Pestalotiopsis-Like Species Causing Gray Blight Disease on *Camellia sinensis* in China

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Abstract

Gray blight of tea, caused by several *Pestalotiopsis*-like species, is one of the most destructive foliar diseases in tea cultivation yet the characteristics of these pathogens have not been confirmed until now. With morphological and multigene phylogenetic analyses, we have identified the gray blight fungi as *Pseudopestalotiopsis camelliae-sinensis*, *Neopestalotiopsis clavispora*, and *Pestalotiopsis camelliae*. Phylogenetic analyses derived from the combined internal transcribed spacer, β -tubulin, and translation elongation factor 1- α gene regions successfully resolved most of the *Pestalotiopsis*-like species used in this study with high bootstrap

supports and revealed three major clusters representing these three species. Differences in colony appearance and conidia morphology (shape, size, septation, color and length of median cells, and length and number of apical and basal appendages) were consistent with the phylogenetic grouping. Pathogenicity tests validated that all three species isolated from tea leaves were causal agents of gray blight disease on tea plant (*Camellia sinensis*). This is the first description of the characteristics of the three species *Pseudopestalotiopsis camelliae-sinensis*, *N. clavispora*, and *Pestalotiopsis camelliae* as causal agents of tea gray blight disease in China.

Gray blight, caused by *Pestalotiopsis*-like species, is one of the most destructive diseases of tea (*Camellia sinensis* (L.) O. Kuntze) (Muraleedharan and Chen 1997; Sanjay et al. 2008), and has been reported to cause 17% production loss in southern India (Joshi et al. 2009) and 10 to 20% yield loss in Japan (Horikawa 1986). *Pestalotiopsis* is a species-rich asexual genus with appendage-bearing conidia in the *Amphisphaeriaceae* (Barr 1975, 1990; Kang et al. 1999; Lee et al. 2006). Because of their roles as both an important plant pathogen and a commonly isolated endophyte, *Pestalotiopsis* spp. have received considerable attention in recent years (Joshi et al. 2009; Wei et al. 2007). Species of *Pestalotiopsis* are primarily thought of as weak or opportunistic pathogens that may cause little damage to ornamental plants (Pirone 1978). However, recent reports confirmed that *Pestalotiopsis* spp. are common phytopathogens that can cause a variety of diseases, including leaf spots, gray blight, severe chlorosis, and various postharvest diseases on a wide range of economic crops such as apple, blueberry, coconut, grapevine, guava, hazelnut, lychee, mango, ginger, and tea plant (Crous et al. 2011; Maharachchikumbura et al. 2012, 2013a, b; Zhang et al. 2012a,b, 2013). The number of known *Pestalotiopsis* spp. is generally increasing, confirming the pathogen's widespread distribution (Hopkins and McQuilken 2000). However, thus far, only several *Pestalotiopsis* spp. have been reported to occur on *C. sinensis*. Among them, *Pestalotiopsis longisetata* has been recorded to cause severe damage to tea production in Japan (Joshi et al. 2009; Takeda 2002) and *P. camelliae* has been reported to cause gray blight disease in China (Chen et al. 2017b).

Pseudopestalotiopsis and *Neopestalotiopsis* are two novel genera segregated from *Pestalotiopsis*. The three genera can be easily distinguished on the basis of combined morphological characteristics and DNA sequence analysis of internal transcribed spacer (ITS), partial β -tubulin, and partial translation elongation factor 1- α (TEF) gene regions (Maharachchikumbura et al. 2014, 2016). Thus far, there are several species recorded in *Pseudopestalotiopsis*: *Pseudopestalotiopsis camelliae*, *P. ignota*, *P. cocos*, *P. indica*, *P. kubahensis*, *P. ampullacea*, *P. chinensis*, and *P. theae* (Lateef et al. 2015; Liu et al. 2017; Maharachchikumbura et al. 2014, 2016). Of these, *P. theae*

and *P. camelliae* are considered to be economically significant species that have been reported in major tea-growing countries all over the world (Agnihothrudu 1964; Maharachchikumbura et al. 2016; Muraleedharan and Chen 1997). *Neopestalotiopsis* spp. having versicolorous median cells can be easily distinguished from *Pseudopestalotiopsis* spp. by gene sequence data and generally darkly colored concolorous median cells (Maharachchikumbura et al. 2014). Thus far, several *Neopestalotiopsis* isolates associated with *C. sinensis* have been reported by Liu et al. (2017). However, due to the unstable tree topologies, *Neopestalotiopsis* isolates were not classified (Liu et al. 2017). The objective of this study was to identify and characterize the causal agents of commonly observed gray blight symptoms on tea leaves and to compare the *Pestalotiopsis*-like species that have gone largely unrecognized.

