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Two novel species of *Pestalotiopsis* on *Picea* and *Tsuga* from temperate forests in the United States

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Abstract: In a 2023 survey evaluating conifers with *Rosellinia* infections, five *Pestalotiopsis* fungal strains were isolated from plant samples obtained from Maine, New Hampshire, and Ohio. Based on a multi locus phylogenetic analysis (ITS, *TUB*, *TEF*), species-specific base pair substitutions, and morphological characterization, the five isolates were determined to constitute two novel species within the genus *Pestalotiopsis*. These two new species are introduced here as *Pestalotiopsis maineana* and *Pestalotiopsis neohantoniensis*. *Pestalotiopsis maineana* shares close phylogenetic affinity to *P. fusiformis*, *P. brachiata*, and *P. hispanica,* while *P. neohantoniensis* shares affinity with *P. daliensis*, *P. chamaeropis,* and *P. hainanensis.* Both new species described herein differ from close relatives based on various morphological characters including the number of basal appendages of the conidia. Further, species-specific base pair substitutions are provided for the *TEF* locus which had the most resolution when compared to the ITS and *TUB* loci. To our knowledge, *P. maineana* is the first species of *Pestalotiopsis* to be isolated and reported from white spruce (*Picea glauca*) while *P. neohantoniensis* is the first species to be isolated and reported from Serbian spruce (*Picea omorika*) and eastern hemlock (*Tsuga canadensis*). Overall, this study provides insight into the microfungi associated with conifers in the United States.

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INTRODUCTION

In the United States (US) forests are economically and ecologically important systems covering approximately 38 % of the land in the country, estimated to be 8 % of the world's total forest area (Renwick 2023). Maine (ME) and New Hampshire (NH) are the two most heavily wooded states in the US with 89 and 83 % forest coverage, respectively (USDA FS 2022a, USDA FS 2022b). The most common type of forest in ME and NH is the maple/beech/birch forest; whereas spruce/fir and white/red pine are the most common softwood forests (Butler 2017, Morin & Lombard 2017). While there are various threats to forests in North America, emerging or re-emerging diseases remain a concern as they can significantly alter existing ecosystem diversity (Guegan *et al.* 2023). Historically, there has been concern for the spread of *Rosellinia* species from nursery planted to naturally regenerative conifers (Shea 1964, Francis 1986). The shift in community diversity caused by host mortality from emerging diseases directly affects forest ecosystems; thus, in 2023 concern for the spread of Rosellinia needle blight between conifer species prompted creation of a surveillance program and the collection of symptomatic plant tissue. The collected plant samples usually yield isolates of *Pestalotiopsis*-like fungi, as well as the target pathogen *Rosellinia*.

The genus *Pestalotiopsis* consists of asexual species with appendage-bearing conidia (Stayaert 1949). The genus is currently placed in the family *Sporocadaceae*, order *Amphisphaeriales*, class *Sordariomycetes*, in the *Ascomycota* (Wijayawardene *et al.* 2020). *Pestalotiopsis* was segregated from *Pestalotia* by Stayaert (1949) based on conidium cell number. More recently, Jaklitsch *et al.* (2016) synonymized *Bartaliniaceae*, *Discosiaceae*, *Pestalotiopsidaceae*, and *Robillardaceae* with *Sporocadaceae* and placed the family within the order *Xylariales*. However, *Amphisphaeriales* was later resurrected over *Xylariales* for the placement of *Sporocadaceae* (Hyde *et al.* 2020, Wijayawardene *et al.* 2020). To date, 445 species of *Pestalotiopsis* have been described (https://www.indexfungorum.org 2025). Species of *Pestalotiopsis* are usually described based on conidial morphology (i.e. apical and basal appendages) paired with gene sequence data. The length, number, position, and branching pattern of the apical or basal appendages are often utilized to differentiate between closely related species (Crous *et al.* 2012, Maharachchikumbura *et al.* 2012). When discerning between species of *Pestalotiopsis* through phylogenetic analyses, current studies use the ITS, *TUB*, and *TEF* gene regions as these have been reported as the most effective in resolving species within

the genus. Maharachchikumbura *et al.* (2012) evaluated 10 loci including ACT, *TUB*, CAL, GPDH, GS, ITS, LSU, RPB 1, SSU and *TEF*; they reported ITS, *TUB*, and *TEF* as providing the most resolution with *TEF* being the most informative locus.

Species of *Pestalotiopsis* vary in lifestyle and host specificity. They have been reported as saprophytes, endophytes, plant pathogens, animal/human pathogens, and as parasymbionts (Maharachchikumbura *et al.* 2011, Li *et al.* 2024, Luo *et al.* 2024). Although historically reported as endophytic and weak/ opportunistic pathogens, some studies have shown some species can be aggressive plant pathogens in various agricultural and horticultural systems, including but not limited to tea (*Camellia sinensis*), coconut (*Cocos nucifera*), pine (*Pinus massoniana*) and hardy ornamentals such as *Calluna vulgaris*, *Erica carnea*, and *Rhododendron* spp. (Hopkins & McQuilken 2000, Sanjay *et al.* 2008, Maharachchikumbura *et al.* 2013, Chen *et al.* 2018, Bhuiyan *et al.* 2021, Li *et al.* 2024). Some species, such as *P. microspora*, have been observed to switch between being endophytic and pathogenic depending on physiological and environmental factors (Lee 1995, Maharachchikumbura *et al.* 2011).

The objectives of this study were to identify and characterize *Pestalotiopsis* species isolated from conifer samples affected by Rosellinia needle blight. Based on a multi locus phylogenetic analysis, species-specific base pair substitutions, and spore morphology, we introduce here two novel species of *Pestalotiopsis* from conifers in the US.

MATERIALS AND METHODS

Sampling, isolation, and morphological characterization

Trees displaying signs and symptoms of Rosellinia needle blight were targeted for collection. Branches were collected from eastern hemlock trees (*Tsuga canadensis*) in Hillsborough County, New Hampshire and Hocking County, Ohio in December of 2023. Branches from white spruce (*Picea glauca*) and Serbian spruce (*Picea omorika*) were collected in Hancock County, Maine in November of 2023. Eleven collected samples were mailed to the Mycology and Nematology Genetic Diversity and Biology Laboratory, U.S. Department of Agriculture, Agricultural Research Service in Beltsville, Maryland, USA, for analysis. Segments of plant tissue no larger than 2 cm^3 adjacent to *Rosellinia* perithecia, were removed from branches and sterilized with 10 % sodium hypochlorite (Clorox™; Oakland, CA, USA) for 3 min and rinsed with sterile distilled water before being air dried. The cut segments included the margin between the plant tissue showing *Rosellinia* symptoms and the adjacent asymptomatic plant tissue. Cut segments were then placed on P4083 Penicillin – Streptomycin – Neomycin antibiotic microbial solution (Sigma-Aldrich; St. Louis, Missouri, USA) supplemented corn meal agar (CMA) (BD Difco; Franklin Lakes, NJ, USA) and incubated at 21–25 °C under an alternating 12-h light and 12-h dark schedule. Following growth of fungi, hyphal tips were transferred to potato dextrose agar (PDA) (BD Difco; Franklin Lakes, NJ, USA) and incubated at 21–25 °C under the same light regimen. Once pure cultures were obtained, agar plugs of isolates were transferred to cryogenic tubes with sterile 20 % MB grade glycerol (USB Corporation; Cleveland, OH, USA) diluted with ultrapure water, for long-term storage. Living cultures were deposited at the Westedijk Fungal Biodiversity Institute culture collection (CBS), while dried specimens were deposited at the US National Fungus Collection (BPI) (Table 1).

For morphological observations, the *Pestalotiopsis* cultures were grown for 21 d on PDA and synthetic nutrient poor agar (SNA; Nirenberg 1981) at 25 °C with 12 h of light and 12 h of darkness. Growth rates and morphological characteristics of colonies were evaluated every 7 d for 3 wk. Colour of the colonies were determined using the Rayner (1970) colour chart. Photographs of colonies on agar plates were taken with a Canon EOS 5D Mark III digital camera (Canon; Tokyo, Japan) in a lighting booth (MK Digital Direct). To induce conidiomatal development, twice autoclaved pine needles were placed on SNA as described by Maharachchikumbura *et al.* (2014). Morphological structures were evaluated on 85 % lactic acid slides after incubation on a warming plate at 50 °C for a minimum of 30 min prior to examination with a Zeiss Axio Imager M2 compound microscope (Zeiss, Jena, Germany). At least 30 measurements were taken for each reported structure. Descriptive statistics are presented and include minimum-maximum, mean, and standard deviation.

