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Article

Identification and Pathogenicity of Pestalotioid Species on *Alpinia oxyphylla* in Hainan Province, China

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Abstract: *Alpinia oxyphylla* is a traditional Chinese medicinal plant, with a medicinal history of more than 1,700 years. Ring leaf blight (RLB) disease, caused by pestalotioid species, is an important disease of *A. oxyphylla*, seriously affecting the yield and quality of fruits. The causal agent of RLB disease has not been systematically identified and characterized so far. In this study, thirty-six pestalotioid strains were isolated from the leaves and stems of *A. oxyphylla* collected from 6 cities of Hainan province, China. Based on multi-locus phylogeny (*ITS*, *tef-1α* and *tub2*) and morphological characteristics analyses, seventeen species belonging to three genera (*Neopestalotiopsis*, *Pestalotiopsis* and *Pseudopestalotiopsis*) were identified and six new species (*N. baotingensis*, *N. oblatespora*, *N. olivaceous*, *N. oxyphylla*, *N. wuzhishanensis* and *N. yongxunensis*) were described. Pathogenicity tests revealed that strains of *Neopestalotiopsis* species caused more severe ring leaf blight on *A. oxyphylla* than strains of *Pestalotiopsis* and *Pseudopestalotiopsis* under wounded inoculation conditions.

Keywords: pestalotioid species; new taxa; *Alpinia oxyphylla*; ring leaf blight; pathogenicity

1. Introduction:

Alpinia oxyphylla is an important Chinese herbal plant, with a medicinal history dating back to 1,700 years ago [1]. As an edible herb, the traditional medicinal effects of *A. oxyphylla* mainly include warming the kidney, solidifying spermatorrhea, arresting polyuria, warming the spleen as well as stopping diarrhea and saliva [2,3]. Moreover, the essential oil of *A. oxyphylla* has various effects including antibacteria, anticancer, antioxidant, vasodilation and improve immunity [4]. *A. oxyphylla* likes to grow in warm and humid environmental conditions and is commonly planted under rubber tree, areca tree and other economic forests as a semi-shade plant [5–7]. *A. oxyphylla* is mainly distributed in southern China, such as Hainan, Guangdong and Guangxi province. Among them, Hainan is the most important planting area for *A. oxyphylla*, accounting for 90% of the total output in China [8–10].

The occurrence of diseases causes serious losses to the production and quality of *A. oxyphylla*. Ring leaf blight (RLB) is an important disease of *A. oxyphylla*, occurring from seedling to fruiting stage, mainly infecting the old leaves. The disease often extends from the leaf edge or tip, forming irregular, reddish-brown spots with alternating dark and light brown, wavy concentric rings and obvious yellow halos around the periphery of the disease spots, on which scattered numerous small black conidiomata of the pathogen. The high temperature and rainy season contribute to the occurrence of RLB disease, and the high incidence of this disease is from August to September. Under suitable conditions, the proportion of diseased plants can reach more than 50%, and the area of the diseased spots can reach 1/3~1/2 of the leaf surface, even the entire leaf, which has an impressive impact to the growth of *A. oxyphylla* [11,12].

The pathogen of RLB disease was first reported as *Pestalotia palmarum* in 1986[11]. Subsequently, the classification status of *P. palmarum* was adjusted to the genus of *Pestalotiopsis*, while the genus of *Pestalotia* and *Pestalotiopsis* was used confusingly in description of *A. oxyphylla* diseases [13]. The ring brown spot (RBS) disease of *A. oxyphylla* was caused by *Pestalosphaeria alpinia*, a species of sexual morphs of pestalotioid fungi [25]. As asexual fungi, most pestalotioid species lack the sexual morphs, *Pestalosphaeria*[26]. Most of pestalotioid species are important plant pathogens, and are also found as commonly endophytes or saprophytes, mainly distributed throughout tropical and temperate regions [14–16]. Pestalotioid species can infect leaves, shoots, flowers, fruits, or other parts of plants, and cause a variety of diseases of multiple economic crops, including leaf spot, gray blight, shoot dieback, trunk diseases, dry flower and fruit rot [15,17–24]. Hence, pestalotioid species causing the disease of *A. oxyphylla* need to be reidentified and characterized based on fungi diversity, molecular systematics and pathogenicity.

The development of molecular phylogenetic analysis overcomes the limitation of overlapping conidial measurements in traditional taxonomy of pestalotioid species [14,15,27,28]. In 2014, two novel genera, *Neopestalotiopsis* and *Pseudopestalotiopsis*, were segregated from *Pestalotiopsis* based on conidial characters and multi-locus phylogenetic analyses. The combined sequences of *ITS*, *tub2* and *tef-1α* genes were used to construct phylogenetic trees, which become an important basis for distinguishing different species within the genus of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis*. Morphologically, *Neopestalotiopsis* can be easily differentiated from *Pestalotiopsis* and *Pseudopestalotiopsis* by their versicolorous median cells of conidia, and *Pseudopestalotiopsis* is different from *Pestalotiopsis* with three darker concolorous median cells [15]. Through these methods, many novel pestalotioid species isolated from different plants have been introduced in recent years [17,20,29–34].

Therefore, the object of this study is to clarify the types, characteristics and pathogenicity of pestalotioid species related to the disease of *A. oxyphylla* in Hainan, China.

2. Materials and Methods

2.1. Sample Collection, Fungi Isolation and Morphological Examination

Fresh leaves and stems of *A. oxyphylla* with typical ring spot were collected from the main planted area at ten towns in six cities of Hainan province, including Baoting, Ledong, Qiongzhong, Sanya, Wanning, and Wuzhishan in 2022. Small pieces (5×5 mm) of leaves or stems were cut from the junction of disease and health areas, disinfected with 3% sodium hypochlorite for 3 min, then 75% ethanol for 30s, subsequently washed with sterilized water for three times. The treated tissue pieces were dried on sterilized blotting paper and then placed on PDA plates (containing 100 μg/mL streptomycin, 50 μg/mL kanamycin and 100 μg/mL ampicillin). The plates were cultured at room temperature and examined daily for 7 days, then the marginal mycelia were transferred to fresh PDA and purified by single-spore culturing.

The pestalotioid strains usually sporulated at room temperature on PDA after 10-20 days. Conidiomata were observed using dissecting microscope (CNOPTEC, SZ680, China) and the characteristics of spores and conidiophores using optical microscope (CNOPTEC, DV320, China). All the morphological characteristics of the spores were photographed and measured at least 30 individuals using OPTPro. The images were progressed by Adobe Photoshop CS6. The pure cultures of isolated fungal strains were stored in the seed health center of China Agricultural University.

2.2. DNA Extraction, Gene Sequencing, and Phylogenetic Analyses

DNA was extracted from fresh fungal mycelia using the Biomed genomic DNA extraction kit (Biomed, Beijing). The partial sequences of three genes (*ITS*, *tef-1α* and *tub2*) were amplified. The PCR was performed according to Table 1 and the PCR products were purified and sequenced at Beijing Tsingke Biotech.

Table 1. PCR primers and procedures used in this study.

Locus	Primes Name	Sequence (5'to 3')	PCR procedures	Reference
ITS	ITS5 ITS4	GGAAGTAAAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	95°C 5 min; 94°C 25 s; 52°C 25 s; 72°C 10 s; repeat 2 to 4 for 35 cycles; 72°C 5 min; 4°C on hold	[35]
tef-1α	EF1-728F EF1-526F EF2	CATCGAGAAGTTCGAGAAGG GTCGTYGTYATYGGHCAYGT GGARGTACCAGTSATCATGTT	95°C 5 min; 94°C 25 s; 52°C 25 s; 72 °C 10 s (15s); repeat 2 to 4 for 35 cycles; 72°C 5 min; 4°C on hold	[36,37]
tub2	T1 Bt2b	AACATGCGTGAGATTGTAAGT ACCCTCAGTGTAGTGACCCTTGGC	95°C 5 min; 94°C 25 s; 55°C 25 s; 72°C 15 s; repeat 2 to 4 for 35 cycles; 72°C 5 min; 4°C on hold	[38,39]

The nucleotide sequences were checked by Chromas2.4.1, then blasted in the NCBI to assess the closest phylogenetic matches. All related sequences by blasted or referenced previous studies were downloaded from GenBank (Table 2). MAFFT v.7(https://mafft.cbrc.jp/alignment/software/) was used to align each locus sequences and MEGA v.11 was used to manually improve the sequences. The three final aligned gene sequences were concatenated by SequenceMatrix [40].

Table 2. The strains information and their genes' accession numbers of pestalotioid species used in this study.

Taxonomic status	Strain No.	Host/Substrate	Origin	Gen Bank Accessions Numbers			References
				ITS	*tub 2*	*tef-lα*	
Neopestalotiopsis acrostichi	MFLUCC 17-1754^{T}	*Acrostichum aureum*	Thailand	MK764272	MK764338	MK764316	[41]
N. acrostichi	MFLUCC 17-1755	*Acrostichum aureum*	Thailand	MK764273	MK764339	MK764317	[41]
N. alpapicalis	MFLUCC 17-2544^{T}	*Rhizophora mucronata*	Thailand	MK357772	MK463545	MK463547	[42]
N. alpapicalis	MFLUCC 17-2545	*Rhizophora mucronata*	Thailand	MK357773	MK463546	MK463548	[42]
N. amomi	HKAS 124563^{T}	*Amomum villosum*	China	OP498012	OP752133	OP653489	[16]
N. amomi	HKAS 124564	*Amomum villosum*	China	OP498013	OP765913	OP753382	[16]

