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Pseudocercospora rizhaoensis sp. nov. Causing Leaf Spot Disease of *Ligustrum japonicum* in China

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Abstract: *Ligustrum japonicum* is a common ornamental tree species in China. However, leaf spot disease has emerged in Rizhao City, Shandong Province of China in recent years. Members of *Pseudocercospora* are usually known as plant pathogens, mainly causing leaf spots and blights. Species of this genus are distinguished mainly based on morphological differences on the host plants, as well as the molecular data. A new species named *Pseudocercospora rizhaoensis* on *Ligustrum japonicum* is introduced herein based on morphology and molecular data of combined ITS, LSU, *act*, *tef1* and *rpb2* sequences. Koch's postulates were confirmed by a pathogenicity test, re-isolation and identification.

Keywords: morphology; Mycosphaerellacea; phylogeny; taxonomy

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1. Introduction

Pseudocercospora Speg. (Mycosphaerellaceae, Mycosphaerellales) is a large cosmopolitan genus of plant pathogenic fungi that are commonly associated with leaf and fruit spots as well as blights on a wide range of plant hosts [1–3]. *Pseudocercospora* typed by *P. vitis* (Lév.) Speg. is distinguished from the other cercosporoid fungi by pale to dark olivaceous caespituli, pigmented conidia with unthickened and not refractive scars on the conidiogenous cells and hila at the basal ends of conidium in vivo [2,3].

Members of *Pseudocercospora* are distributed worldwide, but they are mostly abundant and diverse in tropical and subtropical areas and reproduce mainly by means of conidia [1,4–9]. Some species are associated with important plant diseases; for example, *Pseudocercospora fijiensis* (M. Morelet) Deighton is the causal agent of black Sigatoka leaf diseases of banana in Uganda and Tanzania [10]; *P. actinidiae* Deighton causes sooty spot disease on kiwifruit in Brazil [11]; *P. griseola* (Sacc.) Crous and U. Braun results in bean angular leaf spot disease in Ethiopia [12].

Species of *Pseudocercospora* are distinguished based on the morphology produced on the host plants and sequence data [1]. In addition, host information can also separate species of this genus, which is supported by the overall DNA phylogeny of ITS, LSU, *act* and *tef1* [1]. Subsequently, the *rpb2* locus was recommended to be added to the phylogeny for recognition of species within the genus *Pseudocercospora* [4]. *Ligustrum japonicum* Thunb. of the family Oleaceae Hoffmanns. and Link is native to central and southern Japan and Korea, and widely planted as an ornamental in parks and landscapes in China. During the surveys of plant diseases in Shandong Province, China, a colored cercosporoid fungus with fasciculate conidiophores, slightly thickened and darkened conidial scars and hilum was discovered, which causes a severe foliar disease on *Ligustrum japonicum*. Morphologically and phylogenetically, it was shown to be a species of *Pseudocercospora*. We compared its morphological features and molecular data to the known *Pseudocercospora* species and concluded that this species is new to science. Illustrations and detailed descriptions are provided for this new species herein.

2. Materials and Methods

2.1. Sample Survey, Collection and Fungal Isolation

Diseased leaf samples of *Ligustrum japonicum* were observed and collected in Rizhao City, Shandong Province of China (Figure 1), packed in paper bags and brought to the laboratory for isolation. The infected leaves were first surface-sterilized for 1 min in 75% ethanol, 3 min in 1.25% sodium hypochlorite, and 1 min in 75% ethanol, and then rinsed for 2 min in distilled water and blotted on dry sterile filter paper [13]. Then, the diseased areas of the samples were cut into 0.5 × 0.5 cm pieces using a double-edge blade, and transferred onto the surface of potato dextrose agar plates (PDA; 200 g potatoes, 20 g dextrose, 20 g agar per L) and incubated at 25 °C to obtain pure cultures. The cultures were deposited in the China Forestry Culture Collection Center (CFCC; http://cfcc.caf.ac.cn) and the specimen was deposited in the Herbarium of the Chinese Academy of Forestry (CAF; http://museum.caf.ac.cn).

Figure 1. Symptoms of leaf spots on *Ligustrum japonicum*.

2.2. DNA Extraction, Sequencing and Phylogenetic Analyses

Genomic DNA was extracted from colonies grown on cellophane-covered PDA using a cetyltrimethylammonium bromide (CTAB) method [14]. DNA was checked by electrophoresis in 1% agarose gel, and the quality and quantity were measured using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The nuclear ribosomal DNA internal transcribed spacers (ITS), large subunit rRNA (LSU), actin (*act*), translation elongation factor 1-alpha (*tef1*) and the second lar-gest RNA polymerase subunit (*rpb2*) regions were amplified using the primer pairs ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) [15], LROR (GTA CCC GCT GAA CTT AAG C) and LR5 (TCC TGA GGG AAA CTT CG) [16], ACT-512 (ATG TGC AAG GCC GGT TTC GC) and ACT-783 (TAC GAG TCC TTC TGG CCC AT) [17], EF1-668 (CAT CGA GAA GTT CGA GAA GG) and EF1-1251 (GGA RGT ACC AGT SAT CAT GTT) [17,18] and RPB2-5f2 (GGGGWGAYCAGAAGAAGGC) and fRPB2-7cR (CCCATRGCTTGYTTRCCCAT) [19], respectively. The PCR conditions were set as follows: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, 50 s at 52 °C (ITS and LSU) or 54 °C (*tef1*, *act* and *rpb2*) [20]. The final extension step was done at 72 °C for 7 min. The PCR products were examined by electrophoresis on 1.5% (*w*/*v*) agarose gels stained with ethidium bromide in 1 × TBE buffer. DNA sequencing was performed by the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequences were generated by using SeqMan v.7.1.0 from the DNASTAR Lasergene software suite (DNASTAR Inc., Madison, WI, USA). Reference sequences used in the paper were downloaded from GenBank, and the GenBank accession numbers are listed in Table 1. *Trocophora simplex* was used as the outgroup taxon given its proposed relationship to *Pseudocercospora*. Sequences were aligned using MAFFT v.6 [21] and corrected manually using MEGA 7.0.21. The phylogenetic analyses of the combined loci were performed using the Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML was implemented on the CIPRES Science Gateway portal (https://www.phylo.org, accessed on 3 October 2022) using RAxML-HPC BlackBox 8.2.10 [22] employing a GTRGAMMA substitution model with 1000 bootstrap replicates. Bayesian inference was performed using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v.3.2.6 [23]. The six simultaneous Markov chains were run for 1 M generations; starting from random trees and sampling trees every 100 th generation and 25% of aging samples were discarded, running until the average standard deviation of the split frequencies dropped below 0.01. The phylogram was visualized in FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software, accessed on 4 October 2022) and edited in Adobe Illustrator CS5 (Adobe Systems Inc., San Jose, CA, USA). The nucleotide sequence data of the new taxon were deposited in GenBank, and the GenBank accession numbers of all accessions included in the phylogenetic analyses are listed in Table 1.

