Characterization and Pathogenicity of *Pestalotiopsis*-Like Species Associated With Gray Blight Disease on *Camellia sinensis* in Anhui Province, China

Shuangshuang Wang,¹ Xiaozeng Mi,¹ Zhiran Wu,² Lixin Zhang,^{2,†} and Chaoling Wei^{1,†}

¹ State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei, Anhui 230036, People's Republic of China

² College of Plant Protection, Anhui Agricultural University, Hefei, Anhui 230036, People's Republic of China

Abstract

Gray blight disease, caused by *Pestalotiopsis*-like fungi, is one of the deadliest threats to tea (*Camellia sinensis*) production. However, little information is known about the traits and characteristics of this pathogen. Here, a systematic survey was performed, and a total of 20 representative isolates were obtained from the leaves of tea plants affected by gray blight in two main tea plantations located in Anhui Province, China. Further analyses showed that two isolates were identified as *Neopestalotiopsis ellipsospora*, three isolates were regarded as *Pseudopestalotiopsis chinensis*, one isolate was considered as *Pseudopestalotiopsis camelliae-sinensis*, and the remaining isolates belonged to *Pseudopestalotiopsis*

spp., on the basis of morphological characteristics and multigene phylogenetic analyses of the internal transcribed spacer, β -tubulin, and translation elongation factor 1- α . Pathogenicity tests indicated that there were significant differences in virulence among the *Neopestalotiopsis* and *Pseudopestalotiopsis* isolates when inoculated on the leaves of the tea plant (*C. sinensis* 'Shuchazao'). Furthermore, varied pathogenicity was also observed for the same isolate when inoculated on different varieties of tea plants. To our knowledge, this is the first record of *Neopestalotiopsis ellipsospora* and *Pseudopestalotiopsis chinensis* causing gray blight disease of tea plants in China.

Tea [*Camellia sinensis* (L.) O. Kuntze] is a commercial crop that is widely grown in the tropics and subtropics (Bond 2012). Leaves are raw material for the production of tea, which is one of the three most widely consumed nonalcoholic beverages in the world (Bond 2012). Tea is produced in >50 countries, and China, India, Kenya, Sri Lanka, Vietnam, Turkey, Indonesia, and Myanmar are major producers (Hajiboland 2017). In 2017, world production of tea was 6.1 million tons, with plantation coverage at 4.1 million hectares (Food and Agriculture Organization of the United Nations 2017). However, disease poses a serious threat to tea production around the world. More than 100 diseases have been discovered in China, and about 30 diseases were widespread (Chen and Sun 2013); among these, gray blight is considered one of the most serious foliar diseases on tea plants (Chen et al. 2017, 2018b; Liu et al. 2017; Maharachchikumbura et al. 2016). Gray blight resulted in a 10 to 20% yield loss in Japan (Horikawa 1986) and a 17% production loss in southern India (Joshi et al. 2009), even affecting areas >20 hectares and leading to 50% economic losses in Hubei Province, China (Wang et al. 2017).

Pestalotiopsis-like genera are appendage-bearing conidial asexual coelomycetous fungi belonging to the Sporocadaceae (Liu et al. 2019). *Pestalotiopsis*-like genera consist of a heterogeneous group encompassing *Pestalotiopsis sensu stricto*, *Neopestalotiopsis*, and *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014). The three genera can be separated from each other based on their morphology and the combined DNA sequence data of an internal transcribed sequence (ITS), a partial β -tubulin (*TUB*), and a partial translation elongation factor 1- α (*TEF*) gene (Maharachchikumbura et al. 2014). In addition, these three genera have similar conidia including five cells consisting of three pigmented median cells and branched or unbranched apical appendages. Their differentiation is based on variations in the color of the three pigmented median cells in the conidia. Briefly, genera *Pseudopestalotiopsis* and *Pestalotiopsis* are brown or olivaceous concolorous with distinct conidiophores, and genus *Neopestalotiopsis* is versicolorous (two upper median cells darker than the lower cell) with indistinct conidiophores (Maharachchikumbura et al. 2014). Although the genus *Pseudopestalotiopsis* has similar conidial characteristics compared with the genus *Pestalotiopsis*, *Pseudopestalotiopsis* can be distinguished from *Pestalotiopsis* by a darker-colored concolorous median cell (Maharachchikumbura et al. 2014).

Various studies have reported that *Pestalotiopsis*-like species can cause disease on a variety of hosts, including necrotic lesions on leaves of *Ixora* species, ring spot on *Kadsura coccinea*, leaf spots on *Camellia* species and *Photinia serratifolia*, and fruit rot on apple (Chen et al. 2018b; Hartveld et al. 2014; Tsai et al. 2018; Xie et al. 2018; Yang et al. 2018). Solarte et al. (2018) described that isolates of genera *Neopestalotiopsis* and *Pestalotiopsis* are morphologically and pathogenically diverse but the diversity is not associated with tissue, guava cultivar, or region. Chen et al. (2018b) and Wang et al. (2017) provided evidence on the occurrence and pathogenicity of several *Pestalotiopsis*-like species on *C. sinensis*, although pathogenicity was detected only in one tea cultivar.

The outbreak of gray blight associated with severe lesions was observed in the tea growing season of 2017 in Anhui Province in China. The first symptoms were tiny brown spots, and they extended into large brown circular necrotic lesions, which were similar to the symptoms of gray blight disease described by Hamaya and Horikawa (1982). However, the existence and identity of *Pestalotiopsis*-like species associated with leaf spots on *C. sinensis* in Anhui Province

[†]Corresponding authors: C. Wei; weichl@ahau.edu.cn, and L. Zhang; zhanglixinjoyce@163.com

Funding: This work was supported by grants from the National Key Research and Development Program of China (2018YFD1000601), the Open Fund of the State Key Laboratory of Tea Plant Biology and Utilization (SKLTOF20170108), the National Natural Science Foundation of China (31171608), the Special Innovative Province Construction in Anhui Province (15czs08032), the Special Project for Central Guiding Science and Technology Innovation of Region in Anhui Province (2016080503B024), and the Programme for Changjiang Scholars and Innovative Research Team in University (IRT1101).

*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

The author(s) declare no conflict of interest.

Accepted for publication 29 May 2019.