Materials and Methods

Morphological analysis. More than 80 naturally infected tea leaves were collected from several commercial tea plantations located in the Chongqing district of China. Plant materials were surface disinfected, cut into small pieces (4 mm²), and placed in 9-cm-diameter petri dishes containing 10 ml of potato dextrose agar medium (PDA). All plates were incubated at 25 $\pm$ 2°C with 12 h of darkness. Cultural morphology was examined after 5 to 7 days. The morphological criteria of conidia such as size, shape, color and length of median cells, septation, and number of apical and basal appendages were recorded (Keith et al. 2006; Sutton 1980). Conidial size (length and width) and lengths of median cells and apical and basal appendages were determined by measuring arbitrarily selected conidia from a conidial suspension of each isolate that was prepared in sterile distilled water. Conidial characteristics of all isolates were observed with a U-TV0.5XC-3 microscope (Olympus) and 30 conidial measurements were taken for each isolate.

Polymerase chain reaction amplification and sequencing. The fungal genomic DNA was extracted from fresh mycelium by a cetyltrimethylammonium bromide method, according to the procedure of Than et al. (2008) and Chen et al. (2017a). The mycelium of each isolate was obtained from cultures grown on PDA for 5 days. Five pairs of primers were used for polymerase chain reaction (PCR) amplification (Table 1). The amplified products were separated on 1% agarose gel and purified using the Gel Extraction Kit (Omega Bio-Tek), according to the manufacturer's protocol. The purified PCR products were cloned into the pMD19-T Vector (TaKaRa). The positive transformants were sequenced at the Shanghai Sangon Biotech Company.

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Molecular characterization. To identify the isolates obtained in this study, sequences were subjected to BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the National Center for Biotechnology Information (NCBI) database. Sixteen representative isolates (LB01, LB02, LB05-LB08, and LB11 to LB20) of this study were selected for phylogenetic analyses and their homologous ITS, β -tubulin, and TEF gene sequences of known species in the NCBI GenBank database were retrieved for comparison (Table 2). Phylogenetic trees were built and viewed in Molecular Evolutionary Genetic Analysis software, version 6.0, with both the maximum-parsimony (MP) and neighbor-joining (NJ) methods derived from individual and combined ITS, β -tubulin, and TEF gene sequences. Multiple sequence alignments of each gene were constructed using ClustalW and manually adjusted to allow maximum sequence similarity (Tamura et al. 2013). To determine the confidence values for the grouping within a tree, a bootstrap analysis was performed using 1,000 resamplings of the data (Saitou and Nei 1987; Tamura et al. 2013).

Pathogenicity tests. The pathogenicity of each isolate was tested on leaves of 2-year-old potted ‘Fudingdabai’ tea seedlings that were surfaced disinfected with 75% ethanol. For each isolate, 20 leaves on tea seedlings were inoculated. Conidial suspensions (8 μ l) were placed at wounded and nonwounded sites on the middle of the tea leaves (Chen et al. 2017a; Lin et al. 2002; Than et al. 2008). Wound inoculation involved pinpricking the middle of the leaves (two sites per leaf along the longitudinal axis) and then placing a conidial suspension over each wound. Conidial suspensions were prepared according to the procedure of Chen et al. (2017a). Tea leaves inoculated with only sterile water served as controls. Inoculated tea seedlings were covered with plastic bags to maintain high relative humidity for 2 days and maintained in the greenhouse at $25 \pm 2^\circ\text{C}$ until disease symptoms appeared. All fungal isolates included in pathogenicity tests were reisolated from the diseased tea leaves to confirm their identity with both molecular and morphological methods, as described above. Disease incidence (infected leaves) was assessed 7 to 15 days after inoculation by counting the number of diseased leaves compared with the total inoculated leaves. The virulence of the isolates was evaluated by measuring the diameter of the necrotic lesions which developed on tea leaves 7 days after wound inoculation. Experiments were repeated twice, with similar results. Statistical analysis was carried out with IBM SPSS Statistics 20.0 by one-way analysis of variance and means were compared by Tukey’s honestly significant difference test ($P \leq 0.05$).

Results

Disease survey. During the tea growing season of 2014 and 2015, gray blight was commonly observed on popular tea cultivars in tea plantations of Chongqing, China. Symptoms began as small brown spots that gradually expanded to large, circular to irregular black and brown necrotic lesions surrounded by a dark-brown border; in severe cases, lesions developed on large portions of a single leaf and, eventually, the dead leaves fell from the tea plants (Fig. 1A). Dark acervuli formed on the upper surface of the lesions and the color of necrotic lesions changed to light gray at later stages of disease.