2.3. Morphological Identification and Characterization

The morphology of the new species was studied based on the fruiting bodies formed on the diseased leaves. The fruiting bodies were observed and photographed under a dissecting microscope (M205 C, Leica, Wetzlar, Germany). The conidiogenous cells and conidia were immersed in tap water, and then the microscopic photographs were captured with an Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) equipped with an Axiocam 506 color camera using Differential Interference Contrast (DIC) illumination. For measurements, more than 50 conidia were randomly selected. Culture characteristics were recorded from PDA after 20 d of incubation at 25 °C in the dark.

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

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
Pseudocercospora abeliae	MUCC1674 *	NA	LC599330	LC599407	LC599448	LC599587
P. aeschynomenicola	CPC 25227 = COAD 1972 *	KT290173	KT290146	KT313501	KT290200	NA
P. airliensis	BRIP 58550 *	KM055433	KM055429	NA	KM055436	NA
P. aleuritis	MAFF237174 = MUCC1230 *	NA	LC599331	LC599408	LC599449	LC599588
P. amelanchieris	MAFF 237782 = MUCC885 *	NA	KX462583	KX462550	KX462669	KX462616
P. ampelopsis	CBS 131583 = CPC 11680 *	GU253846	GU269830	GU320534	GU384542	NA
P. angiopteridis	CBS 147385 *	NA	LC599332	LC599409	LC599450	LC599589
P. angolensis	CBS 149.53 *	JQ324941	JQ324975	JQ325011	JQ324988	NA
P. araliae	MUCC 873 *	GU253702	GU269653	GU320361	GU384371	KX462617
P. arecacearum	CBS 118406 *	GU253704	GU269655	GU320363	GU384373	NA
P. assamensis	CBS 122467 *	GU253705	GU269656	GU320364	GU384374	NA
P. avicenniae	CBS 146479 *	NA	GU188047	LC599410	LC599451	LC599590
P. basiramifera	CBS 111072 = CPC 1266 *	GU253709	GU269661	GU320368	DQ211677	NA
P. basitruncata	CBS 114664 = CPC 1202 *	GU253710	DQ267600	DQ147622	DQ211675	NA
P. biophyti	CPC 20020	NA	LC599333	LC599411	LC599452	LC599591
P. bixae	CPC 25244 = COAD 1563 *	KT290180	KT290153	KT313508	KT290207	NA
P. brackenicola	CPC 24695 = COAD 1991 *	KT037565	KT037524	KT037606	KT037484	NA
P. breonadiae	CBS 143489 = CPC 30153 *	MH107959	MH107913	MH107985	MH108026	MH108006
P. bruceae	MUCC 2875 *	NA	LC599334	LC599412	LC599453	NA
P. casuarinae	CBS 128218 *	HQ599604	HQ599603	LC599413	LC599454	NA
P. ceratoniae	CBS 147386 *	NA	LC599335	LC599414	LC599455	LC599592
P. cercidicola	MAFF 237791 = MUCC 896 *	GU253719	GU269671	GU320377	GU384388	KX462618
P. cercidis-chinensis	CBS 132109 = CPC 14481 *	JX901884	GU269670	GU320376	GU384387	LC599593
P. chamaecristae	CPC 25228 = COAD 1973 *	KT290174	KT290147	KT313502	KT290201	NA
P. chiangmaiensis	CBS 123244 *	NG042738	EU882113	KF903544	KF903177	NA
P. chibaensis	MUCC1670E *	NA	KX462584	KX462551	KX462670	KX462619
P. chionanthi-retusi	TUA50 = NCHUPP L1605 *	NA	KX462585	KX462552	KX462671	KX462620
P. cladrastidis	MUCC1494 *	NA	LC599336	LC599415	LC599457	LC599594
P. convoluta	CBS 113377 *	MF951226	DQ676519	NA	NA	MF951617
P. coprosmae	CBS 114639 *	JQ324946	GU269680	GU320386	GU384397	NA
P. cordiana	CPC 2552 *	GU214472	AF362054	GU320387	GU384398	NA
P. corylopsidis	MAFF 237795 = MUCC 908 *	NG069064	GU269684	GU320390	GU384401	KX462621
P. cotini	MAFF410088 = MUCC1415 *	NA	LC599337	LC599416	LC599458	LC599596
P. cotoneastri	MAFF 410089 = MUCC1416 *	NA	KX462586	KX462553	KX462672	KX462622