Table 1. Details of isolates used in the phylogenetic analyses^a

Species	Isolates	Host	Location	GenBank accession number ^b			Reference
				ITS	TUB	TEF	
<i>Pseudopestalotiopsis ampullacea</i>	LC4479	Lauraceae	Jiangxi, China	KX895034	KX895343	KX895229	Liu et al. (2017)
	LC6618*	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Yunnan, China	KX895025	KX895358	KX895244	Liu et al. (2017)
<i>Pseudopestalotiopsis</i> sp. 1	DHL-4	<i>Camellia sinensis</i>	Dongzhi, China	MH699009	MH853795	MH853815	
<i>Pseudopestalotiopsis</i> sp. 2	DXY-4	<i>C. sinensis</i>	Dongzhi, China	MH699010	MH853796	MH853816	
<i>Pseudopestalotiopsis</i> sp. 3	DEC-4	<i>C. sinensis</i>	Dongzhi, China	MH699011	MH853797	MH853817	
<i>Pseudopestalotiopsis</i> sp. 4	DDZ6-2	<i>C. sinensis</i>	Dongzhi, China	MH699020	MH853806	MH853825	
	DDZ-2	<i>C. sinensis</i>	Dongzhi, China	MH699013	MH853799	MH853819	
	DAH3-2	<i>C. sinensis</i>	Dongzhi, China	MH699012	MH853798	MH853818	
	DBH-2	<i>C. sinensis</i>	Dongzhi, China	MH699014	MH853800	–	
	DSP-3	<i>C. sinensis</i>	Dongzhi, China	MH699015	MH853801	MH853820	
<i>Pseudopestalotiopsis</i> sp. 5	DSP-6	<i>C. sinensis</i>	Dongzhi, China	MH699016	MH853802	MH853821	
	DEC-6	<i>C. sinensis</i>	Dongzhi, China	MH699017	MH853803	MH853822	
<i>Pseudopestalotiopsis</i> sp. 5	DDZ6	<i>C. sinensis</i>	Dongzhi, China	MH699021	MH853807	MH853826	
<i>Pseudopestalotiopsis</i> sp. 5	DZC108-2	<i>C. sinensis</i>	Dongzhi, China	MH699018	MH853804	MH853823	
	TYDY-4	<i>C. sinensis</i>	Lujing, China	MH699019	MH853805	MH853824	
<i>Pseudopestalotiopsis</i> sp. 5	DFC-5	<i>C. sinensis</i>	Dongzhi, China	MH699022	MH853808	–	
<i>Pseudopestalotiopsis</i> sp. 5	LC3009	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Fujian, China	KX894935	KX895267	KX895152	Liu et al. (2017)
	LC3487	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Guilin, China	KX894984	KX895315	KX895201	Liu et al. (2017)
	CGMCC 3.9192	<i>Pseudopestalotiopsis camelliae-sinensis</i>	China	JN943622	KU562851	KU562850	Maharachchikumbura et al. (2016)
<i>Pseudopestalotiopsis</i> sp. 5	DAH3-6	<i>C. sinensis</i>	Dongzhi, China	MH699008	MH853794	MH853814	
	LC6695	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Yunnan, China	KX895031	KX895364	KX895249	Liu et al. (2017)
	LC6711	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Yunnan, China	KX895032	KX895365	KX895250	Liu et al. (2017)
	LC3011*	<i>Pseudopestalotiopsis camelliae-sinensis</i>	Fujian, China	KX894937	KX895269	KX895154	Liu et al. (2017)
	EC-4	<i>C. sinensis</i>	Lujing, China	MH699005	MH853791	MH853811	
	FD-6	<i>C. sinensis</i>	Lujing, China	MH699006	MH853792	MH853812	
	SC-3	<i>C. sinensis</i>	Lujing, China	MH699007	MH853793	MH853813	
<i>Pseudopestalotiopsis</i> sp. 5	MFLUCC 12-0121*	Pandanus	Thailand	KJ503812	KJ503815	KJ503818	Song et al. (2014)
	MFLUCC 12-0125	Pandanus	Thailand	KJ503813	KJ503816	KJ503819	Song et al. (2014)
<i>Pseudopestalotiopsis</i> sp. 5	CBS 272.29*	<i>Cocos nucifera</i>	Java	KM199378	KM199467	KM199553	Maharachchikumbura et al. (2014)
<i>Pseudopestalotiopsis</i> sp. 5	CBS 459.78*	<i>Hibiscus rosa-sinensis</i>	India	KM199381	KM199470	KM199560	Maharachchikumbura et al. (2014)
<i>Pseudopestalotiopsis</i> sp. 5	MFLUCC 12-0055*	<i>C. sinensis</i>	Thailand	JQ683727	JQ683711	JQ683743	Maharachchikumbura et al. (2014)
	SC011	<i>C. sinensis</i>	Thailand	JQ683726	JQ683710	JQ683742	Maharachchikumbura et al. (2014)
<i>Pseudopestalotiopsis</i> sp. 5	NBRC 112264*	<i>Averrhoa carambola</i>	Dawei, Myanmar	LC114025	LC114045	LC114065	Nozawa et al. (2017)
	NBRC 112261	–	Dawei, Myanmar	LC114022	LC114042	LC114062	Nozawa et al. (2017)
	NBRC 112255	–	Ho Chi Minh, Vietnam	LC114035	LC114055	LC114075	Nozawa et al. (2017)
<i>Pseudopestalotiopsis</i> sp. 5	NBRC 112252*	<i>Fragaria</i>	Hue, Vietnam	LC114034	LC114054	LC114074	Nozawa et al. (2017)
	NBRC 112257	–	Hue, Vietnam	LC114037	LC114057	LC114077	Nozawa et al. (2017)
<i>Pseudopestalotiopsis</i> sp. 5	NTUCC 17-001.1	<i>Ixora</i>	Taiwan, China	MG816316	MG816326	MG816336	Tsai et al. (2018)
	NTUCC 17-001.2	<i>Ixora</i>	Taiwan, China	MG816317	MG816327	MG816337	Tsai et al. (2018)
<i>Pseudopestalotiopsis</i> sp. 5	NTUCC 17-002.1	<i>Ixora</i>	Taiwan, China	MG816319	MG816329	MG816339	Tsai et al. (2018)
	NTUCC 17-002.2	<i>Ixora</i>	Taiwan, China	MG816320	MG816330	MG816340	Tsai et al. (2018)
<i>Pseudopestalotiopsis</i> sp. 5	MM14-F0015*	–	Dawei, Myanmar	LC324750	LC324751	LC324752	Nozawa et al. (2018)
<i>Pseudopestalotiopsis</i> sp. 5	MM14-F0083*	–	Kawthaung, Myanmar	LC324753	LC324754	LC324755	Nozawa et al. (2018)
<i>Neopestalotiopsis clavisporea</i>	MFLUCC 12-0280	Magnoliaceae	China	JX398978	JX399013	JX399044	Maharachchikumbura et al. (2014)
	MFLUCC 12-0281*	Magnoliaceae	China	JX398979	JX399014	JX399045	Maharachchikumbura et al. (2014)
	CBS 447.73	–	Sri Lanka	KM199374	KM199443	KM199539	Maharachchikumbura et al. (2014)

(Continued on next page)

^a Asterisks indicate the ex-type strains. New submissions are shown in bold. Dashes indicate that information is not available.^b ITS = internal transcribed spacer region, TUB = β -tubulin gene, and TEF = translation elongation factor-1 α gene.

Table 1. (Continued from previous page)