DNA extraction, PCR amplification, sequencing

In preparation for extraction of DNA, single hyphal-tipped isolates were grown on full strength PDA plates for 4 wk at 25 °C with alternating 12 h of light and 12 h of darkness. The hyphal mat was then scraped, and mycelia were transferred to 2 mL polypropylene tubes with 500 µm garnet beads and a 6-mm-diam. zirconium bead (OPS Diagnostics, Lebanon, NJ, USA). The tissue was lysed with a Precellys® Evolution homogenizer (Bertin instruments, France). After lysis, DNA was extracted using the E.Z.N.A. HP Plant & Fungal DNA Kit (OMEGA® Bio-Tek, Norcross, GA, USA) following manufacturer's protocol.

Three loci were amplified, including the internal transcribed spacer (ITS: ITS5/ITS4) (White *et al.*1990), partial beta-tubulin gene (*TUB*: T1/Bt-2b) (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997), and partial translation elongation factor 1-alpha gene (*TEF*: EF1-728/ EF-2) (O'Donnell *et al.* 1998, Carbone & Kohn 1999). Amplification reactions were conducted in 25 µL volumes with 12.5 µL of KAPA2G Robust Hotstart® (Kapa Biosystems, Inc., Wilmington, MA, USA), 1.25 µL of the forward and reverse primers at 10 µM, 1–1.75 µL of DNA at 10–20 ng, and 8 µL of molecular grade H2O. Amplifications were performed following the protocol described by Maharachchikumbura *et al.* (2014) in a C1000 Touch PCR Thermal Cycler (Bio-Rad, Hercules, CA). The PCR products were analysed through capillary electrophoresis using the QIAxcel Advanced System instrument and the QIAxcel ScreenGel software (Qiagen, Hilden, Germany). The PCR products were then purified using ExoSAP-IT Cleanup (Affymetrix, Santa Clara, CA) following the manufacturer's protocol. The BigDye™ v. 3.1 Terminator Cycle sequencing kit was used to sequence amplicons bi-directionally with the Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Taxa used for phylogenetics analyses.

Species	Strain Number	Type	*Host*	Location	GenBank Accession			Reference
					ITS	*TUB*	*TEF*	
Neopestalotiopsis saprophytica	MFLUCC 12-0282	**T**	*Magnolia* sp. (*Magnoliaceae*)	China	JX398982	JX399017	JX399048	Maharachchikumbura *et al.* (2014)
Pestalotiopsis abietis	*CFCC 53011*	**T**	*Abies fargesii* (*Pinaceae*)	China	*MK397013*	*MK622280*	*MK622277*	Gu *et al.* (2021)
P. abietis	*CFCC 53012*		*Abies fargesii* (*Pinaceae*)	China	*MK397014*	*MK622281*	*MK622278*	Gu *et al.* (2021)
P. americana	CBS 111576	**T**	*Leucospermum cunei* × *conocarpodendron* (*Proteaceae*)	USA	MH553961	MH554620	MH554379	Liu *et al.* (2019)
P. anhuiensis	*CFCC 54791*	**T**	*Cyclobalanopsis glauca* (*Fagaceae*)	China	*ON007028*	*ON005056*	*ON005045*	Jiang *et al.* (2022)
P. appendiculata	CGMCC 3.23550	**T**	*Rhododendron decorum* (*Ericaceae*)	China	OP082431	OP185516	OP185509.1	Gu *et al.* (2022)
P. australasiae	*CBS 114126*	**T**	*Knightia* sp. (*Proteaceae*)	New Zealand	*KM199297*	*KM199409*	KM199499	Maharachchikumbura *et al.* (2014)
P. australis	CBS 114193; STE-U 3011	**T**	*Grevillea* sp. (*Proteaceae*)	Australia	KM199332	KM199383	KM199475	Maharachchikumbura *et al.* (2014)
	CBS 111503; STE-U 1770		*Protea neriifoliax susannae* cv. "Pink Ice" (*Proteaceae*)	South Africa	KM199331	KM199382	KM199557	Maharachchikumbura *et al.* (2014)
	CBS 114474; STE-U 1769		*Protea neriifoliax susannae* cv. "Pink Ice" (*Proteaceae*)	South Africa	KM199334	KM199385	KM199477	Maharachchikumbura *et al.* (2014)
	CBS 119350; CMW 20013		*Brabejum stellatifolium* (*Proteaceae*)	South Africa	KM199333	KM199384	KM199476	Maharachchikumbura *et al.* (2014)
P. biciliata	CBS 124463	**T**	*Platanus hispanic* (*Platanaceae*)	Slovakia	KM199308	KM199399	KM199505	Maharachchikumbura *et al.* (2014)
	CBS 236.38		*Paeonia* sp. (*Paeoniaceae*)	Italy	KM199309	KM199401	KM199506	Maharachchikumbura *et al.* (2014)
P. brachiata	LC2988	**T**	*Camellia* sp. (*Theaceae*)	China	NR_175047.1	KX895265.1	KX895150.1	Liu *et al.* (2017)
	LC8188		*Camellia* sp. (*Theaceae*)	China	KY464142.1	KY464162.1	KY464152.1	Liu *et al.* (2017)
	LC8189		*Camellia* sp. (*Theaceae*)	China	KY464143.1	KY464163.1	KY464153.1	Liu *et al.* (2017)
P. brassicae	CBS 170.26	**T**	*Brassica napus* (*Brassicaceae*)	New Zealand	KM199379	—	KM199558	Maharachchikumbura *et al.* (2014)
P. camelliae-oleiferae	CSUFTCC08	**T**	*Camellia oleifera* (*Theaceae*)	China	OK493593	OK562368	OK507963	Li *et al.* (2021)
	CSUFTCC09		*Camellia oleifera* (*Theaceae*)	China	OK493594	OK562369	OK507964	Li *et al.* (2021)
P. chamaeropis	CBS 186.71	**T**	*Chamaerops humilis* (*Arecaceae*)	Italy	KM199326	KM199391	KM199473	Maharachchikumbura *et al.* (2014)
	CBS 113604; STE-U 3078		n/a	n/a	KM199323	KM199389	KM199471	Maharachchikumbura *et al.* (2014)
	CBS 113607; STE-U 3080		n/a	n/a	KM199325	KM199390	KM199472	Maharachchikumbura *et al.* (2014)

Table 1. (Continued).