Cultural characteristics. In total, 16 isolates were selected from more than 80 isolates for further identification based on differences in

field symptoms and cultural morphology on PDA. The isolates were placed into three groups based on colony characteristics. Among these, eight isolates (LB01, LB02, LB05 to LB08, LB15, and LB16), producing conidia with brown or olivaceous concolorous median cells, were identified as the species of *Pseudopestalotiopsis* (Fig. 2A); four isolates (LB11 to LB14), producing conidia with versicolorous median cells, belonged to the genus *Neopestalotiopsis* (Fig. 2B); and the remaining four isolates (LB17 to LB20), producing conidia with light to dark-brown concolorous median cells but without basal appendages, were identified as *Pestalotiopsis* (Fig. 2C). All of the isolates of *Pseudopestalotiopsis* and *Neopestalotiopsis* cultured on PDA grew quickly, attaining 76 to 85 mm in diameter after 5 days at 25°C . Colonies of these two genera were white to creamy; composed of concentric rings; with undulate edge, whitish, dense aerial mycelium on the surface; and white to pale orange color diffused in the reverse side (Fig. 1B and C). By contrast, the colonies of *Pestalotiopsis* (LB17 to LB20) on PDA had no concentric rings, a smooth edge, and thin mycelium (Fig. 1D). The conidiomata of all isolates formed on the surface were black, gregarious, and diffused throughout the culture mediums (Fig. 1B, C, and D).

Conidial morphology. All of the isolates belonging to the genera *Pseudopestalotiopsis* and *Neopestalotiopsis* produced five-celled conidia that were fusiform, straight or slightly curved, with three-colored median cells and two colorless terminal cells as well as two to four hyaline apical appendages (most were three, rarely two or four) and one hyaline basal appendage arising from the apex (Fig. 2A and B). Lengths of basal appendages of the isolates in two groups were similar. However, the isolates in two genera can be clearly and easily differentiated by the size of conidia, the length of apical appendages, and the color and length of median cells. The conidial size of *Pseudopestalotiopsis* isolates ranged from 22.5 to 36.0 μm in length by 4.3 to 8.5 μm in width, and were much longer than those of *Neopestalotiopsis* isolates (Table 3). Lengths of apical appendages of *Pseudopestalotiopsis* were 15.1 to 38.0 μm , distinctly longer than those of the isolates in genus *Neopestalotiopsis*, which were 12.2 to 23.4 μm . The three median cells of isolates in genus *Pseudopestalotiopsis* were concolorous, dark brown or olivaceous (Fig. 2A), with the length ranging from 15.6 to 26.0 μm (Table 3), whereas the median cells of *Neopestalotiopsis* were versicolorous, the upper two cells were dark brown, and the lowest one was pale brown (Fig. 2B), with the length ranging from 11.0 to 17.2 μm (Table 3). In contrast, although the isolates of *Pestalotiopsis* also produced concolorous median cells, the color of median cells was light to dark brown, the basal appendages arising from the apex were absent, and the number of hyaline apical appendages was three to seven (Fig. 2C), all of which distinguished this genus from the other two.

Phylogenetic analysis. Five gene regions of ITS, β -tubulin, TEF, glyceraldehyde-3-phosphate dehydrogenase, and actin were utilized to resolve species delimitation but, due to the failure in PCR amplification or in their ability to resolve species boundaries, only ITS, β -tubulin, and TEF proved to be useful markers for phylogenetic analysis. Sequences obtained in this study were compared with homologous gene sequences retrieved from the NCBI GenBank database (Table 2). Phylogenetic trees were performed by both MP and

Table 1. Primer pairs used to amplify gene regions of *Pestalotiopsis*-like species

Gene ^a	Primer	Gene sequence	Reference
ITS	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al. 1990
	ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	...
β -tubulin	T1	5'-AACATGCGTGAGATTGTAAGT-3'	O'Donnell and Cigelnik 1997
	β t2b	5'-ACCCTCAGTGTAAGTACCCTTGGC-3'	...
TEF	EF1-526F	5'-GTCGYGTGYATYGGHCAYGT-3'	Rehner 2001
	EF1-1567R	5'-ACHGTRCCRATACCACCRATCTT-3'	...
GAPDH	GDF1	5'-GCCGTCAACGACCCCTTCATTGA-3'	Guerber et al. 2003
	GDR1	5'-GGGTGGAGTCGTACTTGACATGT-3'	...
ACT	ACT-512F	5'-ATGTGCAAGGCCGGTTTCGC-3'	Carbone and Kohn 1999
	ACT-783R	5'-TACGAGTCCTTCTGGCCCAT-3'	...

^a ITS = internal transcribed spacer, TEF = translation elongation factor 1- α , GAPDH = glyceraldehyde-3-phosphate dehydrogenase, and ACT = actin.