Table 1. *Cont.*

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
P. crispans	CBS 125999 = CPC 14883 *	GU253825	GU269807	GU320510	GU384518	KX462623
P. crocea	CBS 126004 = CPC 11668 *	JQ324947	GU269792	GU320493	GU384502	NA
P. crousii	CBS 119487	GQ852631	GU269686	GU320392	GU384403	NA
P. cryptomeriicola	MAFF240073 = NBRC 102150 *	NA	LC599338	LC599418	LC599460	LC599598
P. curcumicola	MUCC733 *	NA	LC599339	LC599419	LC599461	LC599599
P. cyathicola	CBS 129520 = CPC 17047 *	JF951159	JF951139	KX462554	KX462673	KX462624
P. cymbidiicola	CBS 115132 *	GU253733	GU269692	GU320397	GU384408	NA
P. dalbergiae	TUA55 *	NA	LC599340	LC599420	LC599462	LC599600
P. daphniphylli	MAFF 410009 = MUCC1399 *	NA	KX462587	KX462555	KX462674	KX462625
P. davidiicola	MAFF 240281 = MUCC296 *	GU253734	GU269693	GU320398	GU384409	KX462626
P. delonicicola	MUCC2869 *	NA	LC599341	LC599421	LC599463	LC599601
P. dingleyae	CBS 114645 *	KX286997	KX287299	NA	NA	KX288454
P. diplusodonii	CPC 25179 = COAD 1476 *	KT290162	KT290135	KT313490	KT290189	NA
P. dodonaeae	CBS 114647 *	JQ324948	GU269697	JQ325013	GU384413	NA
P. dovyalidis	CBS 126002 = CPC 13771 *	GU253818	GU269800	GU320503	GU384513	NA
P. ebulicola	CBS 147387 *	NA	LC599342	LC599422	NA	NA
P. elaeocarpicola	MAFF 237189 = MUCC1236 *	NA	KX462588	KX462556	KX462675	KX462627
P. emmoticola	CPC 25187 = COAD 1491 *	KT290163	KT290136	KT313491	KT290190	NA
P. eriobotryae	MUCC 1007 *	NA	KX462589	KX462557	KX462676	KX462628
P. eriobotryicola	TUA12 = NCHUPPL1601 *	NA	KX462590	KX462558	KX462677	KX462629
P. ershadii	CBS 136114 = CCTU 1206 *	KP717032	KM452867	KM452844	KM452889	MN786459
P. eucalyptorum	CBS 114866 = CPC 11 *	JQ739817	KF901720	KF903474	KF903195	MF951618
P. eumusae	CBS 114824 *	NA	EU514238	NA	NA	NA
P. euonymi-japonici	CGMCC 3.18576 *	NA	MH255812	NA	NA	MH392531
P. eupatoriella	CBS 113372 *	GU253743	GU269704	GU320408	GU384420	NA
P. eupatorii- formosani	TUA59 = NCHUPP L1606 *	NA	KX462591	KX462559	KX462678	KX462630
P. euphorbiacearum	COAD 1537 *	KT290172	KT290145	KT313500	KT290199	NA
P. exilis	CPC 25193 = COAD 1501 *	KT290166	KT290139	KT313494	KT290193	NA
P. farfugii	MUCC978 *	NA	LC599343	LC599423	LC599464	LC599603
P. fijiensis	CBS 120258 = CIRAD 86 *	JQ324952	EU514248	NA	NA	NA
P. flavomarginata	CBS 126001 *	NA	GU269804	GU320507	GU384515	LC599604
P. fori	CBS 113285 *	NA	AF468869	KF903462	NA	KT356874
P. formosana	MUCC2612 *	NA	LC599344	LC599424	LC599466	LC599605
P. forsythiae	MAFF 410087 = MUCC1414 *	NA	LC599345	LC599425	LC599467	NA

Table 1. *Cont.*

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
P. fukuii	MAFF238121 = MUCC1297 *	NA	LC599347	LC599427	LC599469	LC599607
P. fukuokaensis	MAFF 237768 = MUCC 887 *	GU253751	GU269714	GU320418	GU384430	KX462632
P. ginkgoana	R. Kirschner 3563 (TNM) *	NA	JX134048	NA	NA	NA
P. glochidionis	MAFF 237000; MUCC1211 *	NA	LC599348	LC599428	LC599470	LC599608
P. gracilis	CBS 242.94 *	NA	DQ267582	NA	DQ211666	NA
P. griseola f. griseola	CBS 119906 *	NA	DQ289812.	NA	NA	NA
P. griseola f. mesoamericana	CBS 119113 *	NA	DQ289824	NA	NA	NA
P. hachijokibushii	MAFF 238479 *	NA	KX462593	KX462561	KX462680	KX462633
P. haiweiensis	CBS 131584 = CPC 14084 *	GU253821	GU269803	GU320506	GU384514	KX462634
P. hardenbergiae	CBS 147381 *	NA	LC599349	LC599429	LC599471	LC599609
P. heteropyxidicola	CBS 146082 = CPC 38030 *	NA	MN562151	MN556791	NA	NA
P. hiratsukana	MAFF 238300 = MUCC1105 *	NA	KX462594	KX462562	KX462681	KX462635
P. houttuyniae	MAFF 238071 = MUCC1289 *	NA	KX462595	KX462563	KX462682	KX462636
P. humuli	MUCC 742 *	GU253758	GU269725	GU320428	GU384439	KX462637
P. humulicola	CBS 131585 *	JQ324956	GU269723	GU320427	GU384438	NA
P. imazekii	MUCC 1668 *	NA	KX462596	KX462564	KX462683	KX462638
P. indonesiana	CBS 122473 *	NA	GU269735	GU320437	GU384448	NA
P. iwakiensis	MUCC 1736 *	NA	KX462607	KX462574	KX462693	KX462657
P. ixoriana	MUCC2608 *	NA	LC599350	LC599430	LC599472	LC599610
P. izuohshimense	MAFF 238478 = MUCC1336 *	NA	KX462597	KX462565	KX462684	KX462639
P. jagerae	BRIP 58549 *	NA	KM055431	NA	KM055438	NA
P. kadsurae	MUCC 752 *	NA	KX462598	KX462566	KX462685	KX462640
P. kaki	MAFF 238214 *	GU253761	LC512001	LC512007	LC515783	LC515794
P. kakiicola	MAFF 238238 = MUCC 900 *	NA	GU269729	GU320431	GU384442	NA
P. kenyirana	MUCC 2873 *	NA	LC599351	LC599431	LC599473	NA
P. kiggelariae	CBS 132016 = CPC 11853 *	GU253762	GU269730	GU320432	GU384443	NA
P. kobayashiana	MAFF 236999 *	NA	LC511998	LC512004	LC515780	LC515791
P. leandrae-fragilis	COAD 1977 *	NA	KY574288	NA	NA	NA
P. leucadendri	CPC 1869 *	GU214480	GU269842	GU320545	GU384555	NA
P. liquidambaricola	MAFF410455 *	NA	LC599352	LC599432	LC599474	LC599611
P. longispora	CBS 122470 *	NA	GU269734	GU320436	GU384447	NA