Species	Strain Number	Type	*Host*	Location	GenBank Accession			Reference
					ITS	*TUB*	*TEF*	
P. chamaeropis	CBS 237.38		n/a	n/a	KM199324	KM199392	KM199474	Maharachchikumbura *et al.* (2014)
P. chinensis	*MFLUCC 12–0273*	**T**	n/a	*China*	JX398995	—	—	Maharachchikumbura *et al.* (2012)
P. clavata	MFLUCC 12-0268; NN0471340	**T**	*Buxus* sp. (*Buxaceae*)	China	JX398990	JX399025	JX399056	Maharachchikumbura *et al.* (2012)
	MFLUCC 12–0268	**T**	*Buxus* sp. (*Buxaceae*)	China	*JX398990*	*JX399025*	JX399056	Maharachchikumbura *et al.* (2012)
P. daliensis	CGMCC 3.23548 = KUNCC 22-12403	**T**	*Rhododendron decorum* (*Ericaceae*)	China	OP082429	OP185518	OP185511	Gu *et al.* (2022)
P. digitalis	*MFLU 14-0208*	**T**	*Digitalis purpurea* (*Plantaginaceae*)	*New Zealand*	*KP781879*	KP781883	—	Liu *et al.* (2015)
P. disseminata	CBS 143904		*Persea americana* (*Lauraceae*)	New Zealand	MH554152.1	MH554825.1	MH554587.1	Liu *et al.* (2019)
P. endophytica	MFLUCC 18-0932	**T**	*Magnolia garrettii* (*Magnoliaceae*)	Thailand	PQ129358.1	—	MW417119.1	De Silva *et al.* (2021)
P. exudata	*CGMCC 3.23488*	**T**	*Aucuba japonica* (*Garryaceae*)	China	*OR247985*	*OR381060*	*OR361460*	Razaghi *et al.* (2024)
	LC15850		*Aucuba japonica* (*Garryaceae*)	China	*OR247986*	*OR381061*	OR361461	Razaghi *et al.* (2024)
P. ficicrescens	GUCC21556	**T**	*Ficus tikoua* (*Moraceae*)	China	MZ477311	MZ868301	MZ868328	Hyde *et al.* (2023)
	LC12337; CGMCC3.23471		*Oleaceae*	China	OR247980	OR381055	OR361455	Razaghi *et al.* (2024)
	LC2960		*Cleyera japonica* (*Theaceae*)	China	OR247983	OR381058	OR361458	Razaghi *et al.* (2024)
P. fusiformis	LC4365; CGMCC3.23495	**T**	*Rhododendron* sp. (*Ericaceae*)	China	OR247995	OR381070	OR361470	Razaghi *et al.* (2024)
	LC15852		*Rhododendron* sp. (*Ericaceae*)	China	OR247996	OR381071	OR361471	Razaghi *et al.* (2024)
P. fusoidea	*CGMCC 3.23545*	**T**	*Rhododendron delavayi* (*Ericaceae*)	China	*OP082427*	OP185519	OP185512	Gu *et al.* (2022)
P. ganzhouensis	*CGMCC 3.23489*	**T**	*Cinnamomum camphora* (*Lauraceae*)	China	*OR247987*	*OR381062*	*OR361462*	Razaghi *et al.* (2024)
	LC5089		*Cinnamomum camphora* (*Lauraceae*)	China	*OR247998*	*OR381073*	*OR361473*	Razaghi *et al.* (2024)
P. grandis-urophylla	*E72-04*		*Eucalyptus grandis* (*Myrtaceae*)	Brazil	*KU926710*	*KU926718*	KU926714	Carvalho *et al.* (2019)
P. grevilleae	CBS 114127; STE-U 2919	**T**	*Grevilleae* sp. (*Proteaceae*)	Australia	KM199300	KM199407	KM199504	Maharachchikumbura *et al.* (2014)
P. guangxiensis	CFCC 54308	**T**	*Quercus griffithii* (*Fagaceae*)	China	*OK339737*	OK358513	*OK358498*	Jiang *et al.* (2022)
P. guiyangensis	*CFCC 70626*		*Eriobotrya japonicac* (*Rosaceae*)	China	PP784740	PP842617	*PP842629*	Zhang *et al.* (2013)
	CFCC 70630		*Rohdea japonica* (*Asparagaceae*)	China	*PP784741*	PP842618	*PP842630*	Zhang *et al.* (2013)
P. hainanensis	PSHI2004Endo166	**T**	*Podocarpus macrophyllus* (*Podocarpaceae*)	China	DQ334863	DQ137861	—	Liu *et al.* (2007)
	GUCC 23-0314		n/a	n/a	PQ129355.1	PQ148940.1	PQ140990.1	Unpublished (Genbank)
P. hederae	HJAUP C1638.221	**T**	*Hedera helix* (*Araliaceae*)	China	*PP962270*	*PP952234*	*PP952252*	Luo *et al.* (2024)

Table 1. (Continued).

Species	Strain Number	Type	*Host*	Location	GenBank Accession			Reference
					ITS	*TUB*	*TEF*	
P. hederae	*HJAUP C1638.222*		*Hedera helix* (*Araliaceae*)	China	*PP962271*	*PP952216*		Luo *et al.* (2024)
P. heterocornis	PSHI2002Endo303		*C. japonica* (*Theaceae*)	n/a	AY687874.1	DQ137867.1	—	Liu *et al.* (2007)
P. hispanica	CBS 115391 = CPC5193	**T**	*Protea* cv. 'Susara' (*Proteaceae*)	Spain	MH553981	MH554640	MH554399	Liu *et al.* (2019)
P. hollandica	CBS 265.33	**T**	*Sciadopitys verticillata* (*Sciadopityaceae*)	Netherlands	KM199328	KM199388	KM199481	Maharachchikumbura *et al.* (2014)
P. hydei	*MFLUCC 20–0135*	**T**	*Litsea elliptica* (*Lauraceae*)	Thailand	*MW266063*	MW251112	*MW251113*	Huanaluek *et al.* (2021)
P. iberica	*CAA 1004*	**T**	*Pinus radiata* (*Pinaceae*)	Spain	MW732248	MW759035	MW759038	Monteiro *et al.* (2022)
P. intermedia	MFLUCC 12-0259; NN0476420	**T**	Unidentified tree	China	JX398993	JX399028	JX399059	Maharachchikumbura *et al.* (2012)
P. italiana	MFLUCC12-0657	**T**	*Cupressus glabra* (*Cupressaceae*)	Italy	KP781878	KP781882	KP781881	Liu *et al.* (2015)
P. jiangxiensis	LC4399	**T**	*Camellia* sp. *(Theaceae*)	China	KX895009	KX895341	KX895227	Liu *et al.* (2017)
	LC4242		*Eurya* sp. *(Pentaphylacaceae*)	China	KX895035	KX895327	KX895213	Liu *et al.* (2017)
P. kaki	*KNU–PT–1804*	**T**	*Diospyros kaki* (*Ebenaceae*)	Korea	*LC552953*	*LC552954*	LC553555	Das *et al.* (2021)
P. kenyana	*CBS 442.67*	**T**	*Coffea* sp. *(Rubiaceae*)	Kenya	*KM199302*	*KM199395*	*KM199502*	Maharachchikumbura *et al.* (2014)
P. knightiae	CBS 114138; STE-U 2906	**T**	*Knightia* sp. (*Proteaceae*)	New Zealand	KM199310	KM199408	KM199497	Maharachchikumbura *et al.* (2014)
	CBS 111963; STE-U 2905		*Knightia* sp. (*Proteaceae*)	New Zealand	KM199311	KM199406	KM199495	Maharachchikumbura *et al.* (2014)
P. lawsoniae	PSH2000I-057		*Pinus massoniana* (*Pinaceae*)	n/a	AY687871	DQ333577	—	Liu *et al.* (2007)
P. lespedezae	*SY16E*		*Pinus armandii* (*Pinaceae*)	China	*EF055205*	—	*EF055242*	Hu *et al.* 2007
P. leucadendri	CBS 121417	**T**	*Leucadendron* sp. (*Proteaceae*)	South Africa	MH553987	MH554654	MH554412	Liu *et al.* (2019)
P. linearis	MFLUCC 12-0271; NN0471900	**T**	*Trachelospermum* sp. (*Apocynaceae*) & *Tsuga* sp. *(Pinaceae*)	China	JX398992	JX399027	JX399058	Maharachchikumbura *et al.* (2012)
P. lobata	*CGMCC 3.23467 T*	**T**	*Lithocarpus glaber* (*Fagaceae*)	China	*OR247976*	*OR381051*	*OR361451*	Razaghi *et al.* (2024)
	LC15843		*Lithocarpus glaber* (*Fagaceae*)	*China*	OR247977	OR381052	OR361452	Razaghi *et al.* (2024)
P. lushanensis	*LC4344*	**T**	*Camellia* sp. *(Theaceae*)	China	*KX895005*	*KX895337*	*KX895223*	Liu *et al.* (2017)
P. macadamiae	*BRIP 63738b*	**T**	*Macadamia integrifolia* (*Proteaceae*)	Australia	*KX186588*	*KX186680*	*KX186621*	Akinsanmi *et al.* (2017)
P. machiliana	*HJAUP C1790.221*	**T**	*Machilus pauhoi* (*Lauraceae*)	China	*PP962355*	PP952214	PP952253	Luo *et al.* (2024)
	HJAUP C1790.222		*Machilus pauhoi* (*Lauraceae*)	China	*PP962356*	PP952215	PP952254	Luo *et al.* (2024)
P. mainena	CBS 153151	**T**	*Picea glauca* (*Pinaceae*)	United States	PQ568305	PQ573018	PQ573023	This Study
	CBS 153150		*Picea glauca* (*Pinaceae*)	United States	PQ568304	PQ573017	PQ573022	This Study
P. mangifolia	PSHI2002Endo672		*Cameillia sasanqua* (*Theaceae*)	n/a	AY687307.1	DQ333578	—	Liu *et al.* (2007)

Table 1. (Continued).