Table 2. Species isolated from tea plants and the reference isolates used for sequence comparisons and phylogenetic analyses

Species, isolates	Host plant	Geographical origin	GenBank accession number ^z		
			ITS	β-Tubulin	TEF
<i>Pseudopezalotiopsis camelliae-sinensis</i>					
LB01	<i>Camellia sinensis</i>	China	KX757707	KX757720	KY342347
LB02	<i>C. sinensis</i>	China	KX757708	KX757721	KY342348
LB05	<i>C. sinensis</i>	China	KX757711	KX757724	KY342351
LB06	<i>C. sinensis</i>	China	KX757712	KX757725	KY342352
LB07	<i>C. sinensis</i>	China	KX757713	KX757726	KY342353
LB08	<i>C. sinensis</i>	China	KX757714	KX757727	KY342354
LB15	<i>C. sinensis</i>	China	KY319132	KY363537	KY342357
LB16	<i>C. sinensis</i>	China	KY319133	KY363538	KY342358
LC3571	<i>C. sinensis</i>	China	KX894986	KX895317	KX895203
LC3023	<i>C. sinensis</i>	China	KX894943	KX895275	KX895160
LC3022	<i>C. sinensis</i>	China	KX894942	KX895274	KX895159
LC3020	<i>C. sinensis</i>	China	KX894940	KX895272	KX895157
LC3009	<i>C. sinensis</i>	China	KX894935	KX895267	KX895152
LC3010	<i>C. sinensis</i>	China	KX894936	KX895268	KX895153
<i>P. camelliae</i>					
CGMCC 3.9192	<i>C. sinensis</i>	China	JN943622	KU562851	KU562850
<i>P. theae</i>					
MAFF 752011	<i>Thea sinensis</i>	Japan: Tokushima	AB482210	AB453902	AB453858
<i>P. chinensis</i>					
LC6695	<i>C. sinensis</i>	China	KX895031	KX895364	KX895249
LC6711	<i>C. sinensis</i>	China	KX895032	KX895365	KX895250
LC6629	<i>C. sinensis</i>	China	KX895026	KX895359	KX895245
<i>P. smittheae</i>					
MFLUCC 12-0121	<i>Pandanus odoratissimus</i>	Thailand	KJ503812	KJ503815	KJ503818
MFLUCC 12-0125	Living leaves of Pandanus	Thailand	KJ503813	KJ503816	KJ503819
<i>P. cocos</i>					
CBS 272.29	<i>Cocos nucifera</i>	Indonesia	KM199378	KM199467	KM199553
<i>P. ampullaceal</i>					
LC6618	<i>Camellia sinensis</i>	China	KX895025	KX895358	KX895244
<i>P. indica</i>					
CBS 459.78	<i>Hibiscus rosa-sinensis</i>	India	KM199381	KM199470	KM199560
<i>Pseudopezalotiopsis</i> sp. 3-KW-2016					
14	Unknown	Myanmar	LC114034	LC114054	LC114074
<i>Pseudopezalotiopsis</i> sp.					
SC5A7	<i>C. sinensis</i>	China	KX146657	KX146775	KX146716
HUN1A5	<i>C. sinensis</i>	China	KX146646	KX146764	KX146705
GX1D13	<i>C. sinensis</i>	China	KX146645	KX146763	KX146704
<i>Neopezalotiopsis clavispora</i>					
LB11	<i>C. sinensis</i>	China	KX757717	KX757730	KY342359
LB12	<i>C. sinensis</i>	China	KX757718	KX757731	KY342360
LB13	<i>C. sinensis</i>	China	KX757719	KX757732	KY342361
LB14	<i>C. sinensis</i>	China	KY319134	KY363536	KY342362
CBS 447.73	Decaying wood	Sri Lanka	KM199374	KM199443	KM199539
<i>N. samarangensis</i>					
CBS 115451	Unidentified tree	Hong Kong	KM199365	KM199447	KM199556
<i>N. piceana</i>					
CBS 225.30	<i>Mangifera indica</i>	–	KM199371	KM199451	KM199535
CBS 254.32	<i>Cocos nucifera</i>	Indonesia: Sulawesi	KM199372	KM199452	KM199529
CBS 394.48	<i>Picea</i> sp.	United Kingdom	KM199368	KM199453	KM199527
<i>N. rosae</i>					
CBS 101057	<i>Rosa</i> sp.	New Zealand	KM199359	KM199429	KM199523
CBS 124745	<i>Paeonia suffruticosa</i>	United States	KM199360	KM199430	KM199524
<i>N. ellipsospora</i>					
CBS 115113	<i>Ardisia crenata</i>	Hong Kong	KM199343	KM199450	KM199544
<i>N. javaensis</i>					
CBS 257.31	<i>C. nucifera</i>	Indonesia: Java	KM199357	KM199437	KM199543
<i>N. mesopotamica</i>					
CBS 336.86	<i>Pinus brutia</i>	Iraq	KM199362	KM199441	KM199555
CBS 299.74	<i>Eucalyptus</i> sp.	Turkey	KM199361	KM199435	KM199541
<i>Neopezalotiopsis</i> sp.					
CBS 664.94	<i>C. nucifera</i>	The Netherlands	KM199354	KM199449	KM199525
CBS 177.25	<i>Dalbergia</i> sp.	–	KM199370	KM199445	KM199533
CBS 274.29	<i>C. nucifera</i>	Indonesia: Java	KM199375	KM199448	KM199534

(continued on next page)

^z ITS = internal transcribed spacer and TEF = translation elongation factor 1-α.