Species	Isolates	Host	Location	GenBank accession number ^b			Reference
				ITS	TUB	TEF	
<i>N. surinamensis</i>	CBS 111494	Proteaceae	Zimbabwe	JX556232	KM199462	KM199530	Maharachchikumbura et al. (2014)
	CBS 450.74*	Arecaceae	Suriname	KM199351	KM199465	KM199518	Maharachchikumbura et al. (2014)
<i>N. formicarum</i>	CBS 115.83	–	Guba	KM199344	KM199444	KM199519	Maharachchikumbura et al. (2014)
	CBS 362.72*	–	Ghana	KM199358	KM199455	KM199517	Maharachchikumbura et al. (2014)
<i>N. piceana</i>	CBS 225.30	<i>Mangifera indica</i>	–	KM199371	KM199451	KM199535	Maharachchikumbura et al. (2014)
	CBS 254.32	<i>Cocos nucifera</i>	Sulawesi, Indonesia	KM199372	KM199452	KM199529	Maharachchikumbura et al. (2014)
<i>N. ellipsospora</i>	MFLUCC 12-0283*	–	China	JX398980	JX399016	JX399047	Maharachchikumbura et al. (2014)
	MFLUCC 12-0284	–	Thailand	JX398981	JX399015	JX399046	Maharachchikumbura et al. (2014)
	CBS 115113	<i>Ardisia crenata</i>	Hong Kong	KM199343	KM199450	KM199544	Maharachchikumbura et al. (2014)
<i>N. saprophyta</i>	DAH3-1	<i>C. sinensis</i>	Dongzhi, China	MH699004	MH853790	MH853809	
	DZC108-1	<i>C. sinensis</i>	Dongzhi, China	MH699003	MH853789	MH853809	
	CBS 115452	<i>Litsea rotundifolia</i>	Hong Kong	KM199345	KM199433	KM199538	Liu et al. (2017)
<i>N. iranensis</i>	MFLUCC 12-0282*	<i>Magnolia</i>	China	JX398982	JX399017	JX399048	Liu et al. (2017)
	CBS 137767	<i>F. ananassa</i>	Iran	KM074045	KM074056	KM074053	Liu et al. (2017)
<i>N. honoluluana</i>	CBS 137768*	<i>F. ananassa</i>		KM074048	KM074057	KM074051	Liu et al. (2017)
	CBS 111535	<i>Telopea</i>	Hawaii, USA	KM199363	KM199461	KM199546	Maharachchikumbura et al. (2014)
	CBS 114495*	<i>Telopea</i>	Hawaii, USA	KM199364	KM199457	KM199548	Maharachchikumbura et al. (2014)
<i>N. aotearoa</i>	CBS 367.54*	Canvas	New Zealand	KM199369	KM199454	KM199526	Maharachchikumbura et al. (2014)
<i>N. asiatica</i>	MFLUCC 12-0286*	–	China	JX398983	JX399018	JX399049	Maharachchikumbura et al. (2014)
<i>N. eucalypticola</i>	CBS 264.37*	<i>Eucalyptus globulus</i>	–	KM199376	KM199431	KM199551	Maharachchikumbura et al. (2014)
<i>N. javaensis</i>	CBS 257.31*	<i>Cocos</i>	Java	KM199357	KM199437	KM199543	Maharachchikumbura et al. (2014)
<i>N. zimbabweana</i>	CBS 111495*	<i>Leucospermum cuneiforme</i> cv. Sunbird	Zimbabwe	–	KM199456	KM199545	Maharachchikumbura et al. (2014)
<i>N. steyaertii</i>	IMI 192475*	<i>E. viminalis</i>	Australis	KF582796	KF582794	KF582792	Maharachchikumbura et al. (2014)
<i>N. natalensis</i>	CBS 138.41*	<i>Acacia mollissima</i>	South Africa	KM199377	KM199466	KM199552	Maharachchikumbura et al. (2014)
<i>N. protearum</i>	CBS 114178*	<i>L. cuneiforme</i>	Zimbabwe	JN712498	KM199463	KM199542	Maharachchikumbura et al. (2014)
<i>N. samarangensis</i>	CBS 115451	–	China	KM199365	KM199447	KM199556	Maharachchikumbura et al. (2014)
<i>N. umbrinospora</i>	MFLUCC 12-0233*	<i>Syzygium samarangense</i>	Thailand	JQ968609	JQ968610	JQ968611	Maharachchikumbura et al. (2014)
	MFLUCC 12-0285*	–	China	JX398984	JX399019	JX399050	Maharachchikumbura et al. (2014)
<i>N. mesopotamica</i>	CBS 336.86*	<i>Pinus brutia</i>	Iraq	KM199362	KM199441	KM199555	Maharachchikumbura et al. (2014)
	CBS 299.74	<i>Eucalyptus</i>	Turkey	KM199361	KM199435	KM199541	Maharachchikumbura et al. (2014)
<i>N. foedans</i>	CGMCC 3.9123*	Mangrove plant	China	JX398987	JX399022	JX399053	Maharachchikumbura et al. (2014)
	CGMCC 3.9202	<i>Calliandra haematocephala</i>	China	JX398988	JX399023	JX399054	Maharachchikumbura et al. (2014)
<i>N. chrysea</i>	MFLUCC 12-0261*	–	China	JX398985	JX399020	JX399051	Liu et al. (2017)
	MFLUCC 12-0262	–	China	JX398986	JX399021	JX399052	Liu et al. (2017)
<i>N. rosae</i>	CBS 101057*	<i>Rosa</i>	New Zealand	KM199359	KM199429	KM199523	Liu et al. (2017)
	CBS 124745	<i>Paeonia suffruticosa</i>	USA	KM199360	KM199430	KM199524	Liu et al. (2017)
<i>Pestalotiopsis monochaeta</i>	CBS 144.97*	<i>Quercus robur</i>	Netherlands	KM199327	KM199386	KM199479	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis chamaeropsis</i>	CBS 186.71*	<i>Chamaerops humilis</i>	Italy	KM199326	KM199391	KM199473	Maharachchikumbura et al. (2014)

(Continued on next page)

Table 1. (Continued from previous page)

Species	Isolates	Host	Location	GenBank accession number ^b			Reference
				ITS	TUB	TEF	
<i>Pestalotiopsis telopeae</i>	CBS 113606	<i>Telopea</i>	Australia	KM199295	KM199402	KM199498	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis oryzae</i>	CBS 353.69*	<i>Oryza sativa</i>	Denmark	KM199299	KM199398	KM199496	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis kenya</i>	CBS 442.67*	<i>Coffea</i> sp.	Kenya	KM199302	KM199395	KM199502	Maharachchikumbura et al. (2014)
<i>Pestalotiopsis rhodomyrtus</i>	LC3413	<i>C. sinensis</i>	China	KX894981	KX895313	KX895198	Liu et al. (2017)
<i>Pestalotiopsis brachiata</i>	LC8189	<i>Camellia</i>	China	KY464143	KY464163	KY464153	Liu et al. (2017)
<i>Pestalotiopsis knightia</i>	CBS 111963	<i>Knightia</i>	New Zealand	KM199311	KM199406	KM199495	Liu et al. (2017)
<i>Pestalotiopsis parva</i>	CBS 278.35	<i>Leucothoe fontanesiana</i>	–	KM199313	KM199405	KM199509	Liu et al. (2017)
<i>Pestalotiopsis jiangxiensis</i>	LC4242	<i>Camellia</i>	China	KX895035	KX895327	KX895213	Liu et al. (2017)
<i>Pestalotiopsis lushanensis</i>	LC4344*	<i>Camellia</i>	China	KX895005	KX895337	KX895223	Liu et al. (2017)
<i>Pestalotiopsis biciliata</i>	CBS 790.68	<i>Taxus</i>	Netherlands	KM199305	KM199400	KM199507	Chen et al. (2017)
<i>Seiridium phylicae</i>	CPC 19962	<i>Phylica arborea</i>	Tristan da Cunha	LT853092	LT853239	LT853189	Bonthond et al. (2018)

remains unclear, and whether diversity correlates with geographical origins and tea cultivars is still inadequate. In addition, there is currently no information concerning whether the pathogenicity of isolates is specific to the host tissue from which they were originally derived. Thus, the objective of this study was to identify and characterize the causal agents of commonly observed gray blight symptoms on tea leaves and to verify and compare the virulence of *Pestalotiopsis*-like species on leaves of different tea plant cultivars.

Materials and Methods

Isolation of the fungus. In September and October 2017, 52 samples from 35 tea cultivars showing typical gray blight spots were collected from tea plantations located in Lujiang County (31°25'N, 117°09'E) and Dongzhi County (30°05'N, 117°01'E) in Anhui Province.

Isolates were obtained from symptomatic leaves using the procedure described by Photita et al. (2005). Tissues (4 mm²) were removed from the margins of the leaves and surface sterilized using 0.1% HgCl₂ for 2 min, 75% ethanol for 20 s, and rinsing three times with sterile distilled water for 1 min. Samples were then dried on sterilized paper towels and placed onto potato dextrose agar (PDA) plates (three samples per plate). Plates were incubated in a chamber at 28°C under a regime of 12 h of light and 12 h of darkness for 7 days. The growing edges of fungal hyphae developing from the leaf cut surface were transferred aseptically to new PDA plates. Pure colonies were obtained after three to five purifications and stored at 4°C on PDA slants (Guarnaccia et al. 2016; Photita et al. 2005).

Morphological analysis. A total of 20 representative isolates were obtained from hyphal tips of pure cultures. For colony characterization, the isolates were grown on PDA at 28°C with a 12-h photoperiod for 7 days. To obtain conidia, representative isolates were inoculated at 28°C under ultraviolet (UV) light for 7 days, after which the colony color and the shape from each culture of each isolate were recorded. Two replicates were used per isolate.