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N. aotearoa	CBS 367.54T	Canvas	New Zealand	KM199369	KM199454	KM199526	[14]
N. asiatica	MFLUCC 12-0286T	*Prunus dulcis*	China	JX398983	JX399018	JX399049	[15]
N. australis	CBS 114159T	*Telopea* sp.	Australia	KM199348	KM199432	KM199537	[15]
N. brachiata	MFLUCC 17-1555	*Rhizophora apiculata*	Thailand	MK764274	MK764340	MK764318	[41]
N. brasiliensis	COAD 2166T	*Psidium guajava*	Brazil	MG686469	MG692400	MG692402	[43]
N. brasiliensis	CFCC 54341	*Castanea mollissima*	China	MW166229	MW218522	MW199748	[44]
N. camelliae-oleiferae	CSUFTCC81T	*Camellia oleifera*	China	OK493585	OK562360	OK507955	[17]
N. camelliae-oleiferae	CSUFTCC82	*Camellia oleifera*	China	OK493586	OK562361	OK507956	[17]
N. cavernicola	KUMCC 20-0269T	Cave	China	MW545802	MW557596	MW550735	[45]
N. chiangmaiensis	MFLUCC 18–0113	*Pandanus* sp.	Thailand	NA	MH412725	MH388404	[46]
N. chrysea	MFLUCC 12-0261T	Dead leaves	China	JX398985	JX399020	JX399051	[14]
N. chrysea	MFLUCC 12-0262	Dead leaves	China	JX398986	JX399021	JX399052	[14]
N. clavispora	MFLUCC 12-0281T	*Magnolia* sp.	China	JX398979	JX399014	JX399045	[14]
N. clavispora	MFLUCC 12-0280	*Magnolia* sp.	China	JX398978	JX399013	JX399044	[14]
N. cocoes	MFLUCC 15-0152T	*Cocos nucifera*	Thailand	KX789687	NA	KX789689	[41]
N. coffeae-arabicae	HGUP4015	*Coffea arabica*	China	KF412647	KF412641	KF412644	[47]
N. coffeae-arabicae	HGUP4019T	*Coffea arabica*	China	KF412649	KF412643	KF412646	[47]
N. concentrica	CFCC 55162T	*Rosa chinensis*	China	OK560707	OM117698	OM622433	[48]
N. cubana	CBS 600.96T	Leaf litter	Cuba	KM199347	KM199438	KM199521	[15]
N. dendrobii	MFLUCC 14-0106T	*Dendrobium cariniferum*	Thailand	MK993571	MK975835	MK975829	[49]
N. dendrobii	MFLUCC 14-0099	*Dendrobium cariniferum*	Thailand	MK993570	MK975834	MK975828	[49]
N. drenthii	BRIP 72263a	*Macadamia integrifolia*	Australia	MZ303786	MZ312679	MZ344171	[20]
N. drenthii	BRIP 72264aT	*Macadamia integrifolia*	Australia	MZ303787	MZ312680	MZ344172	[20]

N. egyptiaca	CBS 140162^{T}	*Mangifera indica*	Egypt	KP943747	KP943746	KP943748	[50]
N. elaeagni	HGUP10002^{T}	*Elaeagnus pungens*	China	MW930716	MZ683391	MZ203452	[30]
N. elaeidis	MFLUCC 15-0735	*Elaeis guineensis*	Thailand	ON650689	NA	ON734012	[51]
N. ellipsospora	MFLUCC 12-0283^{T}	Dead plant	China	JX398980	JX399016	JX399047	[14]
N. eucalyptorum	CBS 147684^{T}	*Eucalyptus globulus*	Portugal	MW794108	MW802841	MW805397	[18]
N. eucalypticola	CBS 264.37^{T}	*Eucalyptus globulus*	NA	KM199376	KM199431	KM199551	[15]
N. fragariae	ZHKUCC 22-0115	*Fragaria* x *ananassa*	China	ON651146	ON685199	ON685197	[32]
N. foedans	CGMCC 3.9123^{T}	Mangrove plant	China	JX398987	JX399022	JX399053	[14]
N. foedans	CGMCC 3.9178	*Neodypsis decaryi*	China	JX398989	JX399024	JX399055	[14]
N. formicarum	CBS 362.72^{T}	Dead ant	Cuba	KM199358	KM199455	KM199517	[15]
N. formicarum	CBS 115.83	Plant debris	Cuba	KM199344	KM199444	KM199519	[15]
N. guajavae	FMBCC 11.1^{T}	*Psidium guajava*	Pakistan	MF783085	MH460871	MH460868	[52]
N. guajavicola	FMBCC 11.4^{T}	*Psidium guajava*	Pakistan	MH209245	MH460873	MH460870	[52]
N. haikouensis	SAUCC212271^{T}	*Ilexchinensis* sp.	China	OK087294	OK104870	OK104877	[53]
N. hadrolaeliae	COAD 2637^{T}	*Hadrolaelia jongheana*	Brazil	MK454709	MK465120	MK465122	[54]
N. hispanica	CBS 147686^{T}	*Eucalyptus globulus*	Portugal	MW794107	MW802840	MW805399	[18]
N. honoluluana	CBS 114495^{T}	*Telopea* sp.	USA	KM199364	KM199457	KM199548	[15]
N. hydeana	MFLUCC 20-0132^{T}	*Artocarpus heterophyllus*	Thailand	MW266069	MW251119	MW251129	[55]
N. hyperici	HKAS 124561	*Hypericum monogynum*	China	OP498010	OP765908	OP713768	[16]
N. iberica	CBS 147688^{T}	*Eucalyptus globulus*	Portugal	MW794111	MW802844	MW805402	[18]
N. javaensis	CBS 257.31^{T}	*Cocos nucifera*	Indonesia	KM199357	KM199437	KM199543	[15]
N. lusitanica	CBS 147690^{T}	*Eucalyptus globulus*	Portugal	MW794110	MW802843	MW805406	[18]
N. longiappendiculata	CBS 147692^{T}	*Eucalyptus globulus*	Portugal	MW794112	MW802845	MW805404	[18]

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N. macadamiae	BRIP 63737c^{T}	*Macadamia integrifolia*	Australia	KX186604	KX186654	KX186627	[56]
N. macadamiae	BRIP 63742a	*Macadamia integrifolia*	Australia	KX186599	KX186657	KX186629	[56]
N. maddoxii	BRIP 72266a^{T}	*Macadamia integrifolia*	Australia	MZ303782	MZ312675	MZ344167	[56]
N. magna	MFLUCC 12-0652^{T}	*Pteridium* sp.	France	KF582795	KF582793	KF582791	[57]
N. mesopotamica	CBS 336.86^{T}	*Pinus brutia*	Iraq	KM199362	KM199441	KM199555	[15]
N. mesopotamica	CBS 299.74	*Eucalyptus* sp.	Turkey	KM199361	KM199435	KM199541	[15]
N. mianyangensis	HKAS 123211	*Paeonia suffruticosa*	China	OP546681	OP672161	OP723490	[31]
N. musae	MFLUCC 15-0776^{T}	*Musa* sp.	Thailand	KX789683	KX789686	KX789685	[41]
N. natalensis	CBS 138.41^{T}	*Acacia mollissima*	South Africa	KM199377	KM199466	KM199552	[15]
N. nebuloides	BRIP 66617^{T}	*Sporobolus elongatus*	Australia	MK966338	MK977632	MK977633	[58]
N. olumideae	BRIP 72273a^{T}	*Macadamia integrifolia*	Australia	MZ303790	MZ312683	MZ344175	[20]
N. paeoniea	CBS 318.74	*Anacardium occidentale*	Nigeria	MH554031	MH554707	NA	[59]
N. paeonia-suffruticosa	HKAS 123212^{T}	*Paeonia suffruticosa*	China	OP082292	OP235980	OP204794	[31]
N. pernambucana	URM 7148-01^{T}	*Vismia guianensis*	Brazil	KJ792466	NA	KU306739	[60]
N. perukae	FMBCC11.3^{T}	Guava	Pakistan	MH209077	MH460876	MH523647	[52]
N. petila	MFLUCC 17-1737	*Rhizophora mucronata*	Thailand	MK764275	MK764341	MK764319	[41]
N. petila	MFLUCC 17-1738^{T}	*Rhizophora mucronata*	Thailand	MK764276	MK764342	MK764320	[41]
N. phangngaensis	MFLUCC 18-0119^{T}	*Pandanus* sp.	Thailand	MH388354	MH412721	MH388390	[46]
N. piceana	CBS 254.32	*Cocos nucifera*	Indonesia	KM199372	KM199452	KM199529	[15]
N. piceana	CBS 394.48^{T}	*Picea* sp.	UK	KM199368	KM199453	KM199527	[15]
N. photiniae	MFLUCC 22-0129^{T}	*Photinia serratifolia*	China	OP498008	OP752131	OP753368	[16]
N. protearum	CBS 114178^{T}	*Leucospermum cuneiforme*	Zimbabwe	JN712498	KM199463	LT853201	[61]

N. psidii	FMBCC 11.2[T]	*Psidium guajava*	Pakistan	MF783082	MH477870	MH460874	[52]
N. rhapidis	GUCC 21501[T]	*Rhododendron simsii*	China	MW931620	MW980441	MW980442	[34]
N. rhizophorae	MFLUCC 17-1551[T]	*Rhizophora mucronata*	Thailand	MK764277	MK764343	MK764321	[41]
N. rhizophorae	MFLUCC 17 1550	*Rhizophora mucronata*	Thailand	MK764278	MK764344	MK764322	[41]
N. rhododendri	GUCC 21504[T]	*Rhododendron simsii*	China	MW979577	MW980443	MW980444	[34]
N. rhododendricola	KUN-HKAS-123204[T]	*Rhododendron* sp.	China	OK283069	OK274147	OK274148	[62]
N. rosae	CBS 101057[T]	*Rosa* sp.	New Zealand	KM199359	KM199429	KM199523	[15]
N. rosicola	CFCC 51992[T]	*Rosa chinensis*	China	KY885239	KY885245	KY885243	[63]
N. rosicola	CFCC 51993	*Rosa chinensis*	China	KY885240	KY885246	KY885244	[63]
N. samarangensis	CBS 115451	Unidentified Tree	China	KM199365	KM199447	KM199556	[15]
N. saprophytica	MFLUCC 12-0282[T]	*Magnolia* sp.	China	JX398982	JX399017	JX399048	[15]
N. scalabiensis	CAA 1029[T]	*Vaccinium corymbosum*	Portugal	MW969748	MW934611	MW959100	[64]
N. sichuanensis	CFCC 54338[T]	*Castanea mollissima*	China	MW166231	MW218524	MW199750	[44]
N. siciliana	AC46	*Persea americana*	Italy	ON117813	ON209162	ON107273	[65]
N. sonneratae	MFLUCC 17-1744	*Sonneronata alba*	Thailand	MK764279	MK764345	MK764323	[41]
N. sonneratae	MFLUCC 17-1745[T]	*Sonneronata alba*	Thailand	MK764280	MK764346	MK764324	[41]
N. steyaertii	IMI 192475[T]	*Eucalyptus viminalis*	Australia	KF582796	KF582794	KF582792	[15]
N. surinamensis	CBS 450.74[T]	Soil under *Elaeis guineensis*	Suriname	KM199351	KM199465	KM199518	[15]
N. subepidermalis	CFCC 55160	*Rosa chinensis*	China	OK560699	OM117690	OM622425	[48]
N. suphanburiensis	MFLUCC 22-0126[T]	Unknown	Thailand	OP497994	OP752135	OP753372	[16]
N. terricola	HKAS 123213	*Paeonia suffruticosa*	China	OP082294	OP235982	OP204796	[31]
N. thailandica	MFLUCC 17-1730[T]	*Rhizophora mucronata*	Thailand	MK764281	MK764347	MK764325	[41]
N. thailandica	MFLUCC 17-1731	*Rhizophora mucronata*	Thailand	MK764282	MK764348	MK764326	[41]