Table 2. (continued from previous page)

Species, isolates	Host plant	Geographical origin	GenBank accession number ^z		
			ITS	β-Tubulin	TEF
LC6320	<i>Camellia sinensis</i>	China	KX895018	KX895351	KX895237
LC3318	<i>C. sinensis</i>	China	KX894964	KX895296	KX895181
LC6489	<i>C. sinensis</i>	China	KX895020	KX895353	KX895239
LC6288	<i>C. sinensis</i>	China	KX895014	KX895347	KX895233
<i>N. formicarum</i>					
CBS 115.83	Plant debris	Cuba	KM199344	KM199444	KM199519
CBS 362.72	Dead Formicidae (ant)	Ghana	KM199358	KM199455	KM199517
<i>N. aotearoa</i>					
CBS 367.54; ATCC 11763; QM 381	Canvas	New Zealand	KM199369	KM199454	KM199526
<i>Pestalotiopsis camelliae</i>					
LB17	<i>C. sinensis</i>	China	KY319135	KY363539	KY342363
LB18	<i>C. sinensis</i>	China	KY319136	KY363540	KY342364
LB19	<i>C. sinensis</i>	China	KY319137	KY363541	KY342365
LB20	<i>C. sinensis</i>	China	KY319138	KY363542	KY342366
LC3154	<i>C. sinensis</i>	China	KX895176	KX895291	KX895176
LC3346	<i>C. sinensis</i>	China	KX894970	KX895302	KX895187
LC3343	<i>C. sinensis</i>	China	KX894969	KX895301	KX895186
LC3348	<i>C. sinensis</i>	China	KX894971	KX895303	KX895188
CBS 443.62	<i>C. sinensis</i>	Turkey	KM199336	KM199424	KM199512
<i>P. yanglingensis</i>					
LC3412	<i>C. sinensis</i>	China	KX894980	KX895312	KX895197
LC3153	<i>C. sinensis</i>	China	KX894958	KX895290	KX895175
LC3152	<i>C. sinensis</i>	China	KX894957	KX895289	KX895174
<i>Pestalotiopsis</i> sp.					
CBS 263.33	<i>Rhododendron ponticum</i>	The Netherlands	KM199316	KM199414	KM199489
CBS 264.33	<i>Cocos</i> sp.	Indonesia: Sulawesi	KM199322	KM199412	KM199490
<i>P. papuana</i>					
CBS 331.96	Coastal soil	Papua New Guinea	KM199321	KM199413	KM199491
CBS 887.96	<i>Cocos</i> sp.	Papua New Guinea	KM199318	KM199415	KM199492
<i>P. diploclisia</i>					
CBS 115449	<i>Psychotria</i>	Hong Kong	KM199314	KM199416	KM199485
CBS 115585	<i>Diploclisia</i>	Hong Kong	KM199315	KM199417	KM199483
CBS 115587	<i>Diploclisia</i>	Hong Kong	KM199320	KM199419	KM199486
<i>P. malayana</i>					
CBS 102220	<i>Macaranga</i>	Malaysia	KM199306	KM199411	KM199482
<i>P. monochaeta</i>					
CBS 144.97	<i>Quercus robur</i>	The Netherlands	KM199327	KM199386	KM199479
CBS 440.83	<i>Taxus baccata</i>	The Netherlands	KM199329	KM199387	KM199480
<i>P. hollandica</i>					
CBS 265.33	<i>Sciadopitys verticillata</i>	The Netherlands	KM199328	KM199388	KM199481
<i>P. furcata</i>					
LC6601	<i>C. sinensis</i>	China	KX895024	KX895357	KX895243
<i>P. grevilleae</i>					
CBS 114127; STE-U 2919	<i>Grevillea</i> sp.	Australia	KM199300	KM199407	KM199504
<i>P. novae-hollandiae</i>					
CBS 130973	<i>Banksia grandis</i>	Australia	KM199337	KM199425	KM199511
<i>P. oryzae</i>					
CBS 353.69	<i>Oryza sativa</i>	Denmark	KM199299	KM199398	KM199496
CBS 171.26	Unknown	Italy	KM199304	KM199397	KM199494
CBS 111522; STE-U 2083	<i>Telopea</i> sp.	Hawaii	KM199294	KM199394	KM199493
<i>P. telopeae</i>					
CBS 113606; STE-U 3082	<i>Telopea</i> sp.	Australia	KM199295	KM199402	KM199498
CBS 114161; STE-U 3083	<i>Telopea</i> sp.	Australia	KM199296	KM199403	KM199500
<i>P. australasiae</i>					
CBS 114141; STE-U 2949	<i>Protea</i> sp.	Australia	KM199298	KM199410	KM199501
<i>P. biciliata</i>					
CBS 790.68	<i>Taxus</i>	The Netherlands	KM199305	KM199400	KM199507
CBS 124463	<i>Platanus</i>	Slovakia	KM199308	KM199399	KM199505
CBS 236.38	<i>Paeonia</i>	Italy	KM199309	KM199401	KM199506
<i>P. knightiae</i>					
CBS 111963; STE-U 2905	<i>Knightia</i>	New Zealand	KM199311	KM199406	KM199495
<i>P. keniana</i>					
CBS 442.67	<i>Coffea</i> sp.	Kenya	KM199302	KM199395	KM199502
CBS 911.96	Raw material from agar-agar	–	KM199303	KM199396	KM199503