Table 1. *Cont.*

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
P. lonicericola	MUCC 889 = MAFF 237785 *	GU253766	GU269736	GU320438	JQ324999	KX462641
P. luzardii	CPC 25196 = COAD 1505 *	KT290167	KT290140	KT313495	KT290194	NA
P. lyoniae	MAFF 237775 = MUCC 910 *	GU253768	GU269739	GU320441	GU384451	KX462642
P. lythri	CBS 132115 = CPC 14588 *	NA	GU269742	GU320444	GU384454	LC599612
P. macadamiae	CBS 133432 *	KX286998	KX287300	KU878551	KU878504	KX288455
P. macrospora	CBS 114696 = CPC 25538	GU214478	AF362055	GU320447	GU384457	NA
P. madagascariensis	CBS 124155 *	NA	GQ852767	KF253625	KF253265	KX462643
P. maetaengensis	MFLUCC 14-0411 *	NA	MN648323	NA	NA	NA
P. mangifericola	BRIP 52776b *	NA	GU188048	NA	NA	NA
P. manihotii	CPC 25219 = COAD 1534 *	KT290171	KT290144	KT313499	KT290198	NA
P. mapelanensis	CMW40581 *	KM203121	KM203118	KM203127	KM203124	NA
P. marginalis	CBS 131582 = CPC 12497 *	GU253812	GU269794	GU320495	GU384504	NA
P. mazandaranensis	CCTU 1102 = CBS 136115 *	KP717020	KM452854	KM452831	KM452876	LC599613
P. metrosideri	CBS 114294 *	KX286999	KX287301	NA	NA	KX288456
P. microlepiae	BCRC FU30353 *	NA	KR348740	NA	NA	NA
P. musae	CBS 116634 *	GU253775	GU269747	GU320449	GU384459	NA
P. naitoi	MAFF 237906 = MUCC1072 *	NA	KX462599	KX462567	KX462686	KX462644
P. nandinae	MAFF 237633 = MUCC1260 *	NA	KX462600	KX462568	KX462687	KX462645
P. natalensis	CBS 111069 = CPC 1263 *	DQ267576	DQ303077	DQ147620	JQ325000	NA
P. nelumbonicola	BCRC FU30367 *	NA	KY304492	NA	NA	NA
P. neriicola	CBS 138010 = CPC 23765 *	KJ869222	KJ869165	KJ869231	KJ869240	KX462647
P. nodosa	CBS 554.71 *	MF951227	MF951367	NA	NA	MF951620
P. norchiensis	CBS 120738 = CPC 13049 *	GU253780	EF394859	GU320455	GU384464	KX462648
P. ocimi-basilici	CPC 10283 *	NA	GU269754	GU320456	GU384465	NA
P. paederiae	MAFF 239161	NA	KX462603	KX462570	KX462689	KX462651
P. palleobrunnea	CBS 124771 = CPC 13387 *	GQ303319	GQ303288	GU320500	GU384509	KX462652
P. pancratii	CBS 137.94 *	GU253784	GU269759	GU320460	GU384470	NA
P. paranaensis	CPC 24680 = COAD 1987T	KT037563	KT037522	KT037604	KT037482	NA
P. parapseudarthriae	CBS 137996 = CPC 23449 *	KJ869208	KJ869151	KJ869229	KJ869238	NA
P. perae	CPC 25171 = COAD 1465 *	KT290159	KT290132	KT313487	KT290186	NA
P. perrottetiae	CBS 147382 *	NA	LC599353	LC599433	LC599477	LC599614
P. photiniae	MUCC 1661 *	NA	KX462604	KX462571	KX462690	KX462653
P. pini-densiflorae	MUCC 1714 *	NA	LC599354	LC599434	LC599478	LC599615

Table 1. *Cont.*

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
P. planaltinensis	CPC 25189 = COAD 1495 *	KT290164	KT290137	KT313492	KT290191	NA
P. platyceriicola	MUCC2876 *	NA	LC599355	LC599435	LC599479	LC599616
P. plectranthi	CBS 131586 = CPC 11462 *	NG070621	GU269791	GU320492	GU384501	NA
P. plumeriifolii	CPC 25191 = COAD 1498 *	KT290165	KT290138	KT313493	KT290192	NA
P. pothomorphes	CPC 25166 = COAD 1450 *	KT290158	KT290131	KT313486	KT290185	NA
P. profusa	CBS 132306 = CPC 10055 *	GU253787	GU269762	GU320463	GU384473	NA
P. proiphydis	BRIP 58545 *	KM055434	KM055430	NA	KM055437	NA
P. proteae	CBS 131587 = CPC 15217 *	MH877381	GU269808	GU320511	GU384519/	NA
P. pruni-grayanae	MUCC 1715 *	NA	LC599356	NA	LC599481	LC599618
P. pseudomusae	CBS 147147 *	NA	MW063423	MW070772	MW071091	MW070919
P. pseudomyrticola	CBS 145554 = CPC 35448 *	MK876446	MK876405	MK876461	MK876499	MK876490
P. pseudostigminaplatani	CBS 131588 = CPC 11726 *	JQ324963	GU269857	GU320560	GU384568	NA
P. punctata	CBS 132116 = CPC 14734 *	GU253791	GU269765	GU320468	GU384477	MF951622
P. punicae	MAFF236998 = MUCC 1209	NA	KX462606	KX462573	KX462692	KX462655
P. pyracanthae	MAFF237140 = MUCC 1226 *	GU253792	GU269767	NA	GU384479	LC599619
P. pyracanthigena	CBS 131589 = CPC 10808 *	NA	GU269766	GU320469	GU384478	NA
P. ravenalicola	CBS 122468 *	GU253828	GU269810	GU320513	GU384521	NA
P. rhabdothamni	CBS 114872 *	JQ324964	GU269768	GU320471	GU384480	NA
P. rhamnellae	CBS 131590 = CPC 12500 *	GU253813	GU269795	GU320496	GU384505	NA
P. rhapisicola	MAFF305042 = MUCC1484 *	NA	LC599357	LC599436	LC599483	LC599620
P. rhododendri-indici	CBS 131591 = CPC 10822 *	JQ324965	GU269722	GU320426	NA	NA
P. riachueli var. *horiana*	MUCC2141 *	NA	LC599358	LC599437	LC599484	LC599621
P. richardsoniicola	CPC 25248 = COAD 1568 *	KT290181	KT290154	KT313509	KT290208	NA
P. rigidae	CPC 25175 = COAD 1472 *	KT290161	KT290134	KT313489	KT290188	NA
P. rosae	MFLUCC 14-0408 *	MG829063	MG828952	NA	NA	NA
P. rizhaoensis	**CFCC 57581 ***	**NA**	**OP661350**	**OP651770**	**OP651772**	**OP651774**
P. rizhaoensis	**CFCC 57582**	**NA**	**OP661351**	**OP651771**	**OP651773**	**OP651775**
P. sambucigena	CBS 126000 *	GU253809	GU269788	GU320508	GU384498	NA
P. sawadae	MAFF 239714	NA	LC599359	LC599438	LC599485	LC599622
P. schizolobii	CBS 120029 = CPC 12962 *	KF251826	KF251322	KF253628	KF253269	NA
P. sennae-multijugae	CPC 25206 = COAD 1519 *	KT290169	KT290142	KT313497	KT290196	NA
P. serpocaulonicola	CPC 25077 = COAD 1866 *	KT037566	KT037525	KT037607	KT037485	NA