Species	Strain Number	Type	*Host*	Location	GenBank Accession			Reference
					ITS	*TUB*	*TEF*	
P. menhaiensis	*YN3A1*	**T**	*Camellia sinensis* (*Theaceae*)	China	*KU252272*	*KU252488*	KU252401	Wang *et al.* (2019)
P. microspora	*SS1-033I*		*Cornus canadensis* (*Cornaceae*)	Canada	*MT644300*	—	—	Unpublished (Genbank)
P. monochaeta	CBS 144.97	**T**	*Quercus robur* (*Fagaceae*)	Netherlands	KM199327	KM199386	KM199479	Maharachchikumbura *et al.* (2014)
	CBS 440.83; IFO 32686		*Taxus baccata* (*Taxaceae*)	Netherlands	KM199329	KM199387	KM199480	Maharachchikumbura *et al.* (2014)
P. multicolor	CFCC59981	**T**	*Taxus chinensis* (*Taxaceae*)	China	OQ626676	OQ714336	OQ714341	Wang *et al.* (2024)
	CFCC59982		*Taxus chinensis* (*Taxaceae*)	China	OQ771896	OQ779488	OQ779483	Wang *et al.* (2024)
	LC3616; CGMCC 3.23171		*Camellia* sp. (*Theaceae*)	China	KX894990	KX895321	KX895207	Liu *et al.* (2017)
P. neohantoniensis	CBS 153149	**T**	*Tsuga canadensis* (*Pinaceae*)	United States	PQ568303	PQ573016	PQ573021	This study
	CBS 153152		*Picea omrorika* (*Pinaceae*)	United States	PQ568306	PQ573019	PQ573024	This study
	CBS 153153		*Tsuga canadensis* (*Pinaceae*)	United States	PQ568307	PQ573020	PQ573025	This study
P. olivacea	PSHI2002Endo696		*Cameillia sasanqua* (*Theaceae*)	n/a	AY687883	DQ333580	—	Liu *et al.* (2007)
P. oryzae	*CBS 353.69*	**T**	*Oryza sativa* (*Poaceae*)	Denmark	*KM199299*	KM199398	*KM199496*	Maharachchikumbura *et al.* (2014)
P. parva	CBS 278.35	**T**	*Leucothose fontanesiana* (*Ericaceae*)	n/a	KM199313	KM199405	KM199509	Maharachchikumbura *et al.* (2014)
	CBS 265.37; BBA 2820		*Delonix regia* (*Fabaceae*)	n/a	KM199312	KM199404	KM199508	Maharachchikumbura *et al.* (2014)
P. phoebes	*SAUCC230093*	**T**	*Phoebe zhennan* (*Lauraceae*)	China	*OQ692028*	*OQ718803*	*OQ718745*	Zhang *et al.* (2023)
P. pini	*MEAN 1092*	**T**	*Pinus pinea* (*Pinaceae*)	Portugal	*MT374680*	*MT374705*	*MT374693*	Silva *et al.* (2020)
P. piraubensis	*COAD 2165*	**T**	*Psidium guajava* (*Myrtaceae*)	Brazil	*MH627381*	MH643773	*MH643774*	Jayawardena *et al.* (2022)
P. pruni	*CGMCC 3.23507*	**T**	*Prunus cerasoides* (*Rosaceae*)	China	*OR248001*	*OR381076*	OR361476	Razaghi *et al.* (2024)
P. rhododendri	IFRDCC 2399	**T**	*Rhododendron sinogrande* (*Ericaceae*)	China	KC537804	KC537818	KC537811	Zhang *et al.* (2013)
P. rosarioides	CGMCC 3.23549	**T**	*Rhododendron decorum* (*Ericaceae*)	China	OP082430	OP185520	OP185513	Gu *et al.* (2022)
P. rosea	MFLUCC 12-0258; NN0471350	**T**	*Pinus* sp. (*Pinaceae*)	China	JX399005	JX399036	JX399069	Maharachchikumbura *et al.* (2012)
P. rubrae	LC4567; CGMCC 3.23499	**T**	*Quercus rubra* (*Fagaceae*)	China	OR247997	OR381072	OR361472	Razaghi *et al.* (2024)
P. rubrae	LC8233		*Plagiogyria glauca* (*Plagiogyriaceae*)	China	OR248000	OR381075	OR361475	Razaghi *et al.* (2024)
P. scoparia	CBS 176.25	**T**	*Chamaecyparis* sp. (*Cupressaceae*)	n/a	KM199330	KM199393	KM199478	Maharachchikumbura *et al.* (2014)
	CBS296.58		*Picea* rootstock (*Pinaceae*)	Netherlands	MH554026	MH554703	MH554461	Liu *et al.* (2019)
P. sequoia	*MFLUCC 13–0399*	**T**	*Sequoia sempervirens* (*Cupressaceae*)	Italy	*KX572339*	—	—	Hyde *et al.* (2016)

Table 1. (Continued).

Species	Strain Number	Type	*Host*	Location	GenBank Accession			Reference
					ITS	*TUB*	*TEF*	
P. shaanxiensis	*CFCC 54958*	**T**	*Quercus variabilis* (*Fagaceae*)	China	ON007026	ON005054	ON005043	Jiang *et al.* (2022)
P. shandogensis	*JZB340038*	**T**	*Robinia pseudoacacia* (*Fabaceae*)	China	MN625275	*MN626729*	*MN626740*	Unpublished (Genbank)
P. taxicola	CFCC59976	**T**	*Taxus chinensis* (*Taxaceae*)	China	OQ626673	OQ714333	OQ714338	Wang *et al.* (2024)
	CFCC59978		*Taxus chinensis* (*Taxaceae*)	China	OQ771893	OQ779485	OQ779480	Wang *et al.* (2024)
P. telopeae	*CBS 114161*	**T**	*Telopea* sp. (*Proteaceae*)	*Australia*	*KM199296*	*KM199403*	KM199500	Maharachchikumbura *et al.* (2014)
P. terricola	*CBS 141.69*	**T**	Soil	Pacific Islands	MH554004	MH554680	MH554438	Liu *et al.* (2019)
P. trachycarpicola	OP068	**T**	*Trachycarpus fortunei* (*Arecaceae*)	China	*JQ845947*	*JQ845945*	JQ845946	Zhang *et al.* (2012)
P. tumida	CFCC55158	**T**	*Rosa chinensis* (*Rosaceae*)	China	OK560610	OM158174	OL814524	Peng *et al.* (2022)
	CFCC55159		*Rosa chinensis* (*Rosaceae*)	China	OK560613	OM158177	OL814527	Peng *et al.* (2022)
	LC6057; CGMCC 3.23502		n/a	China	OR247999	OR381074	OR361474	Razaghi *et al.* (2024)
P. unicolor	MFLUCC 12-0276; NN0469740	**T**	*Rhododendron* sp. (*Ericaceae*)	China	JX398999	JX399030	—	Maharachchikumbura *et al.* (2012)
	MFLUCC 12-0275; NN0473080		Unidentified tree	China	JX398998	JX399029	JX399063	Maharachchikumbura *et al.* (2012)
P. verruculosa	MFLUCC 12-0274; NN0473090	**T**	*Rhododendron* sp. (*Ericaceae*)	China	JX398996	—	JX399061	Maharachchikumbura *et al.* (2012)
P. yunnanensis	*HMAS 96359*	**T**	*Podocarpus macrophyllus* (*Podocarpaceae*)	China	AY373375	—	—	Wei *et al.* (2013)
P. cangshanensis	*CGMCC 3.23544*	**T**	*Rhododendron delavayi* (*Ericaceae*)	China	OP082426	OP185517	OP185510	Gu *et al.* (2022)

Alignment and phylogenetic analyses

Electropherograms from forward and reverse reads were analysed, edited, and combined with Geneious Prime v. 9 (https://www.geneious.com). Trimmed sequences were aligned with reference sequences obtained from GenBank after using the NCBI BLAST search engine and reviewing relevant literature (Table 1). MAFFT v. 7.490 (Katoh *et al.* 2002, Katoh & Standley 2013) was used to generate alignments for each locus. Alignments for the comparative analysis of species-specific base pair substitutions were deposited in National Agricultural Library AgData Commons (https://doi.org/10.15482/USDA.ADC/28263920) (Tables 2, 3). The nucleotide substitution model for each alignment was estimated using ModelTest-NG (Darriba *et al.* 2019) as implemented by RAxML GUI v. 2.0.10 (Edler *et al.* 2021) based on the Akaike Information Criterion (AICc; Burnham & Anderson 2002). For ITS, *TUB*, *TEF* the best fit models were TPM3uf+I+G4, TrN+I+G4, and TIM2+I+G4, respectively. Maximum likelihood (ML) and Bayesian inference (BI) analyses were generated from the individual and combined data sets. *Neopestalotiopsis saprophytica* (MFLUCC12-0282) was chosen as the outgroup taxa for all analyses. The BI phylogenies were conducted with MrBayes v. 2.24 (Drummond *et al.* 2012) utilizing the GTR GAMMA I (GTR+I+G) model. The ML phylogenies were generated using RAxML (Stamatakis 2014) as implemented by Geneious Prime v. 9. For the BI analysis, the Markov Chain Monte Carlo (BMCMC) was performed with four heated chains for 1000000 generations with trees being sampled every 200th generation. Convergence for each partition was assessed in Geneious Prime v. 9; all parameters had effective sample sizes (ESS) greater than 200. For the ML trees, support for clades were assessed using 1000 nonparametric bootstrap (BS) replicates. Phylogenetic trees were edited using Geneious Prime v.9 (https://www.geneious.com) and Adobe Illustrator CS v.6 (Adobe Systems Inc., USA).