Fig. 1. Symptoms of gray blight on naturally infected tea leaves and the cultural traits of species isolated from symptomatic leaves. **A**, Typical symptoms caused by *Pestalotiopsis*-like species and cultural traits of **B**, *Pseudopezalotiopsis*; **B**, *Neopezalotiopsis*; and **D**, *Pestalotiopsis* isolates on potato dextrose agar at 25°C for 7 days.

Fig. 2. Conidial morphology of **A**, 12 isolates of *Pseudopezalotiopsis*; **B**, 4 isolates of *Neopezalotiopsis*; and **C**, 4 isolates of *Pestalotiopsis* on potato dextrose agar at 25°C for 10 days (scale bar = 20 µm).

Table 3. Conidial characteristics of the *Pestalotiopsis*-like species in the present study^y

Species, isolate	CL (µm)	CW (µm)	Median cells ^z	Middle (µm)	Apical (µm)	Basal (µm)	N
<i>Pseudopezalotiopsis camelliae-sinensis</i>							
LB01	22.5–34.0	5.2–7.8	Con, DB, O	16.5–22.0	25.1–38.0	4.2–9.3	3–4
LB02	24.2–35.0	6.2–8.5	Con, DB, O	17.8–24.1	22.0–36.1	4.8–7.8	3–4
LB05	27.3–36.0	5.4–8.4	Con, DB, O	16.8–24.1	16.2–36.1	4.8–8.1	3–4
LB06	25.6–32.0	5.1–7.8	Con, DB, O	17.1–22.1	25.1–35.2	5.8–8.3	3–4
LB07	23.2–31.0	4.3–7.2	Con, DB, O	17.2–24.1	15.1–38.0	5.6–8.9	3–4
LB08	28.1–33.0	5.7–7.9	Con, DB, O	16.8–24.0	26.0–31.1	5.2–8.2	2–4
LB15	26.7–34.0	5.4–7.4	Con, DB, O	17.2–26.0	25.0–35.0	4.3–7.9	3–4
LB16	24.7–33.1	5.1–7.7	Con, DB, O	15.6–23.1	26.1–37.0	5.1–7.8	3
<i>Neopezalotiopsis clavispora</i>							
LB11	19.8–22.6	6.1–8.9	Ver B, PB	13.5–16.7	12.2–22.5	5.3–8.5	3–4
LB12	18.1–23.6	5.9–8.3	Ver B, PB	12.6–17.2	16.4–19.6	4.8–7.2	3–4
LB13	16.3–22.0	5.8–8.8	Ver B, PB	11.0–15.8	15.5–22.7	5.0–8.2	3
LB14	16.3–23.7	5.5–8.4	Ver B, PB	13.7–17.1	15.8–23.4	4.8–7.4	3–4
<i>Pestalotiopsis camelliae</i>							
LB17	22.1–30.0	3.6–6.2	Con, L, DB	13.5–18.2	14.6–29.1	...	3–7
LB18	19.2–31.0	3.3–6.4	Con, L, DB	12.5–18.9	13.5–28.9	...	3–7
LB19	23.6–33.0	4.3–6.8	Con, L, DB	14.9–22.3	14.3–32.4	...	3–7
LB20	21.1–32.0	3.5–6.7	Con, L, DB	14.5–21.9	15.2–31.8	...	3–7

^y Characteristics: CL = conidia length, CW = conidia width, Middle = length of middle cells, Apical = apical appendage length, Basal = basal appendage length, and N = number of apical appendages.

^z Abbreviations: Con, DB, O = concolorous, dark brown, or olivaceous; Ver, B, PB = versicolorous, the upper two brown, the lowest pale brown; and Con, L, DB = concolorous, light to dark brown.