The colony characteristics were recorded daily for 7 days. Colony color was defined using the color charts of Rayner (1970), and culture characteristics were assessed according to methods described by Maharachchikumbura et al. (2014). Conidial morphologies were observed with a Nikon Eclipse Ti-S inverted microscope after 14 days of incubation and 30 conidial measurements were taken for each isolate. The isolates were identified initially by comparing morphological and cultural characteristics.

PCR amplification and sequencing. Total fungal genomic DNA was extracted from fresh mycelia using a modified protocol of Bai et al. (2015) and Akinsanmi et al. (2017). The mycelia were obtained from culture grown on PDA at 28°C for 7 days. Fresh mycelia (500 mg) were transferred into a 2-ml centrifuge tube containing two 3-mm steel balls and 500 µl of DNA lysis buffer (200 mM of Tris-HCl, pH 8.0, 50 mM of EDTA, 20 mM of NaCl, and 1%

SDS), followed by grinding for 2 min at 30 Hz in a tissue lyser (MM400; Retsch). The mixture was centrifuged at 12,000 × g for 10 min at room temperature, and the supernatant was decanted into a fresh 1.5-ml centrifuge tube. The supernatant containing DNA was precipitated with two volumes of ethanol at –20°C for 20 min. The sample was then centrifuged at 12,000 × g for 10 min, and the supernatant was discarded. The DNA was subsequently washed with 1 ml of 75% ethanol, and the centrifuge tube was inverted onto paper to dry. The genomic DNA was resuspended in 70 µl of double distilled water and visualized by staining with SYBR Green I (Solarbio) on a 1% agarose gel.

PCR amplifications used for the ITS gene region were performed with ITS5 as a forward primer (5'-GGAAGTAAAAGTCGTAA CAAGG-3') and ITS4 as a reverse primer (5'-TCTCTCCGCTTATG ATATGC-3') (White et al. 1990). Primer pairs Bt2A (5'-GGTAACC AAATCGGTGCTGCTTTC-3') and Bt2B (5'-ACCCCTCAGTGTA GTGACCCCTGGC-3') were used for the partial β-tubulin genes (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997). Primer pairs EF1-526F (5'-GTCGTYGTYATYGGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRATACCACCRATCTT-3') were used for *TEF* (Rehner 2001). For the PCR amplifications, a 25-µl reaction volume was prepared, which contained 1.5 µl of template DNA, 12.5 µl of 2× LA Premix Taq (TAKALA), 9.5 µl of double distilled water, and 0.75 µl of each primer (10 µM). PCR programs of ITS, *TUB*, and *TEF* genes were performed as described by Maharachchikumbura et al. (2013a) using an S1000 Thermal Cycler (Bio-Rad). For the ITS region, an initial denaturation step was performed at 95°C for 3 min, followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 52°C for 45 s, elongation at 72°C for 90 s, and extension at 72°C for 10 min. For *TUB*, PCR conditions were an initial step of 3 min at 95°C, followed by 35 amplification cycles of 1 min at 94°C, annealing for 50 s at 55°C, extension for 1 min at 72°C, and a final 10 min at 72°C. For *TEF*, an initial step of 5-min denaturation at 94°C was followed by 10 amplification cycles of 30 s at 94°C, 55 s at 63°C or 66°C (decreasing 1°C per cycle), and 90 s at 72°C, plus 36 cycles of 30 s at 94°C, 55 s at 53°C or 56°C, and 90 s at 72°C, followed by 7 min at 72°C. The PCR products were verified by resolving them on 1.5% agarose electrophoresis gels and staining with SYBR Green I (Solarbio). Purification and sequencing of PCR products using amplification primers was carried out at General Biosystems in Anhui, China.

Phylogenetic analyses. Phylogenetic analyses were conducted with Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0 (Kumar et al. 2016) based on the combined sequences of the ITS, *TUB*, and *TEF* genes. To obtain consensus and high-quantity sequences, the sequences were further checked and aligned using default settings of MAFFT version 7 (<https://mafft.cbrc.jp/alignment/server/>) (Liu et al. 2017; Maharachchikumbura et al. 2014) and were manually adjusted using BioEdit version 7

(Maharachchikumbura et al. 2012). Phylogenetic analyses of the *Pestalotiopsis*-like isolates were performed by the maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods in MEGA 7.0 (Kumar et al. 2016) using the concatenated sequences of three gene loci (ITS+*TUB*+*TEF*) from the selected reference strains (Chen et al. 2017; Liu et al. 2017; Maharachchikumbura et al. 2014, 2016; Nozawa et al. 2017, 2018; Tsai et al. 2018; Song et al. 2014) (Table 1). As the outgroup for the preliminary phylogenetic tree, we selected a *Seiridium* species, which belongs to Sporocadaceae (Liu et al. 2019) and was also used by Maharachchikumbura et al. (2012, 2013b) and Wang et al. (2017). The MP, NJ, and ML methods were performed with the default parameters and the robustness of the phylogenetic trees was tested by bootstrap analysis with 1,000 replicates.

Pathogenicity tests. Pathogenicity tests were performed on detached branches to evaluate the pathogenicity of *Pestalotiopsis*-like isolates from different regions. All identified isolates were cultured on PDA plates at 28°C as described above for 14 days. Spore suspension was obtained from the culture with sterile distilled water by disrupting the spores using a sterile toothpick (Harteveld et al. 2014) and was adjusted to a concentration of 10^7 conidia/ml using a hemocytometer. For the isolates that failed to produce conidia, an agar plug (0.6 cm in diameter) with mycelium was cut from the culture with a sterile cork borer and used directly as inoculum.

The pathogenicity of each isolate was assayed on healthy leaves on detached branches of cultivar Shuchazao from the original collection sites at Lujiang, Anhui Province. Prior to inoculation, the upper and lower surfaces of leaves were disinfected in 75% alcohol, rinsed with sterile water, and air dried. Isolates that failed to produce conidia were inoculated on the wounded leaves with mycelial plugs and sealed with parafilm. Colonized agar discs were prepared from a fresh colony and put onto each tea leaf that had been wounded five

times with a sterile needle (two sites per leaf, avoiding the veins). Controls were inoculated with PDA discs only. For inoculation of conidia, 50 µl of conidial suspension was inoculated onto the upper surface of tea leaves (over the five needle-piercing holes) and wrapped with parafilm to prevent loss of conidial suspension. Controls were performed by 50 µl of sterile water.

The inoculated detached branches were cultured in pure water in Erlenmeyer flasks, covered with plastic bags to provide continuous high humidity in the greenhouse ($25 \pm 2^\circ\text{C}$). After 14 days, pathogenicity on the leaves was recorded and determined by the size of the lesions (Chen et al. 2018a). At least three detached branch replicates were used per isolate. To confirm infection and fulfill Koch's postulates, pieces (4 mm²) of inoculated tea leaves were surface sterilized and plated on PDA plates, and the identity of fungal isolates was confirmed as described above.

Fig. 1. Typical symptoms of gray blight disease on tea plants in the field. The A arrow indicates symptoms with circular or irregular brown lesions. The B arrow indicates severe symptoms with brown necrotic lesions surrounded by a dark-brown border; eventually, lesions developed on large portions of a single leaf.

Fig. 2. Morphological and cultural traits of the representative *Pestalotiopsis*-like isolates obtained from tea plants in this study. **a, b, c, and d**, Colony characteristics without ultraviolet irradiation on PDA after 7 days at 28°C. **a**, Smooth edge with white to creamy color; **b**, lobate edge with concentric rings; **c**, undulating edge with petal arrangement; **d**, smooth edge, whitish with concentric rings. Conidial morphology is as follows: **e**, olivaceous concolorous median cell; **f**, versicolorous conidia; and **g**, dark-colored concolorous median cell. Scale bar = 20 µm.

Pathogenicity of *Pestalotiopsis*-like species on different tea cultivars. To determine the pathogenicity of the *Pestalotiopsis*-like species on different tea cultivars, pathogenicity assays were performed on the leaves of 10 tea cultivars, including Longjing 43, Yunkang 10, Nongkangzao, Baihaozao, Huangshanhuangzhong, Foxiangzao, Tiantaihuang, Shidacha, Huangshandanzhu, and Shidacha 2, which were collected from Dechang tea plantation (31.3°N, 117.2°E) in Shucheng County, Anhui Province.