N. umbrinospora	MFLUCC 12-0285T	Unidentified plant	China	JX398984	JX399019	JX399050	[14]
N. vaccinii	CAA 1059T	*Vaccinium corymbosum*	Portugal	MW969747	MW934610	MW959099	[64]
N. vacciniicola	CAA 1055T	*Vaccinium corymbosum*	Portugal	MW969751	MW934614	MW959103	[64]
N. vheenae	BRIP 72293aT	*Macadamia integrifolia*	Australia	MZ303792	MZ312685	MZ344177	[20]
N. vitis	MFLUCC 15-1265T	*Vitis vinifera* cv. "Summer black"	China	KU140694	KU140685	KU140676	[66]
N. vitis	MFLUCC 15-1270	*Vitis vinifera* cv. "Kyoho"	China	KU140699	KU140690	KU140681	[66]
N. xishuangbannaensis	KUMCC 21-0424T	*Kerivoula hardwickii* (Bat)	China	ON426865	OR025934	OR025973	[67]
N. xishuangbannaensis	KUMCC 21-0425	*Kerivoula hardwickii* (Bat)	China	ON426866	OR025935	OR025974	[67]
N. zakeelii	BRIP 72282aT	*Macadamia integrifolia*	Australia	MZ303789	MZ312682	MZ344174	[20]
N. zimbabwana	CBS 111495T	*Leucospermum cunciforme*	Zimbabwe	NA	KM199456	KM199545	[15]
N. zingiber	GUCC 21001T	*Zingiber officinale*	China	MW930715	MZ683390	MZ683389	[34]
Neopestalotiopsis sp.2	CFCC 54340	*Castanea mollissima*	China	MW166235	MW218528	MW199754	[44]
Neopestalotiopsis sp.2	ZX22B	*Castanea mollissima*	China	MW166236	MW218529	MW199755	[44]
Neopestalotiopsis sp. nov.	GUCC 210003	Unknown	China	MW930717	MZ683392	MZ540914	[34]
Neopestalotiopsis sp.1	CFCC 54337	*Castanea mollissima*	China	MW166233	MW218526	MW199752	[44]
Neopestalotiopsis sp.1	ZX121	*Castanea mollissima*	China	MW166234	MW218527	MW199753	[44]
Pestalotiopsis adusta	ICMP 6088T	Refrigerator door	Fiji	JX399006	JX399037	JX399070	[14]
P. adusta	MFLUCC 10-0146	*Syzygium* sp.	Thailand	JX399007	JX399038	JX399071	[14]
P. aggestorum	LC6301T	*Camellia sinensis*	China	KX895015	KX895348	KX895234	[68]

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P. appendiculata	CGMCC 3.23550T	*Rhododendron* sp.	China	OP082431	OP185516	OP185509	[69]
P. australasiae	CBS 114126T	*Knightia* sp.	New Zealand	KM199297	KM199409	KM199499	[15]
P. australasiae	CBS 11141	*Protea* sp.	New Sout Wales	KM199298	KM199410	KM199501	[15]
P. australis	CBS 114193T	*Grevillea* sp.	New South Wales	KM199332	KM199383	KM199475	[15]
P. biciliata	CBS 124463T	*Platanus×hispanica*	Slovakia	KM199308	KM199399	KM199505	[15]
P. brachiata	LC2988T	*Camellia* sp.	China	KX894933	KX895265	KX895150	[14]
P. brassicae	CBS 170.26T	*Brassica napus*	New Zealand	KM199379	NA	KM199558	[15]
P. camelliae	MFLUCC 12-0277T	*Camellia japonica*	China	JX399010	JX399041	JX399074	[14]
P. camelliae-oleiferae	CSUFTCC08T	*Camellia oleifera*	China	OK493593	OK562368	OK507963	[17]
P. chamaeropis	CBS 186.71T	*Chamaerops humilis*	Italy	KM199326	KM199391	KM199473	[14]
P. chiangmaiensis	MFLUCC 22-0127T	*Phyllostachys edulis*	Thailand	OP497990	OP752137	OP753374	[16]
P. chiaroscuro	BRIP 72970	*Sporobolus natalensis*	Australia	OK422510	OK423752	OK423753	[70]
P. clavata	MFLUCC 12-0268T	*Buxus* sp.	China	JX398990	JX399025	JX399056	[14]
P. colombiensis	CBS 118553T	*Eucalyptus eurograndis*	Colombia	KM199307	KM199421	KM199488	[15]
P. daliensis	CGMCC 3.23548T	*Rhododendron* sp.	China	OP082429	OP185518	OP185511	[69]
P. diploclisiae	CBS 115587T	*Diploclisia glaucescens*	China	KM199320	KM199419	KM199486	[15]
P. diversiseta	MFLUCC 12-0287T	*Rhododendron* sp.	China	NR 120187	JX399040	JX399073	[14]
P. dracaenae	HGUP4037T	*Dracaena fragrans*	China	NA	MT598645	MT598644	[71]
P. dracaenicola	MFLUCC 18-0913T	*Dracaena* sp.	Thailand	MN962731	MN962733	MN962732	[72]
P. dracontomelon	MFUCC 10-0149T	*Dracontomelon dao*	Thailand	KP781877	NA	KP781880	[73]

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P. endophytica	MFLUCC 18-0932	Endophytic on healthy leaves of *Magnolia candoll*	Thailand	NR 172439	NA	MW417119	[74]
P. ericacearum	IFRDCC 2439T	*Rhododendron delavayi*	China	KC537807	KC537821	KC537814	[75]
P. etonensis	BRI P 66615	*Sporobolus jacquemontii*	Australia	MK966339	MK977634	MK977635	[58]
P. formosana	NTUCC 17-009T	dead grass	China	MH809381	MH809385	MH809389	[63]
P. furcata	MFLUCC 12-0054T	*Camellia sinensis*	Thailand	JQ683724	JQ683708	JQ683740	[76]
P. fusoidea	CGMCC 3.23545T	Endophytic in fresh *Rhododendron delavayi* leaves.	China	OP082427	OP185519	OP185512	[69]
P. grevilleae	CBS 114127T	*Grevillea* sp.	Australia	KM199300	KM199407	KM199504	[15]
P. hawaiiensis	CBS 114491T	*Leucospermum* sp.	Hawaii	KM199339	KM199428	KM199514	[15]
P. hispanica	CBS 115391T	*Protea 'Susara'*	Spain	MH553981	MH554640	MH554399	[59]
P. hydei	MFLUCC 20-0135T	*Litsea Petiolata*	Thailand	MW266063	MW251112	MW251113	[55]
P. hydei	GUCC 21-0816	dead twigs	China	OP753660	OP765909	OP753383	[16]
P. hollandica	CBS 265.33T	*Sciadopitys verticillata*	Netherlands	KM199328	KM199388	KM199481	[15]
P. humus	CBS 336.97T	Soil	Papua New Guinea	KM199317	KM199420	KM199484	[15]
P. hunanensis	CSUFTCC15T	*Camellia oleifera*	China	OK493599	OK562374	OK507969	[17]
P. iberica	CAA1006T	*Pinus radiata*	Spain	MW732249	MW759036	MW759039	[77]
P. inflexa	MFLUCC 12-0270T	Unidentified tree	China	JX399008	JX399039	JX399072	[14]
P. intermedia	MFLUCC 12-0259T	Unidentified tree	China	JX398993	JX399028	JX399059	[14]
P. jiangxiensis	LC4399T	*Eurya* sp.	China	KX895009	KX895341	KX895227	[68]
P. jinchanghensis	LC6636T	*Camellia sinensis*	China	KX895028	KX895361	KX895247	[68]

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P. kandelicola	NCYU 19-0355T	*Kandelia candel*	China	MT560722	MT563099	MT563101	[78]
P. kaki	KNU-PT-1804T	*Diospyros kaki*	Korea	LC552953	LC552954	LC553555	[79]
P. kenyana	CBS 442.67T	*Coffea* sp.	Kenya	KM199302	KM199395	KM199502	[15]
P. kenyana	CBS 911.96	raw material from agar-agar	NA	KM199303	KM199396	KM199503	[15]
P. knightiae	CBS 114138T	*Knightia* sp.	New Zealand	KM199310	KM199408	KM199497	[15]
P. knightiae	CBS 111963	*Knightia* sp.	New Zealand	KM199311	KM199406	KM199495	[15]
P. linearis	MFLUCC 12-0271	*Trachelospermum* sp.	China	JX398992	JX399027	JX399058	[14]
P. loeiana	MFLUCC 22-0123	dead leaves	Thailand	OP497988	OP713769	OP737881	[16]
P. lushanensis	LC4344T	*Camellia* sp.	China	KX895005	KX895337	KX895223	[68]
P. macadamiae	BRIP 63738BT	*Macadamia integrifolia*	Australia	KX186588	KX186680	KX186621	[56]
P. malayana	CBS 102220T	*Macaranga triloba*	Malaysia	KM199306	KM199411	KM199482	[15]
P. monochaeta	CBS 144.97T	*Quercus robur*	Netherlands	KM199327	KM199386	KM199479	[15]
P. jesteri	MFLUCC 12-0279T	*Fagraea bodenii*	China	JX399012	JX399043	JX399076	[14]
P. nanjingensis	CSUFTCC16T	*Camellia oleifera*	China	OK493602	OK562377	OK507972	[17]
P. nanningensis	CSUFTCC10T	*Camellia oleifera*	China	OK493596	OK562371	OK507966	[17]
P. neolitseae	NTUCC 17-011T	*Neolitsea villosa*	Taiwan	MH809383	MH809387	MH809391	[63]
P. oryzae	CBS 353.69T	*Oryza sativa*	Denmark	KM199299	KM199398	KM199496	[15]
P. papuana	CBS 331.96T	Coastal soil	Papua New Guinea	KM199321	KM199413	KM199491	[15]
P. photinicola	GZCC 16-0028T	*Photinia serrulata*	China	KY092404	KY047663	KY047662	[80]
P. rhizophorae	MFLUCC 17-0416T	*Rhizophora apiculata*	Thailand	MK764283	MK764349	MK764327	[41]
P. rhodomyrtus	HGUP4230T	*Rhodomyrtus tomentosa*	China	KF412648	KF412642	KF412645	[47]
P. rosarioides	CGMCC 3.23549T	*Rhododendron decorum*	China	OP082430	OP185520	OP185513	[69]

