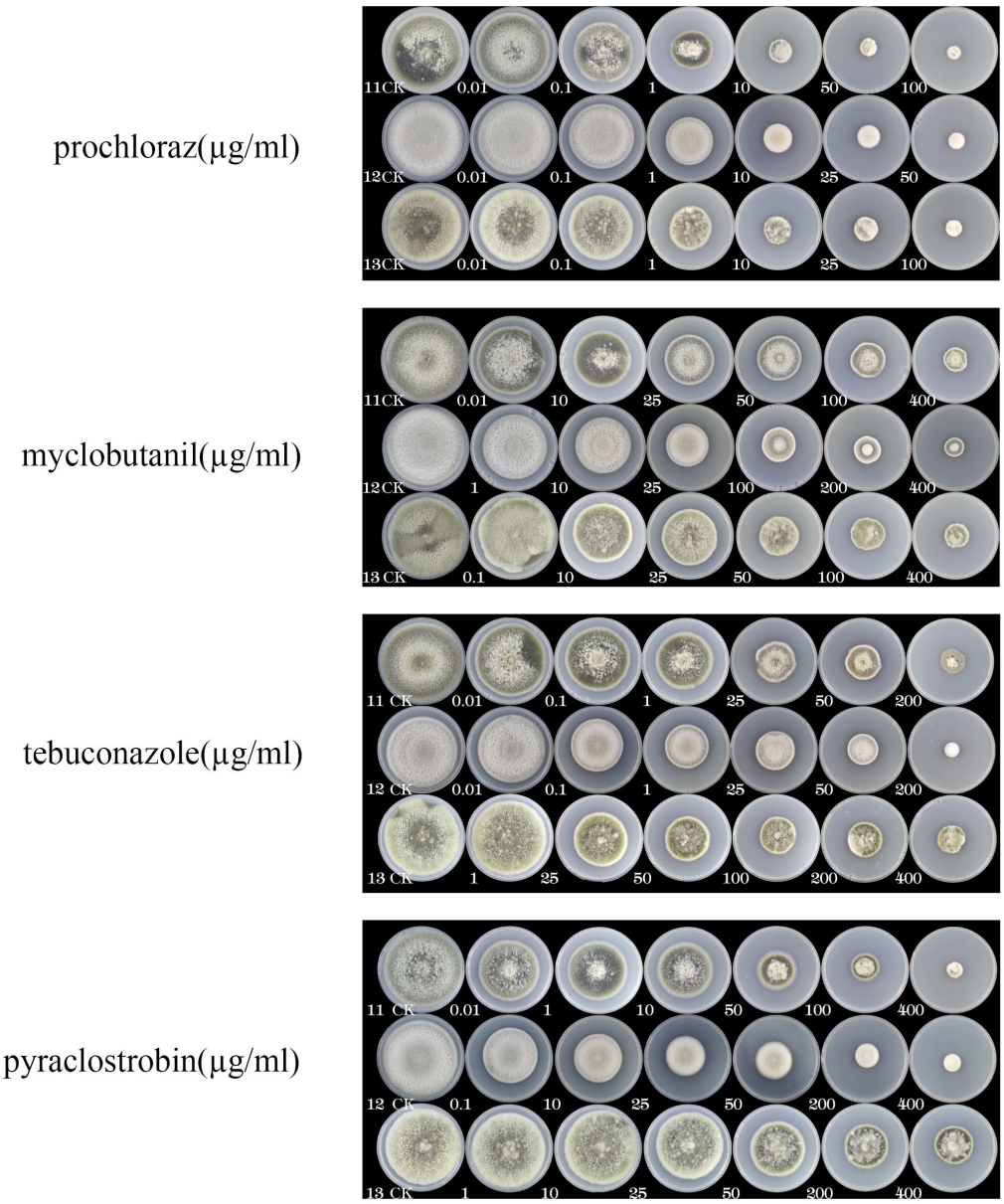

Figure 7. The suppression effect on isolates 11, 12 and 13 on plates by corresponding doses of four different fungicides (prochloraz, myclobutanil, tebuconazole, and pyraclostrobin) based on fresh PDA for 7 days.