Fig. 3. Molecular phylogenetic tree of *Pseudoestalotiopsis* and *Neoestalotiopsis* spp. obtained through maximum-parsimony method based on the combined internal transcribed spacer, β -tubulin, and translation elongation factor 1- α gene sequences from collected isolates in the present study (bold font) and published sequences. *Pseudoestalotiopsis camelliae* CBS 443.62 was used as the outgroup. Numbers in the bootstrap test (1,000 replicates) are shown above the branches and only values above 50% are shown.

Fig. 4. Molecular phylogenetic tree of *Pestalotiopsis* spp. obtained through maximum-parsimony method based on the combined ITS, β -tubulin, and translation elongation factor 1- α gene sequences from collected isolates in the present study (bold font) and published sequences. *Neopestalotiopsis mesopotamica* CBS 336.86 was used as the outgroup. Numbers in the bootstrap test (1,000 replicates) are shown above the branches and only values above 50% are shown.

NJ methods using individual and combined ITS, β -tubulin, and TEF gene sequences. However, some species could not be differentiated from closely related species using any single gene sequence dataset (data not shown). Therefore, the concatenated dataset of ITS, β -tubulin, and TEF were used to resolve all the isolates analyzed in this study. Phylogenetic trees performed by both MP and NJ methods produced similar topologies; thus, only trees constructed by MP method were shown here (Figs. 3 and 4).

The MP tree derived from 54 isolates of *Pseudopezalotiopsis* and *Neopezalotiopsis* indicated that all of the isolates were distinctly separated into two main clusters with high bootstrap supports (Fig. 3). Eight *Pseudopezalotiopsis* isolates in this study (LB01, LB02, LB05 to LB08, LB15, and LB16) were grouped into one cluster containing *P. camelliae-sinensis*, *P. chinensis*, *P. theae*, *P. cocos*, *P. ampullacea*, and *P. indica*, but all isolates were closely related to a new species, *P. camelliae-sinensis* (Liu et al. 2017) obtained from *C. sinensis*; whereas isolates LB11 to LB14 grouped with the other cluster comprising several *Neopezalotiopsis* spp., with a high bootstrap value 100%, and were more related to *Neopezalotiopsis clavispora*. Based on phylogenetic analysis and morphological characteristics, the isolates of *Pseudopezalotiopsis* known to possess concolorous median cells and *Neopezalotiopsis* isolates known to possess versicolorous median cells were described as *P. camelliae-sinensis* and *N. clavispora*, respectively.

The MP tree constructed by the combined ITS, β -tubulin, and TEF genes of 38 *Pestalotiopsis* spp. indicated that the four *Pestalotiopsis* isolates in this study (LB17 to LB20) were grouped together with *Pestalotiopsis camelliae* obtained from tea leaves of *C. sinensis* in China (Liu et al. 2017) and Turkey (Maharachchikumbura et al. 2014), with a high bootstrap value 99% (Fig. 4). Combined with morphological characteristics, the isolates of *Pestalotiopsis* known to possess concolorous median cells but having no basal appendage were identified as *P. camelliae*.

The pathogenicity of *Pestalotiopsis*-like species. Results of pathogenicity tests showed that, with wound inoculation, all isolates in this study were pathogenic on tea leaves (Table 4) and the symptoms induced were similar to those which occur under natural conditions in the field. The percentage of infected tea leaves on wounded inoculated tea leaves for the 16 isolates all ranged from 41.7 to 76.7% by 15 days after inoculation (Table 4). In contrast, symptoms were not usually observed on nonwounded leaves (Table 4), suggesting that

wounding may be necessary for symptom development. All of the representative isolates of *Pseudopezalotiopsis camelliae-sinensis* and *N. clavispora* were highly pathogenic to the artificially inoculated tea leaves and showed no significant difference in lesion size between the two species, whereas the isolates of *Pestalotiopsis camelliae* were less virulent (Table 4). The fungi were reisolated from lesions of the diseased leaves with 100% frequency, and their morphological characteristics and gene sequences were identical to the original ones, which confirmed that all three species (*Pseudopezalotiopsis camelliae-sinensis*, *N. clavispora*, and *Pestalotiopsis camelliae*) are the causal agents for tea gray blight disease.