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P. rosea	MFLUCC 12-0258^{T}	*Pinus* sp.	China	JX399005	JX399036	JX399069	[14]
P. scoparia	CBS176.25^{T}	*Chamaecyparis* sp.	NA	KM199330	KM199393	KM199478	[15]
P. shandogensis	JZB340038^{T}	unknow	China	MN626275	MN626729	MN626740	[81]
P. smilacicola	MFLUCC 22-0125^{T}	*Smilax* sp.	Thailand	OP497991	OP762673	OP753376	[16]
P. suae	CGMCC 3.23546^{T}	*Rhododendron delavayi*	China	OP082428	OP185521	OP185514	[69]
P. telopeae	CBS 114161^{T}	*Telopea* sp.	Australia	KM199296	KM199403	KM199500	[15]
P. telopeae	CBS 114137	*Protea* sp.	Australia	KM199301	KM199469	KM199559	[15]
P. thailandica	MFLUCC 17-1616^{T}	*Rhizophora apiculata*	Thailand	MK764285	MK764351	MK764329	[41]
P. trachycarpicola	OP068^{T}	*Trachycarpus fortunei*	China	JQ845947	JQ845945	JQ845946	[82]
P. unicolor	MFLUCC 12-0276^{T}	*Rhododendron* sp.	China	JX398999	JX399030	NA	[14]
P. verruculosa	MFLUCC 12-0274^{T}	*Rhododendron* sp.	China	JX398996	NA	JX399061	[14]
P. yanglingensis	LC4553^{T}	*Camellia sinensis*	China	KX895012	KX895345	KX895231	[83]
Pseudopestalotiopsis ampullacea	LC6618^{T}	*Camellia sinensis*	China	KX895025	KX895358	KX895244	[68]
Ps. annellata	NTUCC 17-030^{T}	*Camellia sinensis*	China, Taiwan	MT322087	MT321889	MT321988	[21]
Ps. avicenniae	MFLUCC 17-0434^{T}	*Avicennia marina*	Thailand	MK764287	MK764353	MK764331	[41]
Ps. camelliae	CGMCC 3.9192	*Camellia sinensis*	China	NA	KU562851	KU562850	[84]
Ps. camelliae-sinensis	NTUCC 18-031	*Camellia sinensis*	China, Taiwan	MT322047	MT321849	MT321948	[21]
Ps. camelliae-sinensis	LC3490^{T}	*Camellia sinensis*	China	KX894985	KX895316	KX895202	[68]
Ps. chinensis	NTUCC 18-066	*Camellia sinensis*	China, Taiwan	MT322083	MT321885	MT321984	[21]
Ps. chinensis	LC3011^{T}	*Camellia sinensis*	China	KX894937	KX895269	KX895154	[68]
Ps. chinensis	NTUCC 18-038	*Camellia sinensis*	China, Taiwan	MT322055	MT321857	MT321956	[21]

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Ps. cocos	CBS 272.29T	*Cocos nucifera*	Java	MH855069	KM199467	KM199553	[15]
Ps. celtidis	GUCC 21599T	*Celtis sinensis*	China	OL423535	OL439010	OL439012	[33]
Ps. curvatispora	MFLUCC 17-1723	*Rhizophora mucronata*	Thailand	MK764290	MK764356	MK764334	[41]
Ps. curvatispora	MFLUCC 17-1722T	*Rhizophora mucronata*	Thailand	MK764289	MK764355	MK764333	[41]
Ps. dawaina	INPA 2912	*Caryota mitis*	Brazil	MN096659	MN151310	MN151308	[85]
Ps. dawaina	MM14-F0015T	unknown	Dawei, Myanmar	LC324750	LC324751	LC324752	[86]
Ps. gilvanii	INPA 2913T	*Paullinia cupana*	Brazil	MN385951	MN385954	MN385957	[29]
Ps. hydeae	NTUCC 17-003.1	*Diospyros* sp.	China, Taiwan	MG816313	MG816323	MG816333	[87]
Ps. ignota	NN 42909T	*Camellia sinensis*	China	KU500020	NA	KU500016	[84]
Ps. indica	CBS 459.78T	*Hibiscus rosa-sinensis*	India	KM199381	KM199470	KM199560	[15]
Ps. indocalami	GUCC 21600T	*Indocalamus tessellatus*	China	OL423536	OL439011	OL439013	[33]
Ps. ixorae	NTUCC 17-001.1T	*Lxora* sp.	NA	MG816316	MG816326	MG816336	[87]
Ps. kawthaungina	MM14F0083T	unknown	Kawthaung, Myanmar	LC324753	LC324754	LC324755	[86]
Ps. kubahensis	UMAS-KUB-P20T	*Macaranga* sp.	Sarawak, Malaysia	MG818971	NA	NA	[88]
Ps. myanmarina	NBRC 112264T	*Averrhoa carambola*	Dawei, Myanmar	LC114025	LC114045	LC114065	[89]
Ps. rhizophorae	MFLUCC 17-1560T	*Rhizophora apiculata*	Thailand	MK764291	MK764357	MK764335	[41]
Ps. simitheae	KUMCC 17-0255	*Magnolia candolli*	China	MW244023	MW602387	MW273930	[74]
Ps. simitheae	MFLUCC12-0121T	*Pandanus odoratissimus*	Thailand	KJ503812	KJ503815	KJ503818	[90]
Ps. solicola	CBS 386.97T	soil in tropical forest	Papua New Guinea	MH554039	MH554715	MH554474	[59]

Ps. Taiwanensis	NTUCC 17-002.1[T]	*Ixora* sp.	China, Taiwan	MG816319	MG816329	MG816339	[87]
Ps. Thailandica	MFLUCC 17-1724[T]	*Rhizophora mucronata*	Thailand	MK764292	MK764358	MK764336	[41]
Ps. Thailandica	MFLUCC 17-1725	*Rhizophora mucronata*	Thailand	MK764293	MK764359	MK764337	[41]
Ps. Theae	MFLUCC 12-0055[T]	*Camellia sinensis*	Thailand	JQ683727	JQ683711	JQ683743	[14]
Ps. vietnamensis	NBRC 112252	*Fragaria* sp.	Hue, Vietnam	LC114034	LC114054	LC114074	[89]

Holotype strains are marked with [T].

The phylogenetic analyses of the combined sequences were carried out with maximum-likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed on the CIPRES web portal (https://www.phylo.org) using RAxML-HPC BlackBox 8.2.10 with GTRGAMMA substitution model and 1,000 bootstrap replicates [91]. BI analysis was implemented using MrBayes v.3.2.7 [92], and MrModeltest 2.2 [92] was used to seek the best-fit nucleotide substitution models for each gene. Two Markov chain Monte Carlo (MCMC) were run for 1,000,000 generations, and trees were sampled every 1000th generation. The first 25% of trees, standing for the burn-in phase of the analyses, were discarded, and the remaining trees were estimated the posterior probabilities. ML tree and BI tree were viewed using Figtree v.1.4.4. and modified by WPS Office.

The new species can be further confirmed through PHI (Pairwise Homoplasy Index) analysis, which can also be used to analyze the species boundaries and related taxa [93]. The PHI test was completed in SplitsTree v.4 [94,95]，and the value over 0.05 reveals no significant recombination in the dataset. The relationship among closely related species were shown by splits graphs through the LogDet transformation and split decomposition.

2.3. Pathogenicity Test

The pathogenicity of fungi was tested by wound inoculation method. Fresh and healthy leaves of *A. oxyphylla* with 30-40 cm long were collected from the field. The surface of the leaves was disinfected by spraying of 75% ethanol and then washed three times with sterile water. Each fungal isolate was inoculated on 6 sites of a leaf with 3 leave replicates. A piece of mycelial (6 mm diameter), which was taken from the margin of a fresh colony cultured to 2/3 of the PDA plate's diameter, was placed on the wound of leaf injured by sterilized needle. A piece of PDA without mycelium was used as control. The inoculated leaves were placed in a box and cultured in the incubator at 26℃, 600 LUX with 16 h/8 h LED light/dark cycle. After 5 days, disease symptoms were recorded and the lesion area was measured using ImageJ and the data were analyzed by SPSS Statistics 24. The re-isolated fungi from disease lesion were identified based on Koch's postulate.

3. Results

3.1. Phylogenetic Analyses

A total of 36 pestalotioid isolates were obtained from the leaves (32 isolates) and stems (4 isolates) samples of *A. oxyphylla* in six cities of Hainan province. Based on ITS sequence and the color of intermediate cells of conidia, 36 strains were classified into three genera, of which 32 strains belong to *Neopestalotiopsis*, two strains belong to *Pestalotiopsis* and two strains belong to *Pseudopestalotiopsis*.

The phylogenetic tree of *Neopestalotiopsis* contained 145 taxa, with 2 outgroup taxa (*P. colombiensis* and *P. diversiseta*). A total of 1,404 characters including gaps (503 for *ITS*, 469 for *tef-1a*, and 432 for *tub2*) were included in the phylogenetic analysis. For the Bayesian inference, the HKY+G

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model with gamma-distributed rate was selected for *ITS*, HKY+G model with gamma-distributed rate was selected for *tef1-a* and the HKY+I+G model with invgamma-distributed rate was selected for *tub2*. Similar tree topologies were acquired by ML and BI methods, and the best scoring ML tree is shown in Figure 1. The phylogenetic tree analyzed 32 *Neopestalotiopsis* taxa isolated from *A. oxyphylla*, revealed 6 novel species.