RESULTS

Sampling and fungal isolation

Five *Pestalotiopsis*-like strains were isolated from the 11 conifer plant samples; each strain originating from different trees within the United States. Strains C5 (CBS 153149) and C10 (CBS 153153) were isolated from two different eastern hemlock trees in Hillsborough County, New Hampshire and Hocking County, Ohio, respectively. Strain C9 (CBS 153152) was isolated from a Serbian spruce tree in Hancock County, Maine. Lastly, strains C7 (CBS 153150) and C8 (CBS 153151) were obtained from two different white spruce trees located at separate sites in Hancock County, Maine.

Phylogenetic analyses

The nucleotide sequences generated in this study were deposited in GenBank (Table 1). Alignment data sets were generated for ITS, *TUB*, *TEF*, and a concatenation of the three. The ITS alignment contained 116 taxa and consisted of 534 characters, of which 441 were conserved, 91 variable, and 24 parsimony informative. The *TUB* alignment contained 109 taxa, consisting of 785 characters with 545 being conserved, 205 variable, and 117 parsimony informative. The *TEF* alignment contained 102 taxa and consisted of 925 characters of which 680 were conserved, 221 variable, and 137 parsimony informative. For the ITS single gene phylogenetic analysis, species of *Pestalotiopsis* do not resolve into the currently recognized species. The *TUB* single gene tree provides more resolution when compared to the ITS tree, however various species remain unresolved. Comparatively, the *TEF* single gene tree provides the most resolution and is most concordant with the multi-gene phylogenetic analysis. The concatenated phylogenetic reconstruction using both ML and BI analyses indicated that our five isolates belong to two significatively supported

Table 2. *TEF* species-specific base pair substitutions between *Pestalotiopsis maineana sp. nov.* and closely related species.

	Nucleotide position											
Species	24	31	32	33	39	40	55	56	57	61	65	68
P. maineana (CBS153150)	A	—	—	—	C	T	C	C	T	A	G	G
P. maineana (CBS153151)	A	—	—	—	C	T	C	C	T	A	G	G
P. brachiata (LC2988)	A	—	—	—	C	T	C	C	T	A	G	G
P. hispanica (CBS115391)	A	T	C	A	A	T	C	C	T	A	G	G
P. fusiformis (LC4365)	T	—	—	—	T	C	—	—	—	C	A	T

	Nucleotide position											
Species	71	73	80	167	168	236	243	401	421	422	432	440
P. maineana (CBS153150)	A	A	C	A	T	C	A	C	A	T	T	C
P. maineana (CBS153151)	A	A	C	A	T	C	A	C	A	T	T	C
P. brachiata (LC2988)	A	A	G	A	T	C	A	T	T	C	G	T
P. hispanica (CBS115391)	A	A	C	A	T	C	A	T	T	C	G	T
P. fusiformis (LC4365)	T	C	T	G	C	—	G	C	A	T	T	C

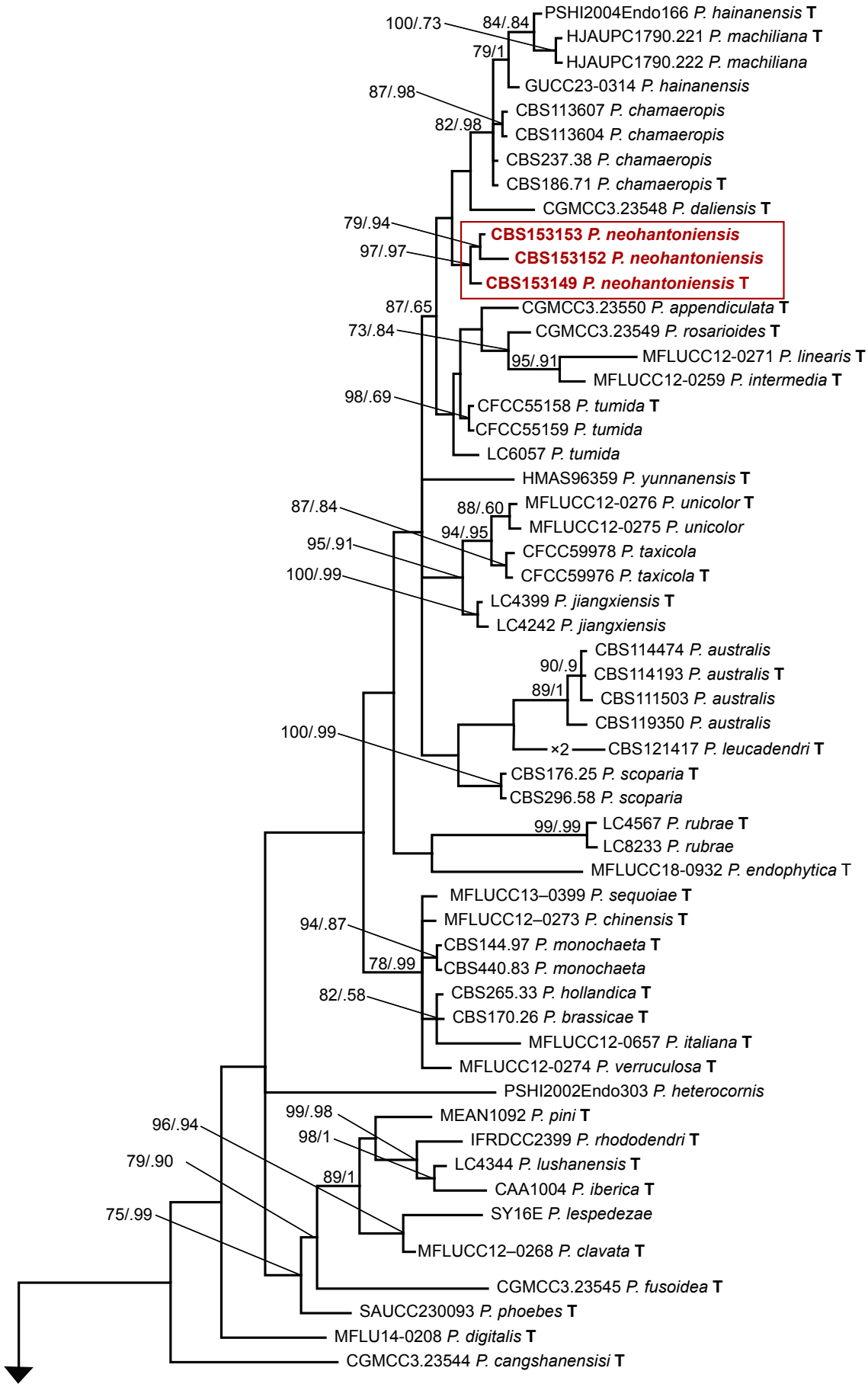

Fig. 1. Phylogenetic tree (ML and BI) based on concatenated sequences of the internal transcribed spacer (ITS), partial beta-tubulin gene (*TUB*), and partial translation elongation factor 1-alpha gene (*TEF*). Bootstrap support values ≥ 70 % and posterior probability values ≥ 0.95 are presented above each node (ML/BI). The two new species reported in this study are in red and "T" indicates the type specimen. *Neopestalotiopsis saprophytica* was used as the outgroup. All isolates are listed in Table 1 with corresponding gene identification numbers and / or accession numbers.