Discussion

Gray leaf blight of tea is an economically important disease caused by *Pestalotiopsis*-like fungi. At this time, there is limited information about the identity of these pathogens in the major tea-growing countries of the world. *Pestalotiopsis* spp., as potent plant pathogens, have gone largely unrecognized using only morphological or molecular data (Hu et al. 2007; Maharachchikumbura et al. 2011, 2012, 2013a,b), because colony morphology such as color, growth rate, and texture tends to be highly variable within species (Jeewon et al. 2003; Keith et al. 2006) and most DNA sequences deposited for *Pestalotiopsis* spp. in GenBank are likely to have been wrongly named or confused (Maharachchikumbura et al. 2011). Furthermore, Hu et al. (2007) showed that the ITS region is less informative than the β -tubulin gene in differentiating *Pestalotiopsis* spp. but Liu et al. (2010) disagreed and indicated that proper analysis and alignment of the ITS region can group *Pestalotiopsis* spp. according to different types of conidia. In 2014, a revision of *Pestalotiopsis* sensu lato was done by Maharachchikumbura et al. (2014) in “*Pestalotiopsis* revisited”, in which they segregated two novel genera from *Pestalotiopsis* (namely, *Neopezalotiopsis* and *Pseudopezalotiopsis*) based on the combined morphological and DNA data. In the present study, although all of the isolates from tea plants could be distinctly separated into three genera by morphological characteristics or any individual ITS, β -tubulin, and TEF sequence data, some species could not be differentiated from closely related species because fewer homologous gene sequences were available for sequence comparison or some of the sequences in this study contained low similarity to other sequences in the NCBI GenBank database. Therefore, combined multigene data of ITS, β -tubulin, and TEF and distinct morphological characteristics may

Table 4. Pathogenicity of 16 representative isolates from tea gray blight

Species, isolate	Infected leaves (%) ^y		Lesion diameter (mm) ^z
	Wounded	Nonwounded	Wounded
<i>Pseudopezalotiopsis camelliae-sinensis</i>			
LB01	60.0 ± 10.0	NS	9.2 ± 0.9 a
LB02	63.0 ± 7.6	NS	9.6 ± 1.1 a
LB05	66.7 ± 12.6	NS	9.9 ± 0.9 a
LB06	61.7 ± 12.6	3.3 ± 5.8	10.5 ± 1.3 a
LB07	73.3 ± 2.9	NS	10.1 ± 0.9 a
LB08	76.7 ± 10.4	1.67 ± 2.9	10.8 ± 1.3 a
LB15	50.0 ± 10.0	NS	10.1 ± 0.9 a
LB16	41.7 ± 10.4	NS	8.7 ± 0.8 a
<i>Neopezalotiopsis clavispora</i>			
LB11	58.3 ± 7.6	NS	9.7 ± 1.0 a
LB12	75.0 ± 5.0	NS	10.5 ± 1.6 a
LB13	70.0 ± 10.0	NS	10.7 ± 0.9 a
LB14	55.0 ± 8.7	NS	8.9 ± 1.0 a
<i>Pestalotiopsis camelliae</i>			
LB17	46.7 ± 10.4	NS	4.7 ± 0.5 b
LB18	51.7 ± 7.6	NS	3.3 ± 0.7 b
LB19	48.3 ± 7.6	NS	5.3 ± 0.9 b
LB20	60.0 ± 10.0	NS	4.9 ± 1.0 b

^y Infected tea leaves were assessed 15 days after inoculation by counting number of disease leaves, compared with the total leaves ($n = 20$). The experiment was conducted twice. NS = no symptoms.

^z Virulence of the isolates was evaluated by measuring the diameter of the typical necrotic lesion on infected tea leaves 7 days after wound or drop inoculation. The mean standard deviation was calculated for three replicates. Data within columns followed by same letter do not differ significantly according to Tukey's honestly significant difference test ($P \leq 0.05$).

provide the best resolution for resolving the *Pestalotiopsis*-like species as compared with any single marker.

In this study, cultural and conidial morphology, multigene sequence data, and pathogenicity tests were combined to confirm that *Pseudopestalotiopsis camelliae-sinensis*, *N. clavispora*, and *Pestalotiopsis camelliae* are all causal agents of tea gray blight, which suggests that this disease may be caused by a species complex. Of the three species, *Pseudopestalotiopsis camelliae-sinensis* and *Pestalotiopsis camelliae* are new species recently reported on *C. sinensis* in several tea-growing countries (Chen et al. 2017b; Liu et al. 2017; Maharachchikumbura et al. 2014). This study reports, for the first time, the characteristics of *Pseudopestalotiopsis camelliae-sinensis*, *N. clavispora*, and *Pestalotiopsis camelliae* causing gray blight on *C. sinensis* in China. The phylogenetic relationships provided more extensive knowledge about the genomic organization in *Pestalotiopsis*-like species responsible for gray blight of tea.

Results of pathogenicity tests in this study showed that symptoms were not usually observed on nonwounded leaves when a conidial suspension was inoculated onto tea leaves (Table 4), suggesting that wounding may be necessary for symptom development. However, in some cases, wounding was not necessary for disease development on ginger and lychee but symptoms were enhanced by wounds (Keith et al. 2006). Further studies on the pathogenic mechanism of pathogens causing tea gray blight disease are underway.

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