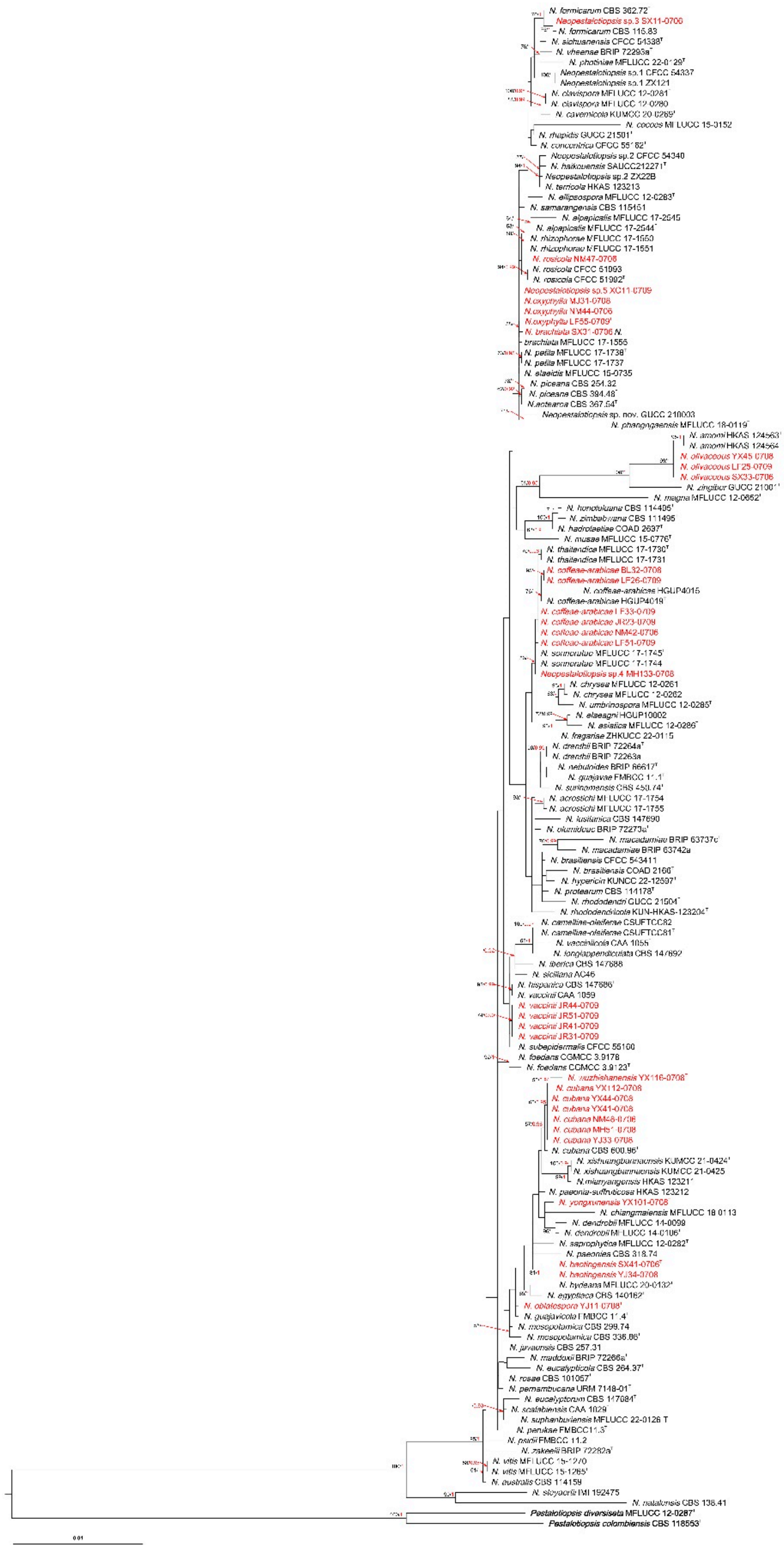

Figure 1. RAxML tree of *Neopestalotiopsis* isolates based on *ITS*, *tef-1α* and *tub2* sequences. The roots of this tree are *Pestalotiopsis diversiseta* MFLUCC 12-0287 and *P. colombiensis* CBS 118553. The strains

isolated in this study are marked in red. Ex-type strains are marked with T. ML bootstrap values ≥50% and BI probabilities (in red) ≥0.90 are displayed at the nodes.

The phylogenetic tree of *Pestalotiopsis* comprised 78 taxa, with the outgroup taxon (*N. cubana* CBS 600.96). A total of 1,475 characters including gaps (505 for *ITS*, 495 for *tef-1a*, and 475 for *tub2*) were included in the phylogenetic analysis. For the Bayesian inference, the GTR + I + G model with invgamma-distributed rate was selected for *ITS*, GTR + G model with gamma-distributed rate was selected for *tef1-a* and the GTR + I + G model with invgamma-distributed rate was selected for *tub2*. Similar tree topologies were obtained by ML and BI methods, and the best scoring ML tree is shown in Figure 2. The phylogenetic tree analyzed two *Pestalotiopsis* strains isolated from *A. oxyphylla*, clustered with the type species of *P. hydei*.

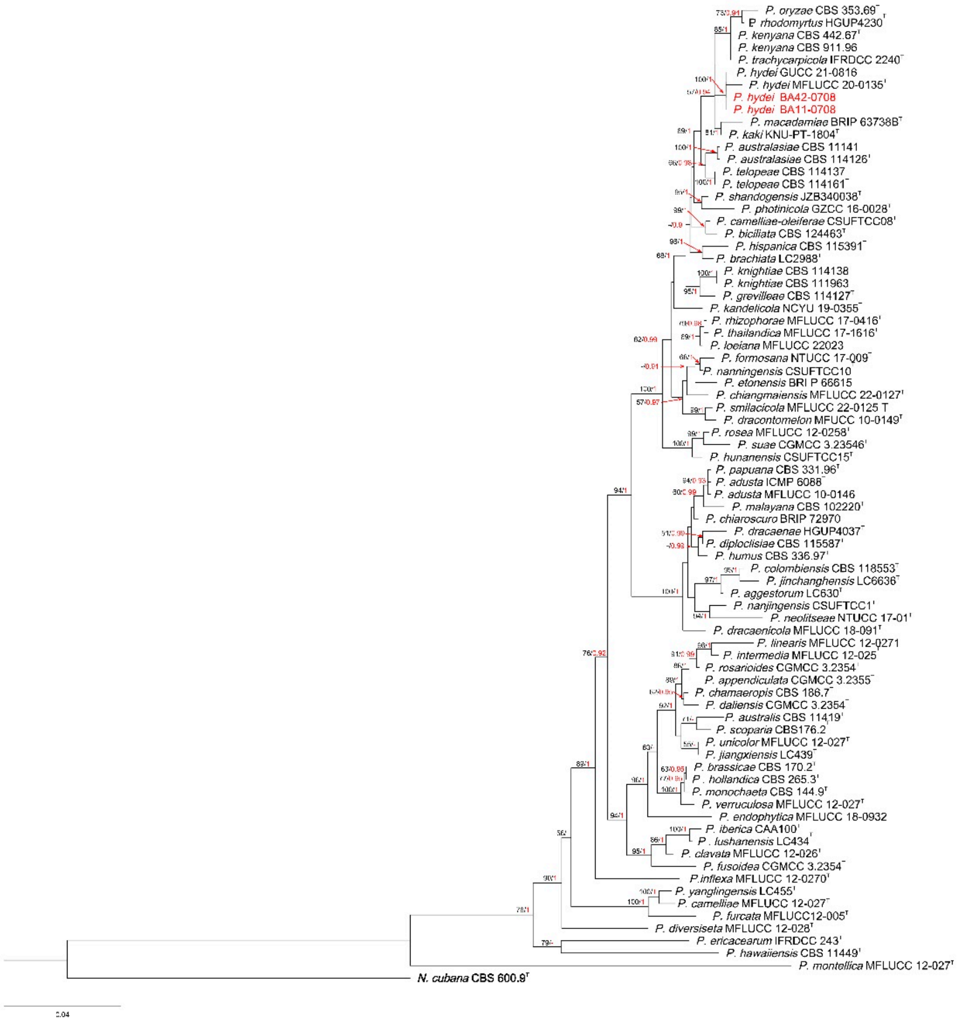

Figure 2. RAxML tree of *Pestalotiopsis* isolates based on *ITS*, *tef-1α* and *tub2* sequences. The root of this tree is *N. cubana* CBS 600.9. The strains isolated in this study are marked in red. Ex-type strains are marked with T. ML bootstrap values ≥50% and BI probabilities (in red) ≥0.90 are displayed at the nodes.

The alignment of *Pseudopestalotiopsis* contained 35 taxa, with *P. trachicarpicola* OP068 as outgroup taxon. A total of 1,392 characters including gaps (521 for *ITS*, 442 for *tef-1a*, and 429 for *tub2*) were included in the phylogenetic analysis. For the Bayesian inference, the HKY+G model with gamma-distributed rate was selected for *ITS*, HKY + G model with gamma-distributed rate was selected for *tef1-a* and the HKY + I model with propinv-distributed rate was selected for *tub2*. Similar tree topologies were obtained by ML and BI methods, and the best scoring ML tree is shown in Figure 3.

The phylogenetic tree analyzed two *Pseudopestalotiopsis* taxa isolated from *A. oxyphylla*, clustered with the type species of *Ps. avicenniae* and *Ps. myanmarina* respectively.

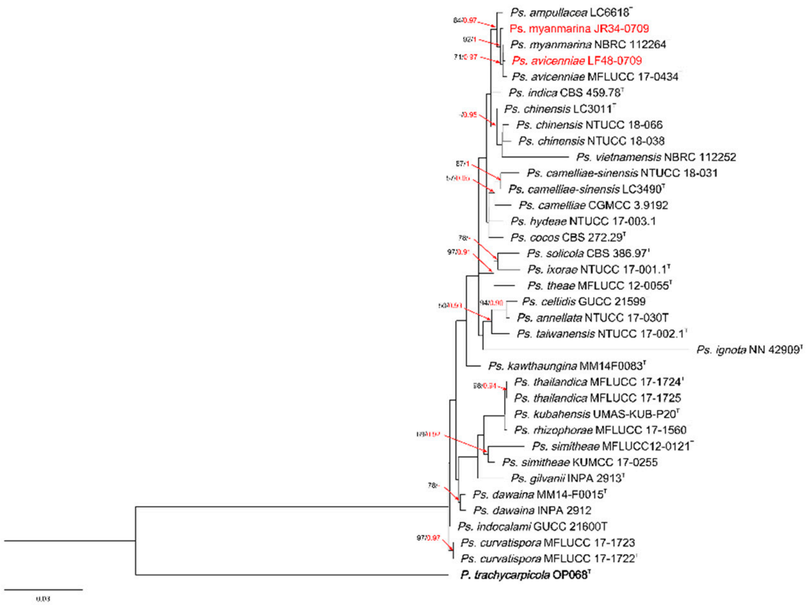

Figure 3. RAxML tree of *Pseudopestalotiopsis* isolates based on *ITS*, *tef-1α* and *tub2* sequences. The root of this tree is *P. trachycaroicola* OP068. The strains isolated in this study are marked in red. Ex-type strains are marked with ^{T}. ML bootstrap values ≥50% and BI probabilities (in red) ≥0.90 are displayed at the nodes.

3.2. PHI Analyses

The result of PHI test indicates no obvious recombination (Φw =0.1064) among *N. baotingensis* SX41-0706, *N. oblatespora* YJ11-0708 and their closely species *N. saprophytica* MFLUCC 12-0282, *N. paeoniea* CBS 318.74, *N. hydeana* MFLUCC 20-0132, *N. egyptiaca* CBS 140162, *N. guajavicola* FMBCC 11.4, *N. mesopotamica* CBS 299.74 (Figure 4a). And there is no significant recombination (Φw =0.0786) between *N. olivaceous* LF25-0709 and its closely species *N. amomi* HKAS 124563, *N. zingiber* GUCC 21001, *N. magna* MFLUCC 12-0652 (Figure 4b). *N. yongxunensis* YX101-0708, *N. wuzhishanensis* YX116-0708 and their closely taxa have no significant recombination according to the PHI test result (Φw =0.1103) (Figure 4c).