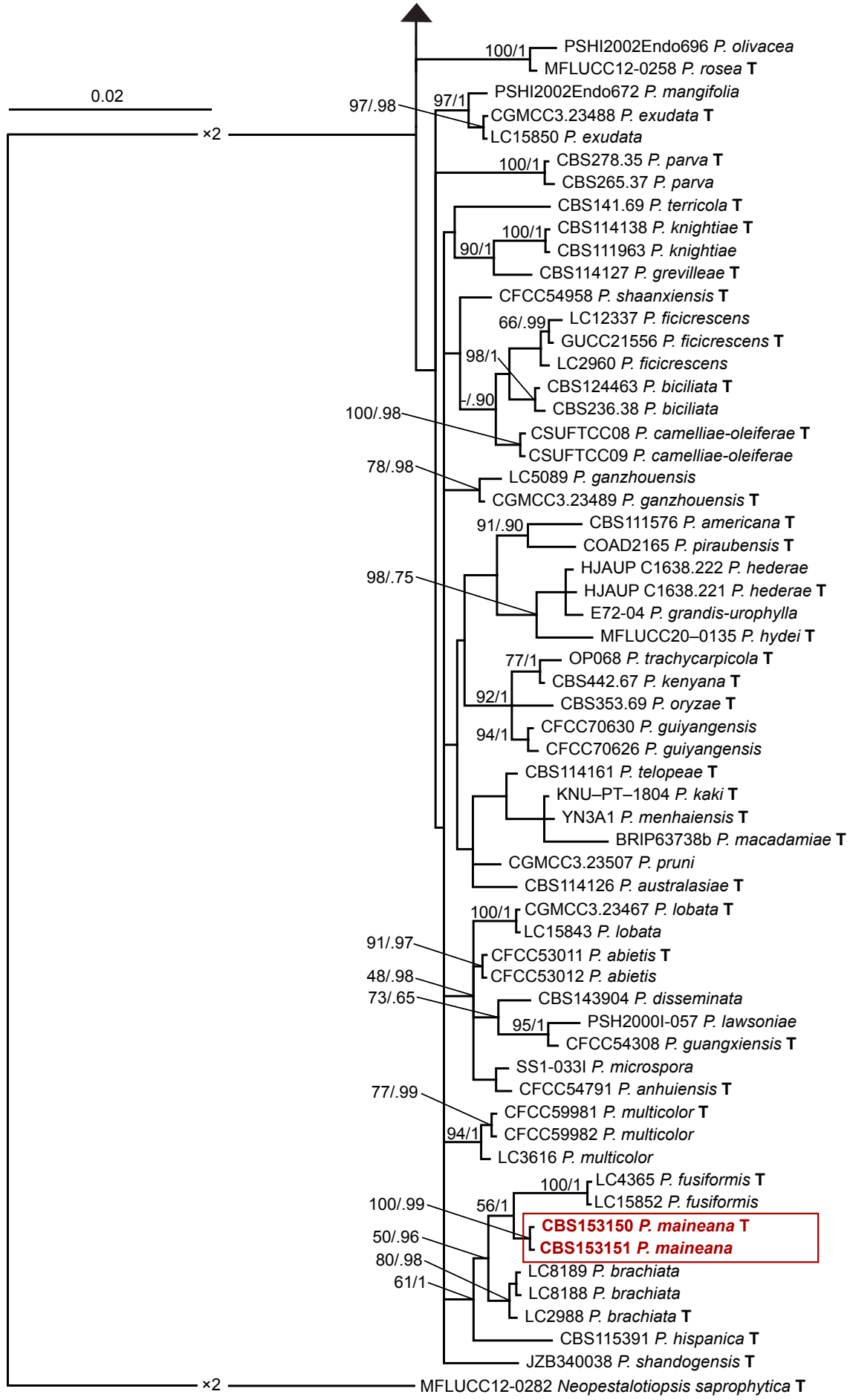

Fig. 1. (Continued).

subclades within the genus of *Pestalotiopsis* (Fig. 1) and are here recognized as *Pestalotiopsis maineana sp. nov*. and *P. neohantoniensis sp. nov. Pestalotiopsis maineana* shares close affinity to *P. fusiformis*, *P. brachiata*, and *P. hispanica*. *Pestalotiopsis neohantoniensis* shares affinity with *P. daliensis*, *P. chamaeropis*, *P. machiliana*, and *P. hainanensis*. Species descriptions, genetic identification, and a comparative morphological analysis of closely related species are presented in the taxonomy section.

TAXONOMY

Pestalotiopsis maineana S.T. Miller & C. Salgado, ***sp. nov.*** MB 857038. Fig. 2.

Etymology: Named after the location where the fungus was collected, Maine, United States of America.

Diagnosis: *Pestalotiopsis maineana* can be diagnosed from the closely related species *P. brachiata*, *P. hispanica*, *P. fusiformis* by nucleotide characters in the *TEF* locus (Table 2). *Pestalotiopsis maineana* can be distinguished from *P. brachiata* by positions 80 (C:G), 401 (C:T), 421 (A:T), 422 (T:C), 432 (T:G), 440 (C:T). From *P. hispanica* by positions 31–33 (- - -:TCA), 401 (C:T), 421 (A:T), 422 (T:C), 432 (T:G), 440 (C:T). From *P. fusiformis* by positions 24 (A:T), 39 (C:T), 40 (T:C), 55–57 (CCT:- - -), 61 (A:C), 65 (G:A), 68 (G:T), 71 (A:T), 73(A:C), 80 (C:T), 167 (A:G), 168 (T:C), 236 (C:-), 243 (A:G).

Description: *Sexual morph*: Undetermined. *Asexual morph*: Conidiomata forming on pine needles or PDA, globose to clavate, scattered or gregarious, exuding black conidial masses. *Conidiophores* indistinct, often reduced to conidiogenous cells. *Conidiogenous cells* discrete, cylindrical or subcylindrical, ampulliform, flask shaped, hyaline, thin-walled, 5.1–18 × 2.1–4.9 µm (av. 10.5 ± 3.69 × 3.1 ± 0.81 µm). *Conidia* fusoid, straight to slightly curved, 4-septate and usually constricted, 21.8–27.9 × 5.4–7.0 µm (av. 25 ± 1.45 × 6.3 ± 0.41 µm); basal cell conic with a truncate base, hyaline, and thin-walled, 4.0–6.4 µm long (av. 5.1 ± 0.69 µm); three median cells doliiform, 14.3–18.4 µm long (av. 15.9 ± 1.08 µm), concolourous, olivaceous to greenish olivaceous with dark septa dividing the cells; apical cell 3.4–6.0 µm long (av. 4.6 ± 0.58 µm), hyaline, subcylindrical with truncate base, thin-walled; with 2–3 tubular apical appendages, arising from the apical or subapical crest, unbranched, filiform, flexuous, 12.4–25.6 µm long (av. 16.4 ± 3.88 µm); basal appendage single, tubular, unbranched 4.4–9.8 µm long (av. 6.4 ± 1.23 µm) (Fig. 2; Table 4).

Fig. 2. *Pestalotiopsis maineana* (ex-holotype culture CBS 153151). **A–D.** Upper and reverse PDA (**A, B**) and SNA (**C, D**) cultures after 7 d of growth. **E.** Conidiomata on SNA. **F.** Conidiomata on PDA. **G–J.** Conidiogenous cells and conidia. **K–N.** Conidia. Scale bars: E = 1 mm; F = 500 µm; G–N = 10 µm.

Culture characteristics: Colonies on PDA flat, entire at the edge, white with flocculent arial mycelia, underside pale luteous in the centre transitioning to white along the margin; after 7 d at 25 °C colonies reach a diameter of 73–80 mm on PDA and 61 mm on SNA. Colonies on SNA flat and colourless (Fig. 2).

Typus: **USA**, Maine, Hancock County, on *Picea glauca* (*Pinaceae*), 20 Nov. 2023, *A. Bergdahl* (**holotype** BPI 977274, culture ex-type CBS 153151; GenBank accession numbers PQ568305, PQ573018, PQ573023).

Notes: *Pestalotiopsis maineana* shares close affinity to *Pestalotiopsis fusiformis*, *P. brachiata*, and *P. hispanica*; however, *P. maineana* differs from *P. fusiformis* and *P. brachiata* based on the number of basal appendages (1 vs 1–2 vs 1–4, respectively) and is somewhat similar to *P. hispanica* in basal appendage number (1 vs 0–1, respectively). *Pestalotiopsis fusiformis* has up to five apical appendages while *P. maineana* has up to three. *Pestalotiopsis mainenana* differs from *P. hispanica* based on the length of the basal appendage (4.4–9.8 µm vs 1.4–4.5 µm), the apical appendage number (2–3 vs 2–4) and length (12.4–25.6 µm vs 2–14 µm), and the width of conidia (5.4–7.0 µm vs 6–9.5 µm) (Table 4).

Pestalotiopsis neohantoniensis S.T. Miller & C. Salgado, ***sp. nov.*** MB 857039. Fig. 3.

Etymology: Named after the location where the fungus was collected, New Hampshire, United States of America.

Diagnosis: *Pestalotiopsis neohantoniensis* can be diagnosed from the closely related species *P. daliensis*, *P. hainanensis*, *P. machiliana*, *P. chamaeropis* by nucleotide characters in the *TEF* locus (Table 3). *Pestalotiopsis neohantoniensis* can be distinguished from *P. daliensis* by positions 11 (G:A), 16 (T:-), 31 (T:C), 33 (C:T), 40–42 (- - -: TCA), 96 (C:T), 205 (T:C), 224 (A:T), 418 (-:C), 478 (T:G). From *P. hainanensis* by positions 16 (T:C), 40–42 (- - -: TCA), 96 (C:T), 154 (T:A), 205 (T:C), 224 (A:T), 411 (C:T), 418 (-:C). From *P. machiliana* by positions 16 (T:C), 40–42 (- - -: TCA), 96 (C:T), 154 (T:A), 205 (T:C), 224 (A:T), 262 (A:T). From *P. chamaeropis* by positions 40–42 (- - -:TCA), 96 (C:T), 154 (T:A), 205 (T:C), 224 (A:T), 411 (C:T), 418 (-:C).