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Table 3. Concentration at 50% of Maximum Effect (EC50 values) of isolates.

Fungicide	EC50 Values (μg/mL)		
	11	12	13
Prochloraz	2.92	3.89	3.98
Myclobutanil	84.54	54.86	17.23
Tebuconazole	34.1	17.23	141.9
Pyraclostrobin	50.21	32.27	421.1

4. Discussion

Celtis sinensis is a common deciduous tree that not only has some value in food and medicine but also plays an important role in urban greening and the economy. However, leaf spot disease deteriorates the appearance of leaves and severely affects the ornamental value of *C. sinensis*. This disease also leads to leaf damage and interferes with photosynthesis, which prevents *C. sinensis* from fully utilizing sunlight for nutrient synthesis, resulting in slow growth of the plant and weakening of the leaves, which in turn affects the health and growth of the whole tree and reduces the quality of the timber [33], resulting in an economic loss to the timber industry, as well as affecting the ecological function of *C. sinensis*. Timely identification of the pathogen responsible for *C. sinensis* leaf spot is essential. By combining morphological identification, molecular analysis, and phylogenetic research [34], *A. alternata* and *A. koreana* were identified as the causal agents of *C. sinensis* leaf spot in China. To our knowledge, this is the first report of *A. alternata* and *A. koreana* causing leaf spot in *C. sinensis*.

The majority of pathogenic species within the *Alternaria* genus are the primary cause of the most detrimental plant diseases, commonly referred to as "leaf spot" or "leaf blight". These diseases are characterized by the development of circular or irregular necrotic lesions on the surface of the leaf, accompanied by a distinct concentric ring-like pattern at the center of the lesions. It is difficult to classify species of *Alternaria* based on spore morphology characteristics. The challenge arises because a considerable number of small-spore *Alternaria* species, commonly referred to as *A. alternata*, exhibit closely similar morphological characteristics [35]. Currently, there is very little research available on *A. koreana* species. The spore size observed in this study closely resembles the findings reported by Romain et al. [36]. However, there is actually a large amount of research available on *Alternaria* spp., and there is considerable variation in spore sizes reported. The spore sizes observed in this study are notably different from those described by Ramirez et al. [37], but they are similar to the description by Sun et al. [38]. Due to its morphological characteristics being susceptible to environmental conditions and the presence of many uncontrollable factors, it is highly prone to variations [39].

Currently, the classification of *Alternaria* species primarily relies on the utilization of morphological characteristics and multigene phylogenetic analysis methods. Lawrence et al [40], Woudenberg et al [41], Grum-Grzhimaylo et al [42] and Ghafri et al [43] used multigene analyses to reconstruct phylogenetic relationships within the genus *Alternaria*, resulting in the division of the genus into 28 clades, each represented by a type specimen. Phylogenetic analysis of *Alternaria* using multiple nucleotide sequences, such as ITS, *mtLSU*, *endoPG*, *TUB*, *mtSSU*, *ATP*, *EF-1α*, *gpd*, *Alt a 1*, *CAL*, *CHS*, *ACT*, *OPA2-1*, *IGS*, *HIS*, *TMA22*, *PGS1*, and *REV3*, is often employed because these genes play a role in the identification of the genus *Alternaria* as well as similar interspecifics. *Alternaria* sect. *Alternaria* contains most of the small-spored *Alternaria* species with concatenated conidia, including important plant, human and postharvest pathogens [44]. *Alternaria* sect. *Alternaria* consists of only 11 phylogenetic species and one species complex [40].

Temperature is typically regarded as the primary environmental factor influencing the prevalence of plant diseases [45]. In this study, the optimal growth temperature for the representative isolates was 25 °C, mycelial growth was stagnant at 5 °C, and growth was weaker at 15 °C, 20 °C, 30 °C, and 35 °C than at 25 °C. This aligns with the findings of prior studies regarding the ideal temperature for the growth of *A. alternata* [46]. Therefore, it is advisable to implement timely pathogen control measures before the optimal growth temperature is reached. Leaf spot of *C. sinensis* tend to occur in March and April, with outbreaks peaking in May and June and continuing through

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October. During this period, the pathogen thrives in suitable environments and produces highly infectious conidia that infect the plant host.