Six representative isolates that had successfully infected the leaves of tea cultivar Shuchazao, including DAH3-1 (*N. ellipsospora*), SC-3 (*Pseudopestalotiopsis chinensis*), DZC108-1 (*N. ellipsospora*), DXY-4 (*Pseudopestalotiopsis* sp. 2), DDZ-6 (*Pseudopestalotiopsis* sp. 6), and DEC-4 (*Pseudopestalotiopsis* sp. 3), were selected and inoculated on the above 10 cultivars of tea plants, respectively. Virulence was measured as described above and three replicates were applied per isolate.

Results

Field observations and symptoms. During September and October of 2017, gray blight disease was commonly observed on leaves of many tea cultivars in two tea plantations of Anhui Province. Symptoms appeared as small spots that usually showed up first on leaf edges and apices. As they expanded, lesions became large circular or irregular brown spots with mycelium growth (Fig. 1, arrow A). In severe cases, the leaves with brown necrotic lesions were surrounded by a dark-brown border, and eventually, lesions developed on large portions of a single leaf, causing it to fall from the tea plants (Fig. 1, arrow B).

Morphology characteristics. Twenty isolates were obtained from 35 cultivars of tea plants from two sites (Lujiang County and Dongzhi County) based on morphological characteristics (Maharachchikumbura et al. 2012). Based on the ITS sequences analyzed by NCBI BLASTn, the results showed that most isolates had 99% sequence homology to the strains of *Pestalotiopsis*-like species in GenBank, whereas several isolates (DEC-6, DSP-3, DDZ-6, DFC-5, TYDY-4, and DBH-2) had 79% sequence homology to this complex of species.

Morphological characteristics were observed after 7 days of incubation, followed by subculturing. The isolates were divided into four groups based on cultural characteristics. Type 1 contained 10 isolates (DAH3-6, SC-3, DBH-2, DDZ-6, DXY-4, DAH3-2, TYDY-4, DAH3-1, DDZ6-2, and FD-6) with white to creamy color and smooth edges (Fig. 2A). Type 2 included one isolate (DZC108-1), which showed light orange color, lobate edges, and concentric rings with yellow centers (Fig. 2B). Type 3 included two isolates (DHL-4 and DDZ-2) with pure white color (Fig. 2C). Type 4 included seven isolates (EC-4, DSP-3, DSP-6, DEC-6, DZC108-2, DEC-4, and DFC-5), which lacked concentric rings (Fig. 2D). The characteristics of colony morphology and sporulation are shown in Table 2.

The conidia showed three phenotypes. One isolate (DHL-4) produced straight to slightly curved conidia with pale brown or olivaceous concolorous (equally pigmented) median cells as well as one to three apical appendages (Fig. 2E). One isolate (DZC108-1) produced versicolorous conidia with the upper two cells darker than

the lowest median cell (Fig. 2F). Fourteen isolates (DAH3-6, SC-3, FD-6, DBH-2, DDZ-6, DAH3-2, DDZ6-2, DXY-4, TYDY-4, DAH3-1, EC-4, DSP-3, DZC-4, and DZC108-2) had dark-colored concolorous median cells (Fig. 2G). Despite the fact that UV light was used to induce sporulation, the remaining isolates (DDZ-2, DSP-6, DEC-6, and DFC-5) still failed to produce spores.

Phylogenetic analyses. Phylogenetic trees were constructed using combined ITS, β -tubulin, and *TEF* gene sequences of 20 isolates in this study and reference strains of *Pestalotiopsis*-like species from GenBank (Table 1). Regarding the varied length of sequences from NCBI, the regions containing many leading or trailing gaps were removed from the ITS, *TUB*, and *TEF* alignment. The sequences for phylogenetic analyses contained 498 nucleotides including gaps for ITS, 418 for *TUB*, and 479 for *TEF*. Phylogenetic trees were constructed using ML, NJ, and MP methods and produced similar topologies; thus, only trees constructed by the ML method are shown (Figs. 3 and 4; Supplementary Fig. S1).

A phylogenetic tree was constructed using combined ITS, *TUB*, and *TEF* sequences of 20 tested isolates and 26 reference sequences. Analysis indicated that all species were separated into three clades (genera *Pestalotiopsis*, *Neopestalotiopsis*, and *Pseudopestalotiopsis*) with high bootstrap support; for the 20 isolates in this study, two belonged to the genus *Neopestalotiopsis* and 18 were grouped into the genus *Pseudopestalotiopsis*.

To identify which species from the above-tested 20 isolates were grouped, further phylogenetic analysis showed that the two *Neopestalotiopsis* isolates (DAH3-1 and DZC108-1) clustered with *N. ellipsospora* CBS 115113 with a bootstrap value of 71% (Fig. 3). For the *Pseudopestalotiopsis* group, 18 isolates clustered into 10 well-defined clades. Some isolates were closely related to the reference species. For example, FD-6, SC-3, and EC-4 formed a clade with *Pseudopestalotiopsis chinensis*, and DAH3-6 formed a clade with three strains of *Pseudopestalotiopsis camelliae-sinensis*. The remaining 14 *Pseudopestalotiopsis* isolates could be separated into individual branches because no reference sequences were present, whereas DDZ-2, DAH3-2, DBH-2, DDZ6-2, and DSP-3 clustered into *Pseudopestalotiopsis* sp. 4 and DEC-6 and DSP-6 clustered into *Pseudopestalotiopsis* sp. 5 with high bootstrap support (Fig. 4).

The pathogenicity of *Pestalotiopsis*-like species. To evaluate the differences in pathogenicity among the 20 isolated isolates, pathogenicity tests were performed on Shuchazao leaves. Our results indicated that isolates displayed different degrees of virulence, although their symptoms were similar to those occurring under natural conditions in the field (Fig. 5). Pathogenicity tests showed that of the 20 isolates, four (DZC108-1, DAH3-1, DAH3-2, and TYDY-4) could not induce lesions. The remaining 16 isolates could be divided into three categories based on the lesion diameter. Leaves attacked by mildly aggressive isolates (DAH3-6, DSP-3, DZC108-2, and DDZ6-2) had small black spots on their surfaces (lesion diameter < 3 mm). Intermediately aggressive isolates (EC-4, FD-6, SC-3, DBH-2, DHL-4, and DXY-4) initially had small black spots but developed significant symptoms on the back of leaves (lesion diameter

Table 2. Morphological characteristics of 20 *Pestalotiopsis*-like isolates obtained from tea plants and grouped according to colony surface morphotypes

Morphotype	Representative strains ^a	Colony color	Colony surface morphotype	Sporulation quantity	Conidial morphology
Type 1	DAH3-6, SC-3, FD-6, DBH-2, DDZ-6, DAH3-2, DDZ6-2, DXY-4, TYDY-4, DAH3-1	White to creamy	Smooth edge, whitish, with concentric rings, reverse similar color, dense aerial mycelium	Medium	Dark-colored concolorous median cells
Type 2	DZC108-1	Light orange	Lobate edge, concentric rings with yellow center, dense aerial mycelium	Abundant	Versicolorous conidia (the upper two cells darker than lowest median cell)
Type 3	DHL-4, DDZ-2*	Pure white	Undulating edge, petal arrangement, scattering, dense aerial mycelium	Medium	Pale brown or olivaceous concolorous
Type 4	EC-4, DSP-3, DSP-6*, DEC-6*, DZC108-2, DZC-4, DFC-5*	White to creamy	Smooth edge, white to creamy, no concentric rings, dense aerial mycelium	Abundant	Dark-colored concolorous median cells

^a Asterisks indicate the isolates that failed to produce conidia.

between 3 and 8 mm). For agar disc inoculation, DFC-5 and DEC-6 were regarded as intermediately aggressive isolates, which developed small lesions on the back of leaves (lesion diameter between 3 and 8 mm), similar to the symptoms in the field or those infected with conidial suspension. Highly aggressive isolates (DDZ-2, DSP-6, DDZ-6, and DEC-4) developed significant symptoms on the back of leaves with circular ash-brown spots after 7 days, resulting in

brown necrotic lesions by 14 days after inoculation (lesion diameter > 8 mm) (Fig. 5).