Table 1. *Cont.*

Species	Isolates	GenBank Accession Numbers				
		LSU	**ITS**	***act***	***tef1***	***rpb2***
P. solani-pseudocapsicicola	CPC 25229 = COAD 1974 *	KT290175	KT290148	KT313503	KT290202	NA
P. sophoricola	CCTU 1037 = CBS 136020 *	KP717027	KM452861	KM452838	KM452883	MW272931
P. sphaerulinae	CBS 112621 *	KF901958	KF901625	NA	KF903215	NA
P. stemonicola	MUCC2874 *	NA	LC599360	LC599439	LC599487	NA
P. stephanandrae	MAFF237799 = MUCC914 *	GU253831	GU269814	GU320516	GU384526	KX462658
P. stranvaesiae	MAFF410090 = MUCC1417 *	NA	LC599361	LC599440	LC599488	LC599623
P. struthanthi	CPC 25199 = COAD 1512 *	KT290168	KT290141	KT313496	KT290195	NA
P. styracina	COAD 2369 *	MH480643	MH397664	MH480641	MH480642	NA
P. symploci	NCHUPP L1685 = CBS142471 *	NA	LC599362	LC599441	LC599489	LC599624
P. tabernaemontanae	CPC 19198 *	NA	LC599363	LC599442	NA	LC599625
P. tereticornis	CBS 125214 = CPC 13299 *	NA	GQ852770	GU320499	GU384508	KX462659
P. terengganuensis	MUCC2871 *	NA	LC599364	LC599443	LC599490	NA
P. tinea	TUA40 = NCHUPP L1603 *	NA	KX462608	KX462577	KX462696	KX462660
P. togashiana	MAFF410006 *	NA	LC599365	LC599444	LC599491	LC599626
P. trichogena	CPC 24664 = COAD 1087 *	KT037560	KT037519	KT037601	KT037479	NA
P. trinidadensis	COAD 1756 *	NA	KT290157	NA	KT290210	NA
P. tumulosa	CBS 121158 *	NA	DQ530217	NA	NA	NA
P. vassobiae	CPC 25251 = COAD 1572 *	KT290182	KT290155	KT313510	NA	NA
P. viburnigena	CBS 125998 = CPC 15249 *	GU253827	GU269809	GU320512	GU320512	NA
P. violamaculans	MUCC 1660 *	NA	KX462610	KX462579	KX462698	KX462662
P. vitis	CBS 132012 =CPC 11595	GU214483	GU269829	GU320533	GU384541	KX462663
P. wulffiae	CPC 25232 = COAD 1976 *	KT290177	KT290150	KT313505	KT290204	NA
P. xanthocercidis	CBS 131593 = CPC 11665Iso *	JQ324971	JQ324983	JQ325026	JQ325005	NA
P. xenopunicae	CBS 147384 *	NA	LC599367	LC599446	LC599493	LC599628
P. xenosyzygiicola	MAFF237986 = MUCC1481 *	NA	KX462611	KX462580	KX462699	KX462664
P. xylopiae	CPC 25173 = COAD 1469 *	KT290160	KT290133	KT313488	KT290187	NA
P. yakushimensis	MAFF237025 = MUCC1214 *	NA	LC599368	LC599447	LC599494	LC599629
P. zambiae	CBS 136423 = CPC 22686 *	NA	KF777175	NA	NA	MF951630
P. zelkovae	MAFF 238237 = MUCC872 *	NA	GU269835	GU320537	GU384547	KX462665
Trochophora simplex	CBS 124744	NA	NA	GU320568	GU384580	KX462666

Note: NA, not applicable. Ex-type strains are marked with *, and strains from the present study are in black bold.

2.4. Pathogenicity Testing

Two isolates of *Pseudocercospora rizhaoensis* (ex-type strain: CFCC 57581; CFCC 52288) were used for inoculations, and agar plugs were used as the negative control. Detached healthy *Ligustrum japonicum* leaves were used for artificial inoculation experiments. The leaves were surface-sterilized with 75% ethanol, rinsing three times in sterile water, and then we waited for the surface moisture to dry. Discs of agar were cut from the actively growing margins of the cultures and these were placed on the non-wounded in vitro leaves. Each group was performed three times and cultured at 25 °C in the dark.

3. Results

3.1. Phylogeny

The combined sequence dataset (ITS, LSU, *tef1*, *act* and *rpb2*) was analyzed to infer the phylogenetic placement of our new isolates within the genus *Pseudocercospora*. The dataset consisted of 193 sequences, including an outgroup taxon, *Trocophora simplex* (CBS124744). A total of 2755 characters, including gaps (510 for ITS, 788 for LSU, 543 for *tef1*, 241 for *act*, and 673 for *rpb2*), were included in the phylogenetic analysis. The best ML tree (lnL = − 33,732.84) revealed by RA×ML is shown in Figure 2. The topologies resulting from ML and BI analyses of the concatenated dataset were congruent (Figure 2). The phylogenetic tree showed that isolates CFCC 57581 and CFCC 57582 from the present study formed into a distinguished clade from the other known *Pseudocercospora* species.

3.2. Taxonomy

Pseudocercospora rizhaoensis Yun Liu, sp. nov. Figure 3.

Mycobank no.: 845997.

Etymology—Named after Rizhao City, where the holotype was collected.

Description—*Leaf spots* amphigenous, circular, scattered, pale brown to brown with reddish brown margin. *Caespituli* hypophyllous, synnematous with blackish brown conidiophores. *Mycelium* internal, hyaline to brown. *Stromata* hypophyllous, substomatal, epidermal, erumpent, well-developed, subglobose to globose, dark brown to blackish, 25–85 µm diam. *Conidiophores* dense, arising from the upper part of stromata, straight to sinuous-geniculated, cylindrical, unbranched, pale brown to brown, paler towards the apex, 4.5–25 × 2–3.5 µm, 0–2-septate, smooth. *Conidiogenous cells* integrated, terminal, proliferating percurrently or sympodially, with unthickened and truncated conidial loci. *Conidia* solitary, holoblastic, cylindrical to obclavate, 15–50.5 × 2.5–4.5 µm, 1–5-septate, hyaline- to pale-colored, smooth, acute at the apex, obconically truncated, unthickened and not darkened at the base.