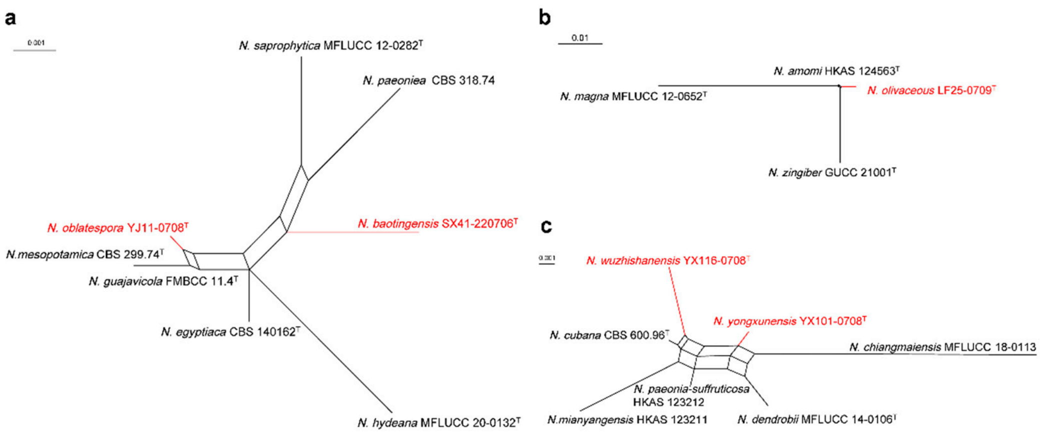

Figure 4. Split graphs showing the result of PHI test of new Neopestalotiopsis species with their most closely related species. The new species in each graph is shown in red font.

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3.3. Taxonomy

Based on multi-locus phylogeny (*ITS*, *tef-1α* and *tub2*) and morphological characteristics analyses, 17 species were identified. Three *Neopestalotiopsis* strains failed to acquire spores and were not been identified to specific species. Six new species were described as below. The conidial dimension of identified isolations in this study and their closely strains are shown in Table 3.

Table 3. The conidial dimension of Pestalotioid species related to this study.

Species	Isolate Number	Conidial Size (μm)	Apical Appendages (μm)		Basal Appendage
			Number	Length	
N. baotingensis	SX41-0706	18-26×5-7.2	2-4	3-30.5	2.5-10
N. Saprophytica	MFLUCC 12-0282	22–30×5–6	2-4	23–35	4-7
N. brachiata	SX31-0706	18.5-25.3×5.5-7.5	1-3	3.7-38.7	2.5-8
N. brachiata	MFLUCC 17-1555	18.5–25×5.5–6	1-3	9.5–33	4–9
N. coffeae-arabicae	BL32-0708	19.2-25.3×5.3-7	2-4	10.9-22.6	1.4-5.4
N. coffeae-arabicae	LF51-0709	17.8-24.2×5-7	2-4	6.6-21.6	2.5-6.8
N. coffeae-arabicae	NM42-0706	17.5-23.8×5.8-7.8	2-4	12.7-31	2.7-9.2
N. coffeae-arabicae	HGUP4019	16-20×5-7	2-4	11-16	3-5
N. cubana	MH51-0708	19.7-30×5-6.8	2-4	15.5-32.2	4-7.5
N. cubana	YX112-0708	21-29×5.6-7.3	2-4	18.7-36.5	3.3-10.3
N. cubana	CBS 600.96	20-25×8-9.5	2-4	21-27	4-7
N. oblatespora	YJ11-0708	18-23.2×5.5-7	2-4	10-26.5	2-9
N. guajavicola	FMBCC 11.4	23.3×6.5	2-3	21.8	4.4
N. olivaceous	LF25-0709	21.5-33.8×5.5-7.7	2-5	9.5-22.5	(0)1.2-4.8
N. amomi	HKAS 124563	18-30×4-7	2-3	7-17	2-5
N. zingiberis	GUCC 21001	21–31×6–9.5	1-3	12-15	0-6
N. oxyphylla	LF55-0709	18.8-23.5×5.3-7.0	2-4	10-25.3	2.5-8
N. aotearoa	CBS 367.54	21–28×6.5–8.5	2-3	5-12	1.5-4
N. elaeidis	MFLUCC 15-0735	10-20×3-7	2-3	10-20	(0)2-6
N. petila	MFLUCC 17-1738	21–26.5×6–7	2-3	22-29	3-8
N. piceana	CBS 394.48	19.5–25×7.5–9	3	21-31	6-23
N. samarangensis	MFLUCC 12-0233	18–21×6.5–7.5	3	12–18	3.5–5.2
N. rosicola	NM47-0708	16.9-24.6×5.5-7.2	2-4	10-25	1.7-7
N. rosicola	CFCC 51992	20.2-25.5×5.5-8	2-4	17-22.8	2-9.5
N. vaccinii	JR31-0709	14.5-20.6×5.5-7.4	2-3	10-22.5	1.3-5.1
N. vaccinii	CAA1059	20.9×6.4	2-3	8.9–25.3	1.7–6.6
N. hispanica	CBS 147686	24.4–25.3×7.2–7.8	3-4	19.5–22.6	5.1–15.5
N. wuzhishanensis	YX116-0708	19.5-26.5×4.5-6.3	1-3	9-20.8	(0)0.8-3.8
N. mianyangensis	UESTCC 22.0006	19–23×5.5–7	3	5.5–11	3–4
N. yongxunensis	YX101-0708	18.2-25.5×5.8-7.5	2-4	10.5-24.7	1.7-7
N. dendrobii	MFLUCC 14-0106	20.5–23×6.5–7.5	2–3	5–6.5	NA
N. paeonia-suffruticosa	CGMCC3.23554	20–23×9–11	3–4	22.5–34	3.5–7.5
P. hydei	BA11-0708	20.3-27.8×4.5-6.6	1-3	3.4-17.2	1-9.5

P. hydei	MFLUCC 20-0135	18-35×3-6	1-3	3-12	2-8
Ps. avicenniae	LF48-0709	20.8-30.7×5.8-7.9	1-3	17.2-33.3	2-7.8
Ps. avicenniae	MFLUCC 17-0434	22.5-26.5×5.5-6	1-3	15.5–28.5	3-4
Ps. myanmarina	JR34-0709	25.4-34.8×5.8-7.4	2-3	18.1-36.9	2.7-7
Ps. myanmarina	NBRC 11226	31-38.5×6.5-9	2-3	22.5-38.5	NA

Isolations in this study are in bold, references see Table 2.

Neopestalotiopsis baotingensis X.F. Cui and Z.G. Hao, sp. nov. (Figure 5)

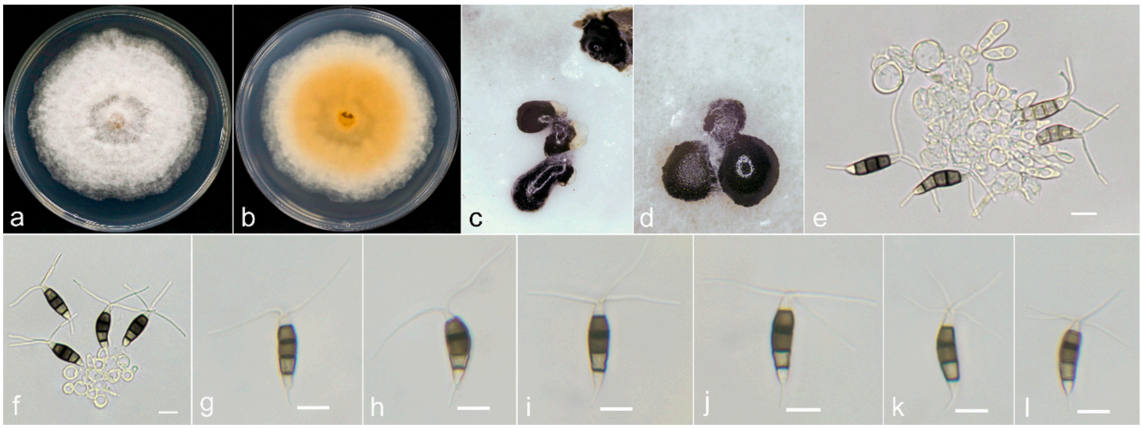

Figure 5. *Neopestalotiopsis baotingensis* (SX41-0706, holotype). (a-b) Colony on PDA (above and reverse), (c-d) Conidiomata on PDA, (e-f) Conidiogenous cells, (g-l) Conidia. Scale bars = 10 μm.

Etymology: named referring to the first collection city of Baoting in Hainan Province.
Holotype: SX41-0706.
Description:

Conidiomata on PDA solitary or aggregated, globose, dark. Conidiophores often degenerated to conidiogenous cells. Conidiogenous cells spherical, hyaline. Conidia, fusiform, straight to slightly curved, 18-26×5-7.2 μm ($\bar{x}$=23.2×6.3 μm), 4 septate; basal cell conical to obtuse, hyaline, thin and smooth-walled, 3.2-6.2 μm long ($\bar{x}$=4.5 μm); three median cells, 12-17.3 μm ($\bar{x}$=14.8 μm), verruculose, versicolor, pale brown to dark brown, septa and periclinal walls darker than the rest of the cell, second cell from base pale brown to brown, paler than the two other cells, 3.2-5.5 μm long ($\bar{x}$=4.4 μm), third cell brown to dark brown, darker than the two other cells, 4-6 μm long ($\bar{x}$=4.9 μm), fourth cell brown to darker brown, 4-6 μm long ($\bar{x}$=5 μm); apical cell 2.5-5 μm long ($\bar{x}$=3.8 μm), cylindric to subcylindric; with 2-4 tubular appendages on the apical cell, often 2-3, arising from the apex of the apical cell, unbranched, 3-30.5 μm long ($\bar{x}$=19.7 μm); single basal appendage, unbranched, tubular, centric, 2.5-10 μm long ($\bar{x}$=6.3 μm). Sexual morph not observed.

Culture characteristics: The colony reached 70 mm diameter on PDA after 4 days growth at room temperature. The colony was off white, dense aerial hyphae on the surface with crenate edge, and its reverse was lemon-yellow.