Evaluating the biological characteristics of pathogens is of utmost significance in preventing and controlling plant disease outbreaks, as it can lay the groundwork for scientifically informed disease prevention and control measures. It is important to detect the presence of pathogens as early as possible to effectively prevent and control plant diseases [47]. Leaf spot of *C. sinensis* caused by *Alternaria* not only affect the health and appearance of plants but also have an impact on their economic value. Fungal leaf spot disease usually spreads under wet conditions and can spread to other healthy *C. sinensis* or plants by wind, raindrops, insects or artificial means, thus spreading the disease further and causing the occurrence of leaf spot disease on large areas of *C. sinensis* as well as the epidemic occurrence of other plant diseases, which disrupts the ecological balance. Therefore, the negative impacts of *C. sinensis* leaf spot on agriculture, forestry, urbanization, ecosystems and the economy are manifold, and measures need to be taken to prevent and manage this plant disease. It is necessary to inhibit the growth of fungal mycelium, thus further controlling the spread and transmission of the disease to prevent greater losses. The temperature experiment conducted in this study conclusively demonstrates the importance of determining the timing for fungicide application before the pathogen reaches its optimal growth temperature.

Different fungicides exert their fungicidal effects through various mechanisms based on their type, chemical structure, etc. [48]. Prochloraz inhibits sterol synthesis in fungal cell membranes, leading to cell wall rupture and cell death. It also interferes with fungal DNA synthesis and protein synthesis, resulting in fungicidal effects [49]. In 2009, researchers conducted in vitro susceptibility tests using six fungicides against the pathogen *Alternaria solani*. The results showed that prochloraz, with EC50 values ranging from 0.03 to 0.11 μg/ml, was effective in inhibiting the growth of the pathogen [50]. This result is consistent with the results of the present study that prochloraz has the best inhibitory effect on *A. alternata* and *A. koreana,* causing leaf spot in *C. sinensis.* However, by comparing the EC50 values, the sensitivity of prochloraz to *A. alternata* and *A. koreana* are much less than that to *A. solani*, and the inhibitory effect is still unsatisfactory. In 2023, the EC50 value of fludioxonil was only 0.089 ± 0.020 in Wang et al.'s study [51], whereas the EC50 value of prochloraz in this study was 2.92, which clearly shows that the inhibitory effect of fludioxonil on *A. alternata* was stronger. Fewer fungicides were selected in this study, and the selection of efficient, environmentally friendly, and economical fungicides needs to be further explored.

5. Conclusions

In this study, we performed the isolation of fungal pathogens and carried out pathogenicity tests. We identified three isolates (11-13) responsible for causing leaf spot in *C. sinensis* through multilocus phylogenetic analyses involving ITS, *GAPDH*, *TEF1-α*, *RPB2*, *Alt a 1* locis, as well as physical attributes. Three isolates were ascertained that *A. alternata* and *A. koreana*. The pathogen's susceptibility to the four fungicides was ascertained through phenotypic experiments conducted on culture medium plates. These sensitivity data can serve as a point of reference for assessing potential changes in the sensitivity of *Alternaria* spp. to various fungicides in the future. To our knowledge, this is the inaugural report that *A. alternata* and *A. koreana* causing leaf spot in *C. sinensis* in China and worldwide. This finding will yield valuable insights for future investigations focused on the prevention and treatment strategies for this recently emerged disease.

Author Contributions: Q.-Q.W., X.-Y.Z., Y.W., and Y.-J.Z. designed the study and were involved in writing the paper; Q.-Q.W. and Y.-J.Z. were responsible for sample collections; Q.-Q.W. was responsible for pathogenicity tests; Q.-Q.W., X.-Y.Z., and Y.W. were involved in morphological identification; Q.-Q.W., X.-Y.Z. and Y.W. were involved in phylogenetic analyses; Q.-Q.W., X.-Y.Z., Y.W., and Y.-J.Z. contributed to planning and editing of the paper. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All data generated or analyzed during this study are included in this article.

Acknowledgments: The authors would like to thank those who provided assistance and advice for this study.

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

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