Culture characteristics—*Colonies* on PDA flat, spreading, with flocculent aerial mycelium, edge entire, mouse grey, reaching 60 mm diam. after 20 d at 25 °C.

Material examined—CHINA, Shandong Province, Rizhao City, Beijinglu Street, on diseased leaves of *Ligustrum japonicum*, 7 August 2021, Yun Liu (CAF800066 *holotype*; *ex-type living culture*, CFCC 57581); Ibid. Shandong Province, Rizhao City, Shijiu Street, on diseased leaves of *Ligustrum japonicum*, 12 August 2021, Yun Liu (culture CFCC 57582).

Notes—Two isolates of *Pseudocercospora* from leaf spots of *Ligustrum japonicum* clustered into a well-supported clade distinguished from the other members within this genus (Figure 2), which is proposed as *P. rizhaoensis* herein. Phylogenetically, *P. rizhaoensis* is close to *P. eupatoriella* Crous and Den Breeÿen and *P. ginkgoana* R. Kirschner; however, these species can be distinguished by their hosts (*P. rizhaoensis* on *Ligustrum japonicum* vs. *P. eupatoriella* on *Chromolaena odorata* R. M. King and H. Robinson vs. *P. ginkgoana* on *Ginkgo biloba* L.) [1,24]. Morphologically, conidia of *P. rizhaoensis* is similar to those of *P. eupatoriella*, but wider than those of *P. ginkgoana* (2.5–4.5 µm in *P. rizhaoensis* vs. 2–2.5 µm in *P. ginkgoana*) [1,24].

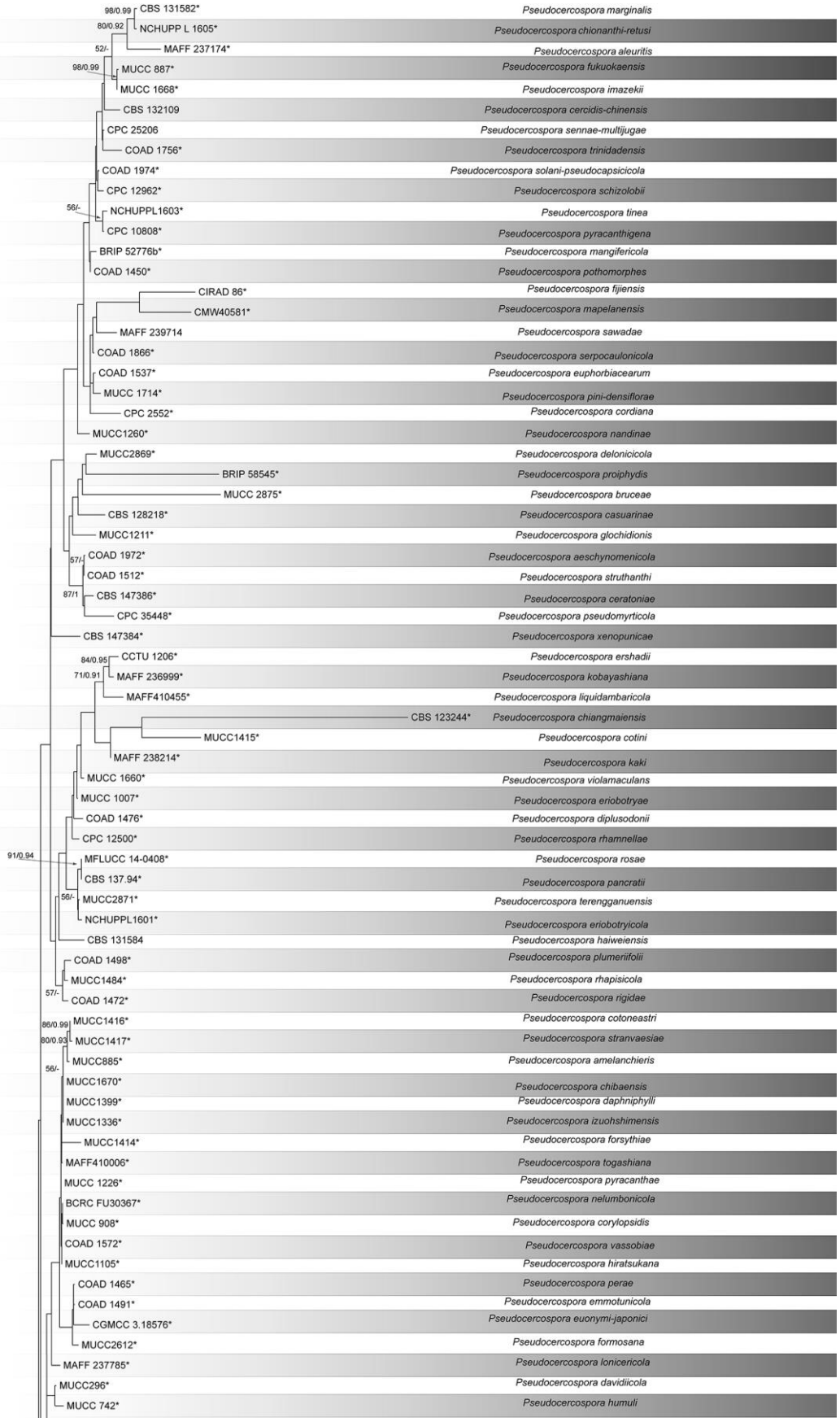

Figure 2. *Cont*.