To test the pathogenicity variation on different tea varieties, 10 cultivars of tea plants were selected to be inoculated by six representative isolates involved in genera *Pseudopestalotiopsis* and *Neopestalotiopsis*. The results indicated that a given isolate differed in virulence on different cultivars (Table 3). For example,

Fig. 3. Maximum-likelihood phylogram based on combined sequences of the internal transcribed spacer, β -tubulin, and translation elongation factor-1 α gene of the two *Neopestalotiopsis* isolates in this study, with 37 selected strains of different species in the phylogenetic clades of the genus *Neopestalotiopsis*. The numbers in the bootstrap test (1,000 replicates) are shown above the branches, and only values above 50% are shown. Isolates from this study are in bold and ex-type strains are indicated with an asterisk. *Pestalotiopsis monochaeta* CBS 144.97 was used as the outgroup.

DXY-4 (*Pseudopestalotiopsis* sp. 2) showed significant virulence on Nongkangzao, Tiantaihuang, and Yunkang 10, whereas there were few to no symptoms on Baihao, Huangshanhuangzhong 2, and Huangshandanzhu (Fig. 6; Table 3). On the other hand, both DAH3-1 (*N. ellipsospora*) and DZC108-1 (*N. ellipsospora*) showed no symptoms on 10 tea cultivars (Fig. 5; Table 3). In addition, there were also significant differences within the same genus: DEC-4 (*Pseudopestalotiopsis*

sp. 3) was able to induce lesions with a diameter between 3 and 8 mm on cultivars Baihao and Huangshanhuangzhong 2, whereas DXY-4 (*Pseudopestalotiopsis* sp. 2), DDZ-6 (*Pseudopestalotiopsis* sp. 6), and SC-3 (*Pseudopestalotiopsis chinensis*) could not cause any disease symptoms at all (Table 3).

In all symptomatic leaves, Koch's postulates were satisfied by re-isolation and morphological comparison with a respective isolate. No

Fig. 4. Maximum-likelihood phylogram based on combined sequences of the internal transcribed spacer, β -tubulin, and translation elongation factor-1 α gene of the 18 *Pseudopestalotiopsis* isolates in this study, with 25 selected strains of different species in the phylogenetic clades of the genus *Pseudopestalotiopsis*. The numbers in the bootstrap test (1,000 replicates) are shown above the branches, and only values above 50% are shown. Isolates from this study are in bold and ex-type strains are indicated with an asterisk. *Pestalotiopsis biciliata* CBS 790.68 was used as the outgroup.

symptoms were observed on the control leaves, and the target pathogens were not reisolated.

Discussion

Pestalotiopsis-like species are relatively important plant pathogenic fungi that can infect a wide range of hosts to cause gray blight disease, although they can only infect wounded or stressed plants (Chen et al. 2018a; Tsai et al. 2018). A large-scale outbreak of gray blight disease occurred in Anhui tea plantations in 2017 and inflicted huge financial losses. Since gray blight disease poses a threat to the production of tea leaves by causing tea leaf loss and reduced tea material quality, it is imperative to uncover the identities and biology of the main species that cause this disease in tea plants.

To our knowledge, this is the first study documenting the pathogenicity relationships of different isolates from symptomatic leaves of

gray blight disease from tea plants located in two areas of Anhui Province. Based on the results, the diversity of fungal species associated with symptoms is similar to that reported from other tea growing areas (Chen et al. 2018a; Liu et al. 2017) and is also consistent with reports of fungi isolated from gray blight disease (Solarte et al. 2018; Tsai et al. 2018). A total of 20 representative isolates from two tea growing fields were identified and characterized. Molecular and morphological characterization, along with pathogenicity testing, demonstrated that tea gray blight was caused by more than one species, which agrees with previous studies (Chen et al. 2018a; Liu et al. 2017; Solarte et al. 2018). Some studies suggested that colony characteristics were variable and not reliable as means of identification; in contrast, conidial characteristics were more stable and reliable (Jeewon et al. 2003; Maharachchikumbura et al. 2012; Tejesvi et al. 2007). *Pestalotiopsis* species had pale brown or

Fig. 5. Typical symptoms observed in pathogenicity tests on leaves of cultivar Shuchazao caused by 20 representative *Pestalotiopsis*-like isolates 14 days after wound inoculation. **A**, Inoculation by spores (1×10^7 conidia/ml). **B**, Inoculation by agar plug (0.6 cm in diameter).

Table 3. Pathogenicity tests on leaves of 10 different tea cultivars^a

Species	Isolate	Cultivar									
		Bailhao	Longjing 43	Nongkangzao	Huangshan-huangzhong 2	Tiantaihuang	Huangshandanzhu	Shidacha	Yunkang 10	Shidacha 2	Foxiangzao
<i>Pseudopestalotiopsis</i> sp. 2	DXY-4	–	+	++	–	++	–	+	++	+	+
<i>Pseudopestalotiopsis</i> sp. 3	DEC-4	+	++	+	+	+	–	+	+	+	++
<i>Pseudopestalotiopsis</i> sp. 6	DDZ-6	–	+	++	–	++	–	++	+	++	++
<i>Pseudopestalotiopsis chinensis</i>	SC-3	–	++	++	–	–	–	++	+	++	++
<i>N. ellipsospora</i>	DAH3-1	–	–	–	–	–	–	–	–	–	–
	DZC108-1	–	–	–	–	–	–	–	–	–	–

^a The infected leaves were evaluated after 14 days of inoculation. The minus sign (–) represents no difference with the control, the plus sign (+) represents the lesion diameter between 3 and 8 mm, and two plus signs (++) represent the lesion diameter >8 mm.

olivaceous concolorous (equally pigmented) median conidial cells, whereas *Neopestalotiopsis* species had versicolorous conidia with the upper two cells darker than the lowest median cell, and *Pseudopestalotiopsis* species had dark-colored concolorous median cells (Maharachchikumbura et al. 2014). However, our findings showed inconsistent results when we took into account both morphological and phylogenetic analyses. For example, DAH3-1 and DZC108-1 could be classified as *N. ellipsozona* based on the *Neopestalotiopsis* phylogenetic tree. Within this subclade, DAH3-1 has dark-colored concolorous median cells, whereas DZC108-1 has versicolorous conidial cells (the upper two median cells are darker than the lowest one) and *N. ellipsozona* has pale brown concolorous median conidial cells (Maharachchikumbura et al. 2012). DHL-4 was separated into individual branches in the phylogenetic tree of *Pseudopestalotiopsis*. However, DHL-4 has pale brown or olivaceous coloration, whereas all other species in our study belong to the genus *Pseudopestalotiopsis*, which has dark-colored concolorous median cells. This is similar to the results of previous reports that the conidial morphology might not be sufficient to distinguish the members of *Pestalotiopsis*-like genera (Jeewon et al. 2003; Liu et al. 2010; Maharachchikumbura et al. 2011, 2012). Thus, it will be necessary to develop more loci and analyze their sequences to clarify the *Pestalotiopsis*-like genera and species.

Combined sequence analyses of ITS, *TUB*, and *TEF* genes resolved most *Pestalotiopsis*-like species used in this study successfully with high bootstrap support. Tsai et al. (2018), Solarte et al. (2018), and Nozawa et al. (2017) previously showed that a combination of ITS, *TUB*, and *TEF* genes gave better species resolution. The

multilocus DNA sequence analysis gave better species resolution than that for a single gene, and at least two genes should be used to resolve species in the genus *Pestalotiopsis* (Hu et al. 2007; Liu et al. 2010; Maharachchikumbura et al. 2012). The phylogenetic tree in our study was derived from a combination of ITS, *TUB*, and *TEF*, and results indicated that the 20 isolates could be divided into two genera with high bootstrap support.