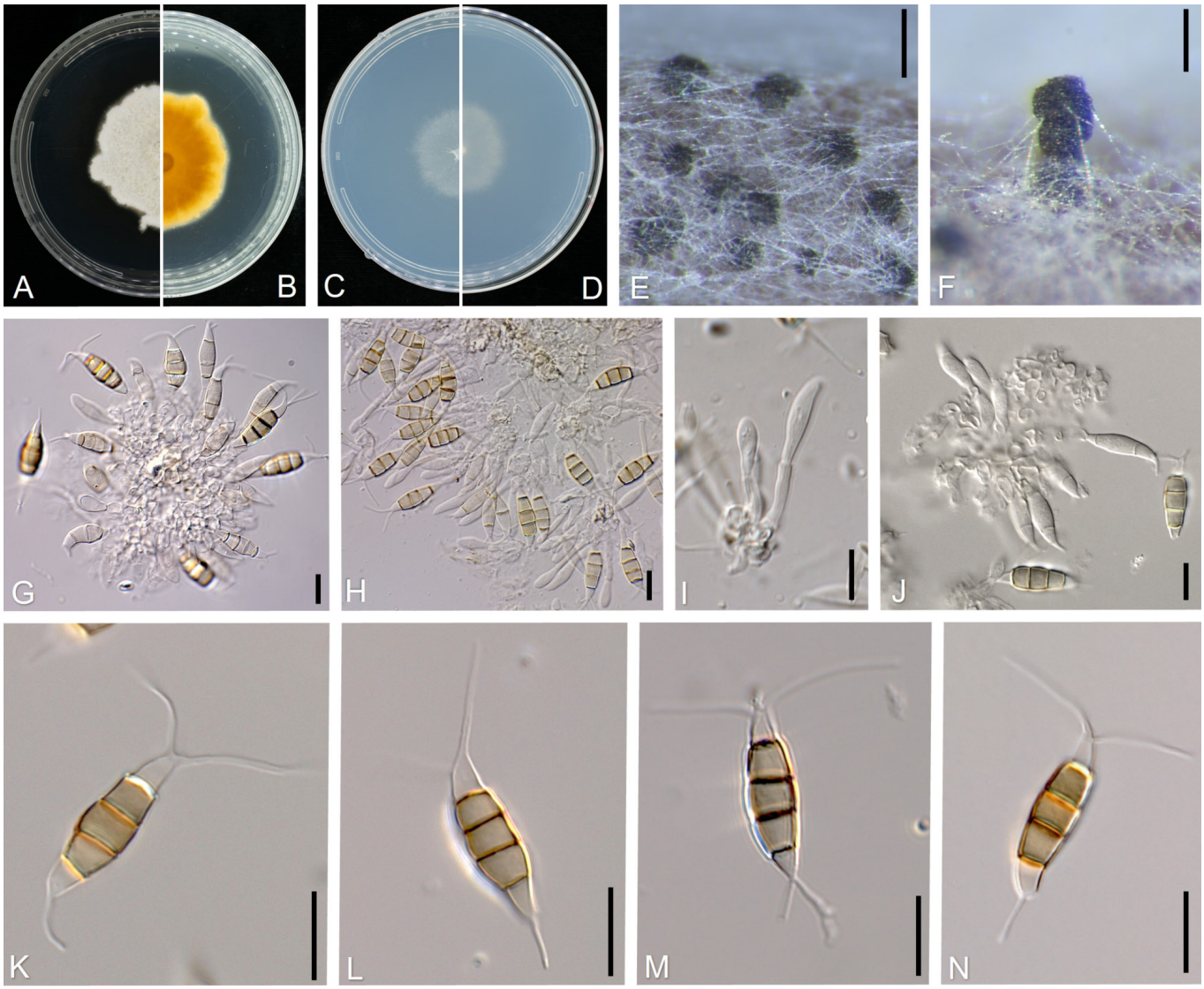

Fig. 3. *Pestalotiopsis neohantoniensis* (ex-holotype culture CBS 153149). **A–D.** Upper and reverse PDA (**A, B**) and SNA (**C, D**) cultures after 7 d of growth. **E, F.** Conidiomata on pine needle. **G–J.** Conidiogenous cells and conidia. **K–N.** Conidia. Scale bars: E = 200 µm; F = 100 µm; G–N = 10 µm.

Description: *Sexual morph*: Undetermined. *Asexual morph*: *Conidiomata* forming on pine needles, globose to clavate, scattered or gregarious, covered by aerial mycelia, exuding dark black conidial masses. *Conidiophores* indistinct, often reduced to conidiogenous cells. *Conidiogenous cells* discrete, cylindrical or subcylindrical, ampulliform, hyaline, 6.2–20 × 1.9–5.8 µm (av. 12.9 ± 3.470 × 3.71 ± 1.2 µm). *Conidia* fusoid, ellipsoid, usually straight but sometimes curved, 4-septate, usually constricted at septa, 19.2–25.5 × 5.1–7.6 µm (av. 22.2 ± 1.43 × 6.6 ± 0.54 µm); basal cell conic with a truncate base, hyaline, and thin-walled, 3.1–6.6 µm long (av. 4.2 ± 0.64 µm); three median cells doliiform, 12–15.9 µm long (av. 14.1 ± 0.91 µm), concolourous, olivaceous to olivaceous buff with dark septa dividing the cells; apical cell 3.3–6.0 µm long (av. 4.6 ± 0.62 µm) hyaline, subcylindrical with truncate base, thin-walled; with 1–4 (usually 1–3) tubular apical appendages, arising from the apical or subapical crest, unbranched (occasionally branched), filiform, flexuous, 10.9–26 µm long (av. 17.9 ± 3.9 µm); basal appendage 1–2 (usually 1), tubular, centric, unbranched (occasionally branched), 3.8–13.2 µm long (av. 7.4 ± 2.4 µm) (Fig. 3; Table 4).

Culture characteristics: Colonies on PDA are flat with round edges; white with flocculent aerial mycelia, underside luteous to amber with a pure yellow margin on day 7, and after 14 d the centre darkens while the outer mycelia remain pure yellow with a white margin. Colonies reach a diameter of 35 mm after 7 d and 50 mm after 14 d on PDA at 25 °C; on SNA colonies are flat and colourless reaching 32 mm after 7d and 55.8 mm after 14 d at 25 °C (Fig. 3).

Typus: **USA**, New Hampshire, Hillsborough County, on *Tsuga canadensis* (*Pinaceae*), 12 Dec. 2023, *W. Davidson* (**holotype** BPI 911275, culture ex-type CBS 153149; GenBank accession numbers PQ568303, PQ573016, PQ573021).

Notes: *Pestalotiopsis neohantoniensis* shares close affinity to *Pestalotiopsis daliensis*, *P. hainanensis*, *P. machiliana*, and *P. chamaeropis*; however, *P. neohantoniensis* is the only species that has up to 2 basal appendages (usually 1) and up to 4 apical appendages (usually 1–3) when compared to *P. daliensis*, *P. hainanensis*, *P. machiliana* and *P. chamaeropis* (Table 4). *Pestalotiopsis neohantoniensis* exhibited two basal appendages in 7.5 % of observed spores (n = 40) (Fig. 3M). *Pestalotiopsis neohantoniensis* differs from *P. daliensis* and *P. hainanensis* based on the length of the basal appendage (3.8–13.2 µm vs 10–16 µm vs absent, respectively). *Pestalotiopsis neohantoniensis* has wider conidia cells when compared to *P. daliensis* (5.1–7.6 µm vs 4–5 µm). Further, *P. neohantoniensis* has longer apical appendages when compared to *P. hainanensis* (10.9–26 µm vs 1–10 µm) (Table 4).

DISCUSSION

Historically, species of *Pestalotiopsis* were often named based on the host from which they were isolated; however, host association is unreliable for delimiting species of *Pestalotiopsis* when compared to DNA sequence-based analyses and morphological characterization (Jeewon *et al.* 2004, Crous *et al.* 2012, Maharachchikumbura *et al.* 2012). When analysing the ITS, *TUB*, and *TEF* sequence data individually, *TEF* was the most effective for species delimitation within our analysis while ITS and *TUB* did not resolve all the known taxonomic relationships of species. Maharachchikumbura *et al.* (2012) reported *TEF* and *TUB* to be the most favourable markers when comparing 10 loci, including *ACT*, *TUB*, *CAL*, *GPDH, GS*, ITS, LSU, *RPB1*, SSU and *TEF.* The concatenated phylogenetic analysis indicates that our five isolates belong to two well supported subclades within *Pestalotiopsis*. Considering morphological characteristics, we found the appendages of the conidia to be helpful for discerning between closely related species, as has been reported in previous studies (Crous *et al.* 2012, Maharachchikumbura *et al.* 2012).