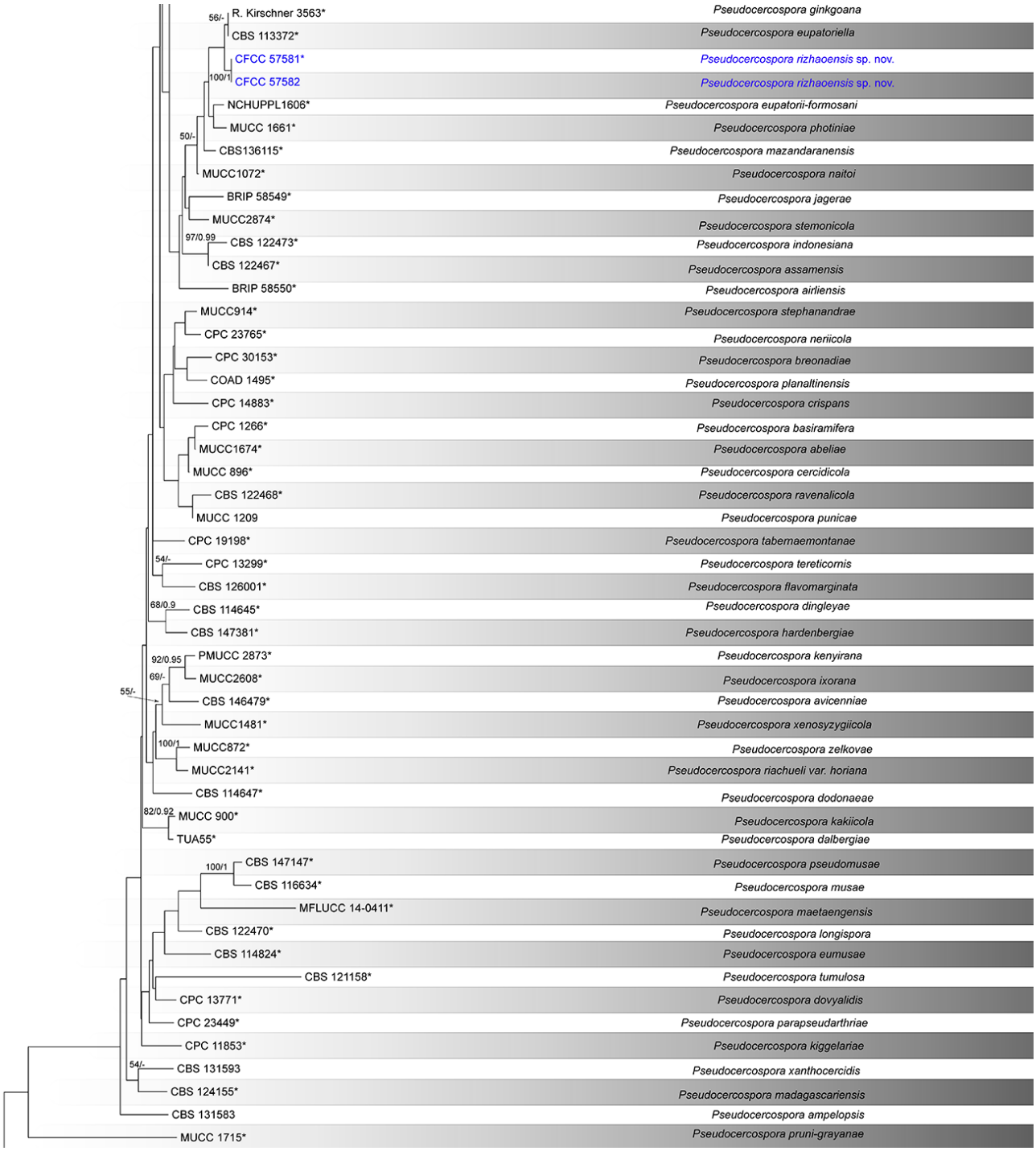

Figure 2. *Cont*.