Material examined: China, Hainan Province, Baoting city, Shiling Town, Shuixian village, from leaf spots of *A. oxyphylla*, 6 July 2022, X.F. Cui and Z.G. Hao (SX41-0706, holotype); ex-type, Hainan Province, Wuzhishan city, Shuiman Town, Yongxun village, from spots on base stem of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (YJ34-0708);

Notes: Two strains of *Neopestalotiopsis baotingensis* were isolated from two cities of Hainan, SX41-0706 and YJ34-0708, clustered with well-supported (ML=81%, BI=1). *N. baotingensis* is closed related to *N. saprophytica* (MFLUCC 12-0282) in the phylogenetic analysis. The conidiophores of *N. baotingensis* often degenerated to conidiogenous cells, while that of *N. saprophytica* unbranched or irregularly branched; *N. baotingensis* is shorter than *N. saprophytica* (*N. baotingensis* 18-26 μm, $\bar{x}$=23.2 μm *vs. N. saprophytica* 22–30 μm, $\bar{x}$=24.9 μm); and *N. baotingensis* has shorter apical appendages (*N.*

baotingensis 3-30.5 μm, $\bar{x}$=19.7 μm *vs. N. saprophytica* 23-35 μm, $\bar{x}$=27.3 μm). Additionally, there are 18 bp difference of *ITS~tef-1α~tub2* between *N. baotingensis* and *N. saprophytica* (4/452 in *ITS*; 16/784 in *tef-1α*; and 1/448 in *tub2*). The PHI test about *N. baotingensis* reveals that there is no obvious recombination between *N. baotingensis* and its closely taxa. Therefore, *N. baotingensis* is classified as a new species in this study.

Neopestalotiopsis oblatespora X.F. Cui and Z.G. Hao, sp. nov. (Figure 6)

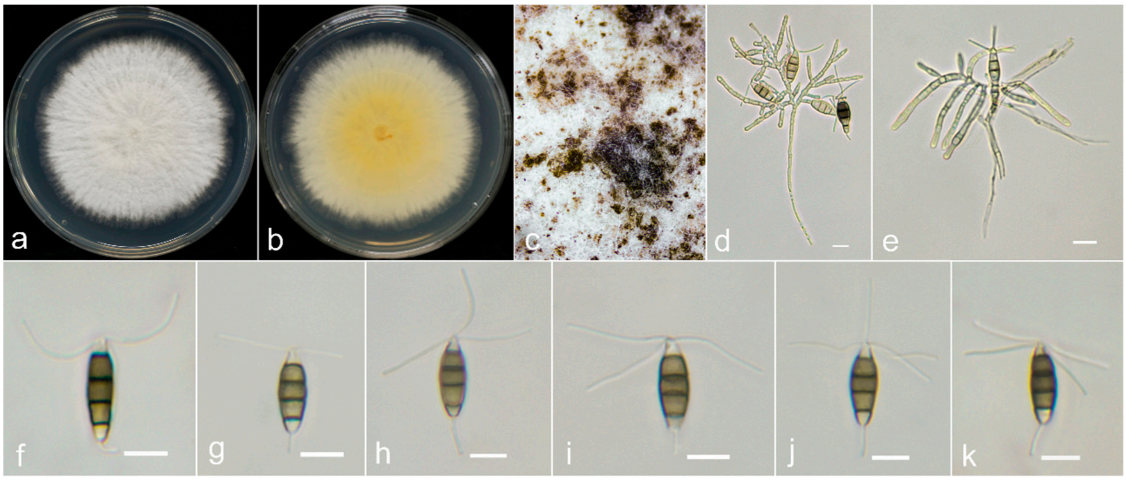

Figure 6. *Neopestalotiopsis oblatespora* (YJ11-0708, holotype). (**a-b**) Colony on PDA (above and reverse), (**c**) Conidia pile on PDA, (**d-e**) Conidiophores, (**f-k**) Conidia. Scale bars = 10 μm.

Etymology: named referring to spore morphology.

Holotype: YJ11-0708.

Description:

Conidiomata not observed on PDA. Conidiophores often monopodial branched, colorless. Conidia, oblate, straight, scarcely curved, 18-23.2×5.5-6.7 μm ($\bar{x}$=20.2×6.2 μm), 4 septate; basal cell conical to subcylindrical, pale brown or hyaline, thin and smooth-walled, 2.5-4.5 μm long ($\bar{x}$=3.2 μm); three median cells, 12-15 μm ($\bar{x}$=13.6 μm), nearly concolor or versicolor, brown to dark brown, septa and periclinal walls darker than the rest of the cell, second cell from base brown to dark brown, 3.7-6 μm long ($\bar{x}$=4.7 μm), third cell dark brown, 3-5 μm long ($\bar{x}$=4.2 μm); fourth dark brown, 3.5-5.3 μm long ($\bar{x}$=4.4 μm); apical cell 2.5-4 μm long ($\bar{x}$=3.2 μm), conical, hyaline, thin and smooth-walled; 2-4 tubular appendages on the apical cell (often 3), arising from the apex of the apical cell, unbranched, 10-26.5 μm long ($\bar{x}$=18 μm); single basal appendage, unbranched, tubular, centric or lateral, 2-9 μm long ($\bar{x}$=5.6 μm). Sexual morph not observed.

Culture characteristics: The colonies reached 70 mm diameter after 4 days on PDA at room temperature, serrated edge, off white, sparse aerial hyphae on the surface appearing radiant, turning grey after sporulation.

Material examined: China, Hainan Province, Wuzhishan city, Shuiman Town, Yongxun village, from the spots on base stem of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (YJ11-0708);

Notes:

Based on multigene analyses, *Neopestalotiopsis oblatespora* is closely related to *Neopestalotiopsis guajavicola* (FMBCC 11.4), only 2 bp difference between them (1/476 in *ITS*, 1/378 in *tef-1α*). However, *N. oblatespora* is distinct from *N. guajavicola* with sporulation structure (branched conidiophores of *N. oblatespora vs.* conidiomata of *N. guajavicola*); smaller spore (*N. oblatespora*: 18-23×5.5-6.7 μm, $\bar{x}$=20.2×6.2 μm *vs. N. guajavicola* 21.7-24.9×6-7 μm, $\bar{x}$=23.3×6.5 μm); shorter apical appendages (*N. oblatespora*: 10-26.5 μm, $\bar{x}$=18 μm vs. *N. guajavicola* : 19.1-24.5 μm, $\bar{x}$=21.8 μm); additionally, having 2-4 apical appendages, while *N. guajavicola* carrying 2-3 appendages. Moreover, *N. oblatespora* has no significant recombination with its closely taxa according to the PHI test. Therefore, *N. oblatespora* is classified as a new species at present study.

Neopestalotiopsis olivaceous X.F. Cui and Z.G. Hao, sp. nov. (Figure 7)

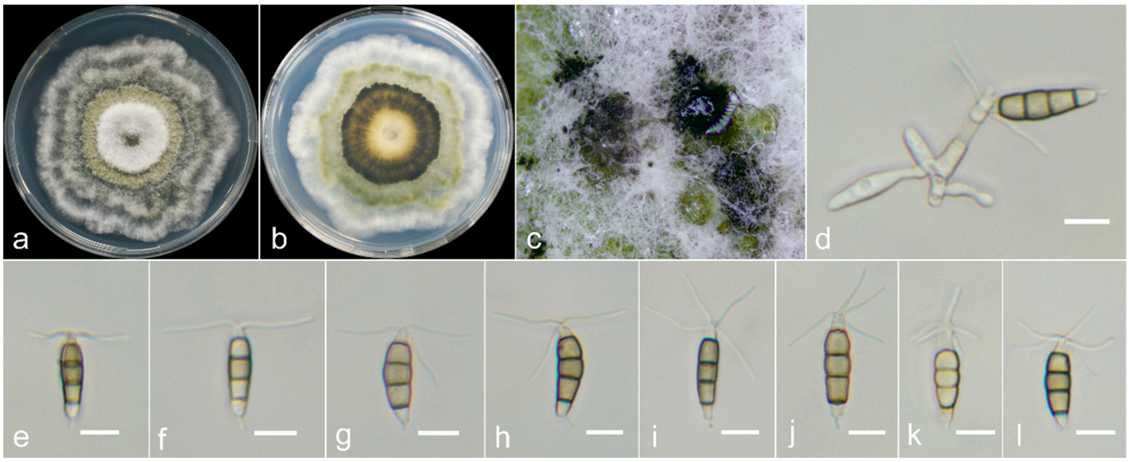

Figure 7. *Neopestalotiopsis olivaceous* (LF25-0709, holotype). (a-b) Colony on PDA (above and reverse), (c) Conidia pile on PDA, (d) Conidiophores, (e-l) Conidia. Scale bars = 10 μm.

Etymology: named referring to the color of colony.

Holotype: LF25-0709.

Description:

Conidiomata not observed on PDA. Conidia sometimes aggregate into globose, dark green. Conidiophore branches, with spore scars. Conidia, fusiform, straight to obviously irregular curved, 21.5-33.8×5.5-7.7 μm ($\bar{x}$=26.5×6.3 μm), 4 septate; basal cell conical, hyaline or pale olive, smooth, thin-walled, 2.7-6.2 μm long ($\bar{x}$=4.5 μm); three median cells 14 to 21.7 μm long ($\bar{x}$=17 μm), pale olivaceous to olivaceous, concolorous, wall rugose, septa darker than the rest of the cell; second cell from base, pale olivaceous to olivaceous, 3.3 to 8.5 μm long ($\bar{x}$=5.9 μm); third cell, pale olivaceous to olivaceous, 4 to 6.5 μm long ($\bar{x}$=5.1 μm); fourth cell, pale olivaceous to olivaceous, 4 to 6.5 μm long ($\bar{x}$=5.4 μm); apical cell 3.5 to 5.5 μm long ($\bar{x}$=4.5 μm), hyaline, conic to acute; with 2 to 5 (often 3-4) tubular appendages on the apical cell, inserted at different loci in a crest at the apex of the apical cell, unbranched, 9.5 to 22.5 μm ($\bar{x}$=14 μm) long; single basal appendage, occasionally no, unbranched, tubular, centric or lateral, 1.2 to 4.8 μm ($\bar{x}$=2.4 μm) long. Sexual morph not observed.

Culture characteristics: The colonies reached 70 mm diameter on PDA after 7 days growth at room temperature. Colonies appeared circular, white above, medium dense, aerial hyphae on the surface flat; and its reverse was olivaceous, gradually deepened over time.

Material examined: China, Hainan Province, Qiongzhong city, Changzheng Town, Luofan village, from leaf spots of *A. oxyphylla*, 9 July 2022, X.F. Cui and Z.G. Hao (LF25-0709, holotype); ex-type, Hainan Province, Baoting city, Shiling Town, Shuixian village, from leaf spots of *A. oxyphylla*, 6 July 2022, X.F. Cui and Z.G. Hao (SX33-0706); Hainan Province, Wuzhishan city, Shuiman Town, Yongxun village, from leaf spots of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (YX45-0708).