When comparing *Pestalotiopsis* species which have historically been reported on spruce or hemlock trees, the two new species described herein differ morphologically and on DNA sequence data (where available). *Pestalotiopsis stevensonii*, *P. gravesii,* and *P. heterocornis* have all been reported from either Norway spruce (*Picea abies*), red spruce (*Picea rubens*), or dragon spruce (*Picea asperata*)

Table 3. *TEF* species-specific base pair substitutions between *Pestalotiopsis neohantoniensis sp. nov.* and closely related species.

	Nucleotide position														
Species	11	16	31	33	40	41	42	96	154	205	224	262	411	418	478
P. neohantoniensis (CBS153149)	G	T	T	C	—	—	—	C	T	T	A	A	C	—	T
P. neohantoniensis (CBS153152)	G	T	T	C	—	—	—	C	T	T	A	A	C	—	T
P. neohantoniensis (CBS153153)	G	T	T	C	—	—	—	C	T	T	A	A	C	—	T
P. daliensis[a]	A	—	C	T	T	C	A	T	T	C	T	A	C	C	G
P. hainanensis (PSHI2004Endo166)	G	C	T	C	T	C	A	T	A	C	T	A	T	C	T
P. machiliana (HJAUPC1790.221)	G	C	T	C	T	C	A	T	A	C	T	T	N/A	N/A	N/A
P. chamaeropis (CBS186.71)	G	C	T	C	T	C	A	T	A	C	T	A	T	C	T

[a] CGMCC3.23548 = KUNCC22-12403

while *P. linearis* was described from *Tsuga* sp. (Nag Raj 1993, Kobayashi 2007, Ge *et al.* 2009, Chandelier *et al.* 2010, Maharachikumbura *et al.* 2012). There is no sequence data available for *P. stevensonii* and *P. gravesii*; however, morphological differences are prominent. In fact, *P. stevensonii* likely does not belong to the *Pestalotiopsis* genus. As discussed by Maharachchikumbura *et al.* (2014), Nag Raj (1993) grouped *P. stevensonii* within *Pestalotiopsis* due to his broad concept of the genus which included 3-septate, and 2-central celled conidial forms; therefore, *P. stevensonii* likely belongs in the genus *Truncatella* (Adrianova & Minter 2012). *Pestalotiopsis gravesii* has been documented as having 3–4 apical appendages with one located at the apical cell apex and the other 2–3 appendages located on the basal part of the apical cell pointing horizontally/backwards towards the basal cell (Kobayashi 1974). Comparatively, both *P. maineana* and *P. neohantoniensis* have apical appendages which arise from the apical or subapical crest and not on the lower portion of the apical cell. Furthermore, the apical appendages for *P. maineana* and *P. neohantoniensis* do not point towards the basal cell. Sequence data is available for *P. heterocornis* and *P. linearis* and based on the multi-gene phylogeny, both belong to subclades separate from *P. maineana* and *P. neohantoniensis* within *Pestalotiopsis*.

The host specificity of *P. maineana* and *P. neohantoniensis* is unclear. If they are specific to conifers or if they occur on other hosts is not yet known. To our knowledge, *P. maineana* is the first species to be found associated with white spruce (*Picea glauca*) while *P. neohantoniensis* is the first species of *Pestalotiopsis* found associated with Serbian spruce (*Picea omorika*) and eastern hemlock (*Tsuga canadensis*). As discussed by Razaghi *et al.* (2024), it is common for very closely related species of *Pestalotiopsis* to differ in host specificity; one species may have a broad host range while a close relative can remain specialized. The species closely related to *P. maineana* include *P. fusiformis*, *P. brachiata*, and *P. hispanica. Pestalotiopsis fusiformis* and *P. brachiata* have only been reported on one host thus far, *Rhododendron* sp. and *Camellia* sp., respectively, whereas *P. hispanica* has been reported on *Protea* sp., *Dianthus caryophyllus*, *Peristrophe japonica*, and *Cryptomeria japonica* (Liu *et al.* 2017, Liu *et al.* 2019, Fan 2023, Sun 2023, Hsu *et al.* 2024, Razaghi *et al.* 2024). Comparatively, of the species closely related to *P. neohantoniensis,* two (*P. daliensis* and *P. hainanensis*) have only been reported on one host, *Rhododendron decorum* and *Podocarpus macrophyllus* var. *maki*, respectively (Liu *et al.* 2007, Gu *et al.* 2022). The other closely related species, *P. chamaeropis*, has been reported on over eight hosts including *Chamaerops humilis*, *Camellia sinensis*, *Eurya nitida*, *Prostanthera rotundifolia*, *Vaccinium corymbosum*, *Vandopsis gigantea*, *Dianthus caryophyllus,* and *Vitis vinifera* (Maharachchikumbura *et al.* 2014, Moslemi & Taylor 2015, Ran *et al.* 2017, Jayawardena *et al.* 2018, Chen *et al.* 2021, Qiu *et al.* 2022, Santos *et al.* 2022, Hsu *et al.* 2024). It is important to note that the plant samples collected in this study, from which the two new species were isolated, were collected from conifers with Rosellinia needle blight, which may have biased these results. It is uncertain if *P. maineana* and *P. neohantoniensis* are generalists or specialists; however, previous studies have reported that species of *Pestalotiopsis* are usually not specific to one host plant (Liu *et al.* 2007).

Table 4. Comparison of conidia spores from *Pestalotiopsis* species which are closely related to the two species described in this study.

Species	Conidia Size (µm)				Apical appendages		Basal appendages		References
		Length							
		Apical cell (µm)	Median cells (µm)	Basal cell (µm)	Number	Length (µm)	Number	Length (µm)	
P. maineana (CBS153151) Type	21.8–27.9 × 5.4–7.0	3.4–6.0	14.3–18.4	4.0–6.4	2–3	12.4– 25.6	1	4.4–9.8	This study
P. fusiformis[a]	18–25 × 5.5–8	3–5.5	12–15	3.5–7	2–5	13–25	1–2	3–11	Razaghi *et al.* (2024)
P. brachiata[b]	23.5–25 × 6.5–8	3.5–6	14–16.5	4–6.5	2–3	16–28.5	1–4	5.5–9.5	Liu *et al.* (2017)
P. hispanica[c]	16.5–29 × 6–9.5	2–4.5	14–18.5	3–5	2–4	2–14	0–1	1.4–4.5	Liu *et al.* (2019)
P. neohantoniensis (CBS153149) Type	19.2–25.5 × 5.1–7.6	3.3–6.0	12.0–15.9	3.1–6.6	1–4	10.9–26	1–2	3.8–13.2	This Study
P. daliensis[d]	23–26 × 4–5	4–6	13–16	4–6	2–3	13–22	1	10–16	Gu *et al.* (2022)
P. hainanensis[e]	19–22 × 5–6	n/d	13–15	n/d	1–3	1–10	absent	absent	Liu *et al.* (2007)
P. machiliana[f]	18.6–27.2 × 5.6–7.4	3–4.8	12.5–17.3	3–5.2	2–3	12.9–22.5	1	4.5–10.2	Luo *et al.* (2024)
P. chamaeropis[g]	21–28 × 6–9.5	4–6	15–18.5	5–6.5	2–3	13–24	1	4–8.5	Maharachchikumbura *et al.* (2014)

[a] CGMCC3.23495 = LC4365
[b] CGMCC3.18151 = LC2988
[c] CBS115391 = CPC5193
[d] CGMCC3.23548 = KUNCC22-12403
[e] PSHI2004Endo166
[f] HJAUPC1790.221
[g] CBS186.71

Pestalotiopsis maineana and *P. neohantoniensis* were asymptomatic when discovered and if they are endophytes or if they cause disease is not known. They may be pathogenic depending on physiological and environmental triggers like *P. microspora* who Lee *et al.* (1995) reported as being able to switch between endophytic and pathogenic lifestyles (Maharachchikumbura *et al.* 2011). Inoculation assays in future studies are needed to help clarify if they pose a threat to conifer species. Furthermore, surveys investigating the microfungi of different tree species within temperate forests of the US would help explain host specificity, distribution, and disease severity/ incidence (if any) of the newly described species.

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