This study helped identify the main *Pestalotiopsis*-like species affected in Anhui Province. For the genus *Neopestalotiopsis*, the phylogenetic tree was conducted including 20 ex-types. The results showed that DAH3-1 and DZC108-1 were grouped into one cluster with *N. ellipsozona* CBS 115113 with a bootstrap value of 71%, whereas *N. ellipsozona* MFLUCC12-0283 (ex-type) and *N. ellipsozona* MFLUCC12-0284 were grouped into another cluster with 99% bootstrap support. A similar result was reported by Maharachchikumbura et al. (2014), who found that *N. ellipsozona* CBS 115113 clustered with *N. ellipsozona* MFLUCC12-0283 (ex-type) and *N. ellipsozona* MFLUCC12-0284 with low bootstrap support (<50%). This suggested that strain CBS 115113 and the two isolates (DAH3-1 and DZC108-1) in this study might belong to another novel species within the group of genus *Neopestalotiopsis*. In the *Pseudopestalotiopsis* analysis, FD-6, SC-3, and EC-4 gathered well with three reference *Pseudopestalotiopsis chinensis* isolates including the ex-type LC3011, indicating that they truly belong to *Pseudopestalotiopsis chinensis*. The remaining isolates were regarded as *Pseudopestalotiopsis* sp., whereas isolates DDZ-2, DAH3-2, DBH-2, DDZ6-2, and DSP-3 always clustered into one branch in all analyses without reference sequences. The same topology also included DEC-6

Fig. 6. DXY-4 artificially inoculated diverse tea cultivars: **a**, Baihaozao; **b**, Foxiangzao; **c**, Huangshanzhong 2; **d**, Huangshandanzhu; **e**, Longjing 43; **f**, Nongkangzao; **g**, Shidacha; **h**, Shidacha 2; **i**, Shuchazao; **j**, Tiantaihuang; and **k**, Yunkang.

and DSP-3, DZC108-2, and TYDY-4. The results of clustering might indicate that the five isolates (DDZ-2, DAH3-2, DBH-2, DDZ6-2, and DSP-3) belong to *Pseudopestalotiopsis* sp. 4, two isolates (DEC-6 and DSP-6) belong to *Pseudopestalotiopsis* sp. 5, and the other two isolates (DZC108-2 and TYDY-4) belong to *Pseudopestalotiopsis* sp. 7. Further studies of *Pseudopestalotiopsis* species are therefore required to reveal whether the less informative loci lead to this poorly resolved phylogram.

In studies by McQuilken and Hopkins (2004), *Pestalotiopsis*-like fungi could infect varieties of plants with typical symptoms including browning of foliage, stems, roots, and leaves owing to their host inconsistencies. Some studies of gray blight disease resulting from *Pestalotiopsis*-like species revealed that isolates were pathogenic on wound-inoculated tea leaves (Chen et al. 2017; Hamaya and Horikawa 1982; Liu et al. 2017). Chen et al. (2017) tested the pathogenicity of *Pseudopestalotiopsis camelliae-sinensis*, *N. clavispora*, and *Pestalotiopsis camelliae* on tea cultivar Fudingdabai, and Wang et al. (2017) tested *Pestalotiopsis* pathogenicity on *C. sinensis* var. Taicha No. 12; however, none of these cultivars were included in our study. Pathogenicity tests with the 20 isolates on Shuchazao showed that virulence differed within the same genus, indicating that the *Pestalotiopsis*-like species showed considerable variation in their pathogenicity and virulence to leaves within a species complex.

In order to compare the virulence among different tea varieties, two *Pestalotiopsis*-like genera including six isolates DAH3-1 (*N. ellipsospora*), SC-3 (*Pseudopestalotiopsis chinensis*), DZC108-1 (*N. ellipsospora*), DXY-4 (*Pseudopestalotiopsis* sp. 2), DEC-4 (*Pseudopestalotiopsis* sp. 3), and DDZ-6 (*Pseudopestalotiopsis* sp. 6) were selected for infection of 10 tea cultivars. The results indicated that the pathogenicity of a given *Pestalotiopsis*-like isolate varies among different tea varieties and that isolates within the same species also showed variability in pathogenicity. Pathogenicity may be isolate dependent rather than species dependent. It is worth noting that DAH3-1 (*N. ellipsospora*) and DZC108-1 (*N. ellipsospora*) showed no significant symptoms on 11 tea cultivars in this study. Hence, we presume that *N. ellipsospora* might be an endophyte or epiphyte. Cultivar Huangshandanzhu inoculated with conidia from the six representative isolates also did not show any typical symptoms, so we propose that it may be resistant to gray blight disease. Further research using more cultivars and isolates is needed to fully understand and document cultivar specificity among the *Pestalotiopsis*-like species causing gray blight disease of tea plants. This finding was also consistent with previous results (Chen et al. 2018a; Hamaya and Horikawa 1982; Joshi et al. 2009; Maharachchikumbura et al. 2013a; Yamada et al. 2016), indicating that there is more than one species causing gray blight disease on tea plants.

To our knowledge, this is the first study to provide evidence that *N. ellipsospora* and *Pseudopestalotiopsis chinensis* are capable of causing gray blight disease on tea plants in China. We provide evidence for diversity in both the *Pseudopestalotiopsis* and *Neopestalotiopsis* spp. associated with gray blight disease on *C. sinensis* in China. In conclusion, this study provides an initial assessment of differences in the pathogenicity and cultivar specificity of each gray blight disease isolate in tea plants in Anhui Province. Because of the variability in pathogenicity and aggression of *Pestalotiopsis*-like isolates, care must be taken when selecting tea germplasm for resistance to gray blight disease. Our results will serve as a foundation for further research toward improved targeted control strategies. These findings are of great epidemiological importance for regions where gray blight disease has not yet been reported.