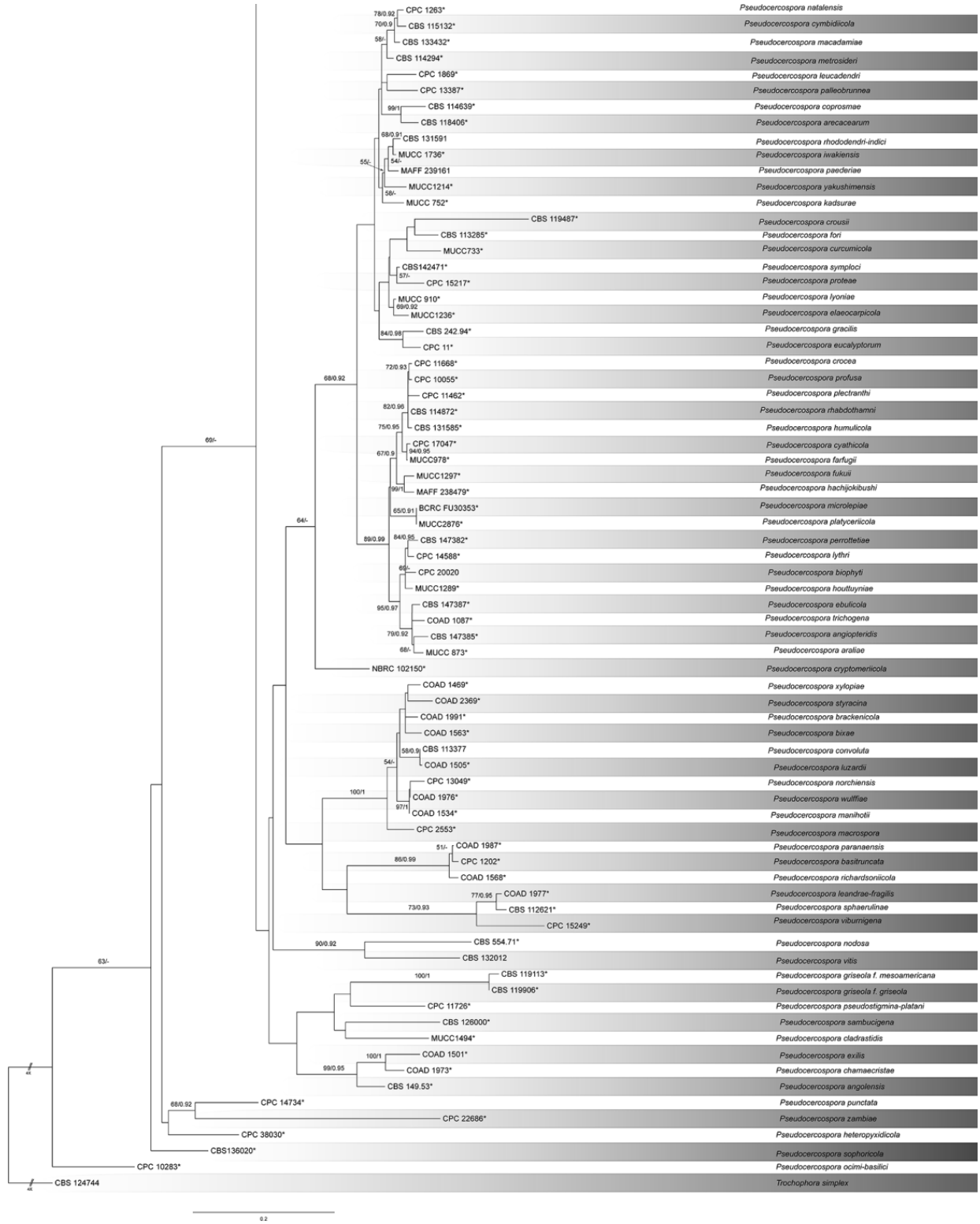

Figure 2. Phylogram of *Pseudocercospora* resulting from a maximum likelihood analysis, based on a combined matrix of ITS, LSU, *act*, *rpb2* and *tef1*. Numbers above the branches indicate ML bootstraps (**left**, ML BS $\geq$ 50%) and Bayesian Posterior Probabilities (**right**, BPP $\geq$ 0.90). Ex-type strains are marked with *, and the new species proposed in the present study is marked in blue.

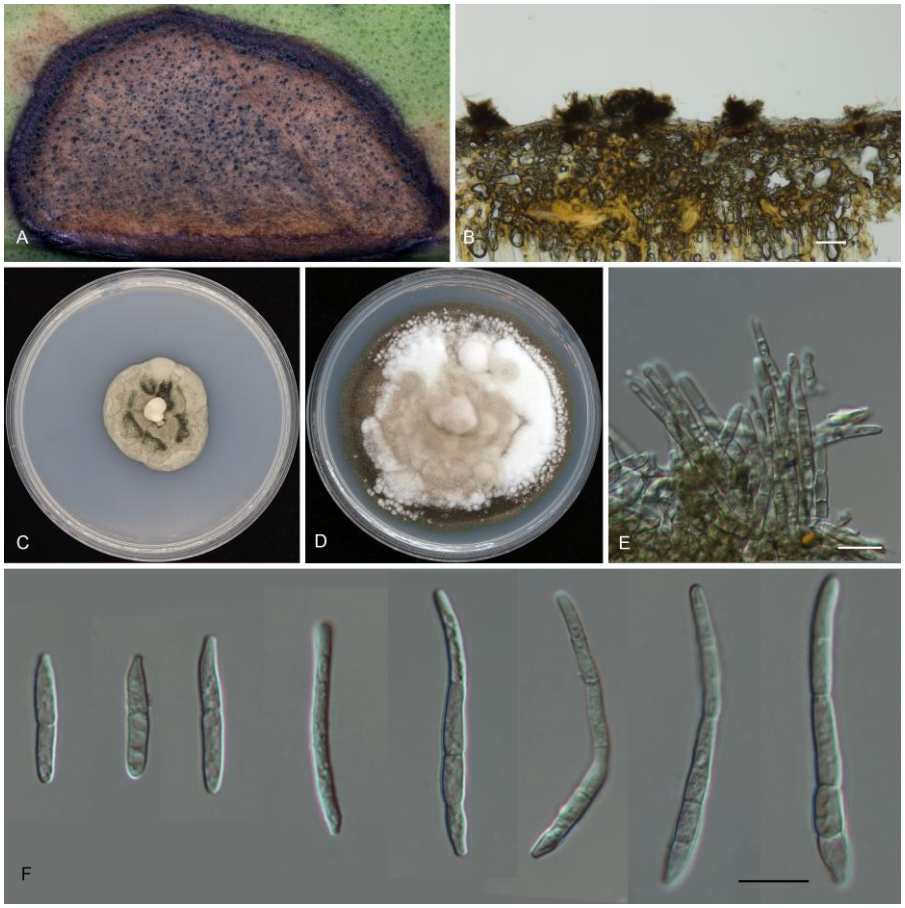

Figure 3. Morphology of *Psedocercospora rizhaoensis*. (**A**) Disease symptom on the host leaves; (**B**,**E**) stroma and conidiophores; (**C**) colony on PDA plates at 10 d; (**D**) colony on PDA plates at 20 d; (**F**) conidia. Scale bars: (**B**) = 100 μm; (**E**,**F**) = 10 μm.

3.3. Pathogenicity Tests

Similar leaf spot symptoms were reproduced on the *Ligustrum japonicum* leaves after inoculated 20 days, while no symptoms were observed on the control leaves. The respective inoculated fungi were re-isolated from leaves' lesions and were identical to *Ligustrum japonicum* by using morphological characteristics and phylogeny.

4. Discussion

The genus *Psedocercospora* was previously considered as an anamorphic state of *Mycosphaerella* or having mycosphaerella-like teleomorphs, but it is now treated as a genus based on phylogeny and morphology [1,25–28]. Now members within this genus are distinguished from each other based on combined approaches of host association, conidia characters and gene sequences [1].

In the pathogenicity test, the leaves after inoculating those isolates showed the same symptoms as disease that occurred in the field, and those isolates could be re-isolated from the lesions. Based on those data, *Psedocercospora rizhaoensis* is considered as the causal agent of the *Ligustrum japonicum* leaf spot disease in China.

Pseudocercospora ligustri was recorded causing *Ligustrum japonicum* leaf spots in the USA and *Ligustrum japonicum* 'Howardii' leaf spots in China [29,30]. For this fungus species, no DNA data are available from the type material (IMI 91224 collected in the USA) [29], and three genes, namely ITS, *act* and *tef1*, were sequenced based on the samples collected in China (JS1201, JS1202 and JS1203) [30]. Morphologically, conidiophores of *Psedocercospora rizhaoensis* are narrower than those of *P. ligustri* (2–3.5 μm in *P. rizhaoensis* vs. 3–4.2 μm

in *P. ligustri*) [29,30]. In addition, *P. rizhaoensis* differs from *P. ligustri* in the sequence data (3/470 in ITS, 3/222 in *act* and 1/309 in *tef1*) [30].

As shown in Figure 2, several species were not well-distinguished in the phylogram based on combined loci of ITS, LSU, *act*, *rpb2* and *tef1*. This may be caused by the absence of sequence data for those species (Table 1). Chen et al. [25] demonstrated that ITS is the genus DNA barcode, and the *act*, *rpb2* and *tef1* genes are the species DNA barcode. More phylogenetic analyses to infer the species relationships are necessary in following studies by employing more genes.

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