Literature Cited

- Akinsanmi, O. A., Nisa, S., Jeff-Ego, O. S., Shivas, R., and Drenth, A. 2017. Dry flower disease of *Macadamia* in Australia caused by *Neopestalotiopsis macadamiae* sp. nov. and *Pestalotiopsis macadamiae* sp. nov. *Plant Dis.* 101: 45-53.
- Bai, Q., Zhai, L. F., Chen, X. R., Hong, N., Xu, W. X., and Wang, G. P. 2015. Biological and molecular characterization of five *Phomopsis* species associated with pear shoot canker in China. *Plant Dis.* 99:1704-1712.
- Bond, T. J. 2012. The origins of tea, coffee and cocoa as beverages. Pages 1-24 in: Teas, Cocoa and Coffee: Plant Secondary Metabolites and Health, 1st ed., A. Crozier, H. Ashihara, and F. T. Barberán, eds. Blackwell Publishing Ltd., Oxford, UK.
- Bonthond, G., Sandoval-Denis, M., Groenewald, J. Z., and Crous, P. W. 2018. *Seiridium* (*Sporocadaceae*): an important genus of plant pathogenic fungi. *Persoonia Mol. Phylogeny Evol. Fungi* 40:96-118.
- Chen, Y. J., Zeng, L., Shu, N., Jiang, M. Y., Wang, H., Huang, Y. J., and Tong, H. R. 2018a. *Pestalotiopsis*-like species causing gray blight disease on *Camellia sinensis* in China. *Plant Dis.* 102:98-106.
- Chen, Y. J., Zeng, L., Shu, N., Wang, H., and Tong, H. R. 2017. First report of *Pestalotiopsis camelliae* causing grey blight disease on *Camellia sinensis* in China. *Plant Dis.* 101:1034.
- Chen, Y. J., Zeng, L., Shu, N., Wang, H., and Tong, H. R. 2018b. Occurrence of *Pestalotiopsis lushanensis* causing gray blight disease on *Camellia sinensis* in China. *Plant Dis.* 102:2654.
- Chen, Z. M., and Sun, X. L. 2013. Concise identity handbook of major plant diseases and insect pests of tea, 1st Ed. China Agriculture Press, Beijing, China.
- Food and Agriculture Organization of the United Nations. 2017. FAOSTAT. <http://www.fao.org/faostat/en/#data/QC>
- Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.
- Guarnaccia, V., Vitale, A., Cirvilleri, G., Aiello, D., Susca, A., and Epifani, F. 2016. Characterisation and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. *Eur. J. Plant Pathol.* 146:963-976.
- Hajiboland, R. 2017. Environmental and nutritional requirements for tea cultivation. *Folia Hort.* 29:199-220.
- Hamaya, E., and Horikawa, T. 1982. Gray blight of tea plant caused by *Pestalotia longiseta* Spegazzini. *Study Tea* 62:21-27.
- Harteveld, D. O. C., Akinsanmi, O. A., and Drenth, A. 2014. Pathogenic variation of *Alternaria* species associated with leaf blotch and fruit spot of apple in Australia. *Eur. J. Plant Pathol.* 139:789-799.
- Horikawa, T. 1986. Yield loss of new tea shoots due to grey blight caused by *Pestalotia longiseta* Spegazzini. *Bull. Shizuoka Tea Exp. Stn.* 12:1-8.
- Hu, H. L., Jeewon, R., Zhou, D. Q., Zhou, T. X., and Hyde, K. D. 2007. Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: Evidence from rDNA and β -tubulin gene phylogenies. *Fungal Divers.* 24:1-22.
- Jeewon, R., Liew, E. C. Y., Simpson, J. A., Hodgkiss, I. J., and Hyde, K. D. 2003. Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. *Mol. Phylogenet. Evol.* 27:372-383.
- Joshi, S. D., Sanjay, R., Baby, U. I., and Mandal, A. K. A. 2009. Molecular characterization of *Pestalotiopsis* spp. associated with tea (*Camellia sinensis*) in southern India using RAPD and ISSR markers. *Indian J. Biotechnol.* 8:377-383.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870-1874.
- Liu, A. R., Chen, S. C., Wu, S. Y., Xu, T., Guo, L. D., Jeewon, R., and Wei, J. G. 2010. Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy. *Mol. Phylogenet. Evol.* 57:528-535.
- Liu, F., Bonthond, G., and Groenewald, J. Z. 2019. *Sporocadaceae*, a family of coelomycetous fungi with appendage-bearing conidia. *Stud. Mycol.* 92: 287-415.
- Liu, F., Hou, L., Raza, M., and Cai, L. 2017. *Pestalotiopsis* and allied genera from *Camellia*, with description of 11 new species from China. *Sci. Rep.* 7:1-19.
- Maharachchikumbura, S. S. N., Chuakeatirote, E., Guo, L. D., Crous, P. W., McKenzie, E. H. C., and Hyde, K. D. 2013a. *Pestalotiopsis* species associated with *Camellia sinensis* (tea). *Mycotaxon* 123:47-61.
- Maharachchikumbura, S. S. N., Guo, L. D., Cai, L., Chuakeatirote, E., Wu, W. P., Sun, X., Crous, P. W., Bhat, D. J., McKenzie, E. H. C., Bahkali, A. H., and Hyde, K. D. 2012. A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. *Fungal Divers.* 56:95-129.
- Maharachchikumbura, S. S. N., Guo, L. D., Chuakeatirote, E., Bahkali, A. H., and Hyde, K. D. 2011. *Pestalotiopsis*—morphology, phylogeny, biochemistry and diversity. *Fungal Divers.* 50:167-187.
- Maharachchikumbura, S. S. N., Guo, L. D., Liu, Z. Y., and Hyde, K. D. 2016. *Pseudopestalotiopsis ignota* and *Ps. camelliae* spp. nov. associated with grey blight disease of tea in China. *Mycol. Prog.* 15:22-28.
- Maharachchikumbura, S. S. N., Hyde, K. D., Groenewald, J. Z., Xu, J., and Crous, P. W. 2014. *Pestalotiopsis* revisited. *Stud. Mycol.* 79:121-186.
- Maharachchikumbura, S. S. N., Zhang, Y., Wang, Y., and Hyde, K. D. 2013b. *Pestalotiopsis anacardiacearum* sp. nov. (Amphisphaeriaceae) has an intricate relationship with *Penicillaria jocosatrix* the mango tip borer. *Phytotaxa* 99:49-57.
- McQuilken, M. P., and Hopkins, K. E. 2004. Biology and integrated control of *Pestalotiopsis* on container grown ericaceous crops. *Pest Manag. Sci.* 60:135-142.
- Nozawa, S., Ando, K., Phay, N., and Watanabe, K. 2018. *Pseudopestalotiopsis dawaina* sp. nov. and *Ps. kawthaungina* sp. nov.: Two new species from Myanmar. *Mycol. Prog.* 17:865-870.
- Nozawa, S., Yamaguchi, K., Van Hop, D., Phay, N., Ando, K., and Watanabe, K. 2017. Identification of two new species and a sexual morph from the genus *Pseudopestalotiopsis*. *Mycoscience* 58:328-337.

- O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- Photita, W., Taylor, P., Ford, R., Hyde, K., and Lumyong, S. 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Divers.* 18:117-133.
- Rayner, R. W. 1970. A Mycological Colour Chart. Commonwealth Mycological Institute and British Mycological Society, Kew, UK.
- Rehner, S. A. 2001. Primers for elongation factor 1- α (EF1- α).
- Solarte, F. A., Muñoz, C. G., Maharachchikumbura, S. S. N., and Álvarez, E. 2018. Diversity of *Neopestalotiopsis* and *Pestalotiopsis* spp., causal agents of guava scab in Colombia. *Plant Dis.* 102:49-59.
- Song, Y., Tangthirasun, N., Maharachchikumbura, S. S., Jiang, Y., Xu, J., Hyde, K. D., and Wang, Y. 2014. Novel *Pestalotiopsis* species from Thailand point to the rich undiscovered diversity of this chemically creative genus. *Cryptogam., Mycol.* 35:139-149.
- Tejesvi, M. V., Nalini, M. S., Mahesh, B., Prakash, H. S., Kini, K. R., Shetty, H. S., and Subbiah, V. 2007. New hopes from endophytic fungal secondary metabolites. *Bol. Soc. Quim. Mex.* 1:19-26.
- Tsai, I., Maharachchikumbura, S. S., Hyde, K. D., and Ariyawansa, H. A. 2018. Molecular phylogeny, morphology and pathogenicity of *Pseudopestalotiopsis* species on *Ixora* in Taiwan. *Mycol. Prog.* 17:941-952.
- Wang, Z. H., Zhao, Z. X., Hong, N., Ni, D., Cai, L., Xu, W. X., and Xiao, Y. N. 2017. Characterization of causal agents of a novel disease inducing brown-black spots on tender tea leaves in China. *Plant Dis.* 101:1802-1811.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. W. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. N. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
- Xie, J., Wei, J. G., Huang, R. S., Wei, J. F., Luo, J. T., Yang, X. H., and Yang, X. B. 2018. First report of ring spot on *Kadsura coccinea* caused by *Neopestalotiopsis clavispora* in China. *Plant Dis.* 102:2032.
- Yamada, K., Sonoda, R., and Ishikawa, K. 2016. Population genetic structure of QoI-resistant *Pestalotiopsis longiseta* isolates causing tea gray blight. *Plant Dis.* 100:1686-1691.
- Yang, Q. Q., Li, F., Guo, Q. Q., and Liang, W. X. 2018. First report that *Pestalotiopsis clavispora* causes leaf spots on *Photinia serratifolia* in China. *Plant Dis.* 102:1457.