Notes: Three strains of *Neopestalotiopsis olivaceous* were isolated from three cities of Hainan, LF25-0709, SX33-0706 and YX45-0708, clustered with well-supported (ML=99%, BI=1). *N. olivaceous* clusters a sister group with *N. amoni* (HKAS 124563) and *N. zingiberis* (GUCC 21001). Molecularly, *N. olivaceous* can be differed from *N. amoni* (HKAS 124563) and *N. zingiberis* (GUCC 21001) according to *ITS~ tef-1α~tub2* (1/471 of *ITS*, 6/347 of *TEF* with *N. amoni*; 3/447 of ITS, 14/722 of TUB and 13/358 of *TEF* with *N. zingiberis*). Morphologically, *N. olivaceous* is distinguished with longer conidia (21.5-33.8 μm of *N. olivaceous vs.* 18-30 μm of *N. amoni* and 21-31 μm of *N. zingiberis*), different numbers of apical appendages (2-5 tubular appendages of *N. olivaceous vs.* 2-3 of *N. amoni* and 1-3 of *N. zingiberis*) and longer apical appendages (*N. olivaceous* 9.5-22.5 μm *vs. N. amoni* 7-17 μm and *N. zingiberis* 12-15 μm). The result of PHI test showed no significant recombination among *N. olivaceous* and its closely taxa. Thus, *N. olivaceous* is classified as a new species at present study.

Neopestalotiopsis oxyphylla X.F. Cui and Z.G. Hao, sp. nov. (Figure 8)

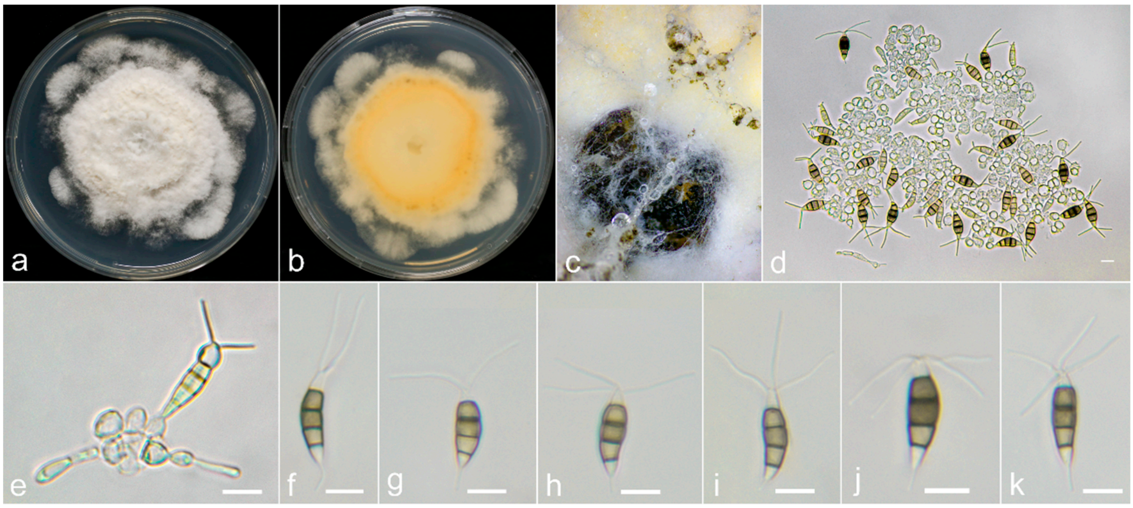

Figure 8. *Neopestalotiopsis oxyphylla* (LF55-0709, holotype). (**a-b**) Colony on PDA (above and reverse), (**c**) Conidiomata on PDA, (**d-e**) Conidiogenous cells, (**f-k**) Conidia. Scale bars = 10 μm.

Etymology: named referring to the host species, *Alpinia oxyphylla*.

Holotype: LF55-0709.

Description:

Conidiomata solitary or aggregated, globose, dark, often immersed in PDA. Conidiophores distinct, often degenerated to conidiogenous cells. Conidiogenous cells spherical, hyaline. Conidia, fusiform, straight to slightly curved, 18.8-23.5×5.3-7.0 μm ($\bar{x}$=21×6.2 μm), 4 septate; basal cell conical to subcylindrical, hyaline, thin and smooth-walled, 2.3-5 μm long ($\bar{x}$=3.9 μm); three median cells, 11.3-15 μm ($\bar{x}$=13 μm), versicolor, brown to dark brown, septa and periclinal walls darker than the rest of the cell, wall with verrucae; second cell from base pale brown, paler than the other two cells, 3.3-5.2 μm long ($\bar{x}$=4.1 μm), third cell dark brown, darker than the other two, 3.5-5.0 μm long ($\bar{x}$=4.1 μm), fourth pale brown to brown, 3.7-5.4 μm long ($\bar{x}$=4.4 μm); apical cell 2.8-5 μm long ($\bar{x}$=3.8 μm), conic to acute, hyaline, thin and smooth-walled; with 2-4 tubular appendages on the apical cell (often 2-3), arising from the apex of the apical cell, occasionally branched, flexuous, 10-25.3 μm long ($\bar{x}$=18.6 μm); single basal appendage, unbranched, tubular, centric, 2.5-8 μm long ($\bar{x}$=5 μm). Sexual morph not observed.

Culture characteristics: The colonies reached 70 mm diameter after 9 days on PDA at room temperature, edge circular, off white, dense, central aerial hyphae on the surface raised, with filiform margin; fruit bodies black; reverse similar in color.

Material examined: China, Hainan Province, Qiongzhong city, Changzheng Town, Luofan village, from leaf spots of *A. oxyphylla*, 9 July 2022, X.F. Cui and Z.G. Hao (LF55-0709, holotype); ex-type, Hainan Province, Wuzhishan city, Maoyang Town, Maohui village, from base stem spots of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (MJ31-0708); ex-type, Hainan Province, Baoting city, Nanmao Shengli Farm, from leaf spots of *A. oxyphylla*, 6 July 2022, X.F. Cui and Z.G. Hao (NM44-0706).

Notes:

Based on multigene analyses, *Neopestalotiopsis oxyphylla* is closely related to *N. brachiata* (MFLUCC 17-1555), *N. elaeidis* (MFLUCC 15-0735), *N. petila* (MFLUCC 17-1738), *N. aotearoa* (CBS 367.54) and *N. piceana* (CBS 394.48), only 0-2 bp difference among them. However, *N. oxyphylla* is distinct from *N. elaeidis* with larger spore (*N. oxyphylla*: 18.8-23.5×5.3-7.0 μm, $\bar{x}$=21×6.2 μm *vs. N. elaeidis* 10-20×3-7 μm, $\bar{x}$=16×5.5 μm) and thinner spore (*N. oxyphylla*: 5.3-7.0 μm *vs. N. aotearoa*: 6.5–8.5 μm and *N. piceana* 7.5–9 μm); *N. oxyphylla* has different numbers of apical appendages (*N. oxyphylla:* 2-4; *N. brachiata:* 1-3; *N. aotearoa*, *N. elaeidis* and *N. petila:* 2-3; *N. piceana:* 3), and shorter apical appendages(*N. oxyphylla:* 10-25.3 μm *vs. N. brachiata:* 9.5-33; *N. petila*: 22-29 μm, *N. piceana*: 21-31 μm), but longer than *N. aotearoa* (5-12 μm). In addition, *N. oxyphylla* has shorter basal appendage (*N.*

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oxyphylla: 2.5-8 μm vs. *N. piceana*: 6-23 μm). Therefore, *N. oxyphylla* is classified as a new species at present study.

Neopestalotiopsis wuzhishanensis X.F. Cui and Z.G. Hao, sp. nov. (Figure 9)

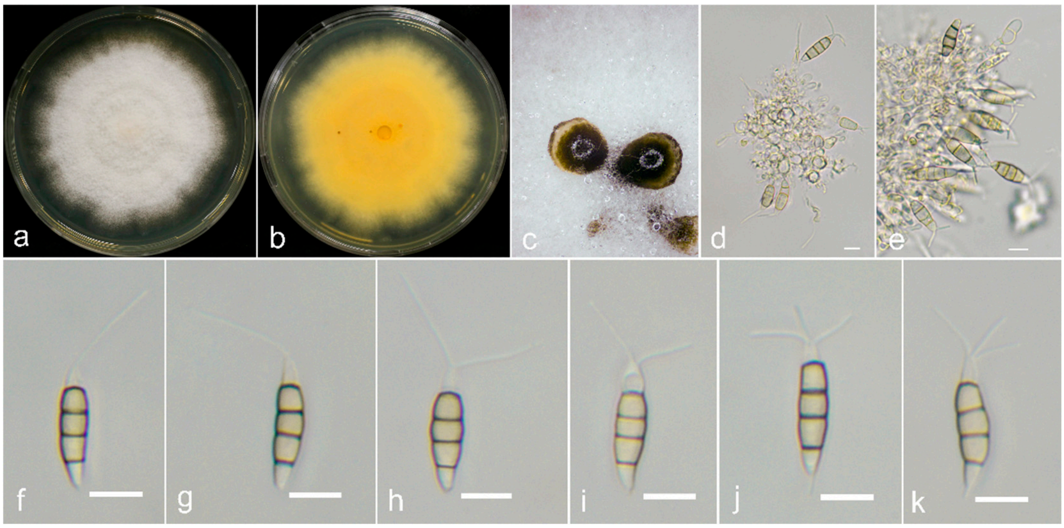

Figure 9. *Neopestalotiopsis wuzhishanensis* (YX116-0708, holotype). (**a-b**) Colony on PDA (above and reverse), (**c**) Conidiomata on PDA, (**d-e**) Conidiogenous cells, (**f-k**) Conidia. Scale bars = 10 μm.

Etymology: named referring to the first collection city of Wuzhishan in Hainan province.

Holotype: YX116-0708.

Description:

Conidiomata on PDA solitary, globose, dark. Conidiophores often degenerated to conidiogenous cells. Conidiogenous cells unclear. Conidia, fusiform, straight, scarcely curved, 19.5-26.5×4.5-6.3 μm ($\bar{x}$=22.4×5.2 μm), 4 septate; basal cell conical to subcylindrical, hyaline, thin and smooth-walled, 2.8-5.5 μm long ($\bar{x}$=4.2 μm); three median cells, 12.8-16 μm ($\bar{x}$=14.4 μm), nearly concolor, pale brown, hyaline, septa and periclinal walls darker than the rest of the cell; second cell from base pale brown , 4-6.2 μm long ($\bar{x}$=5.1 μm), third cell pale brown, 3.5-5.2 μm long ($\bar{x}$=4.4 μm), fourth pale brown, 3.8-6.3 μm long ($\bar{x}$=4.7 μm); apical cell 2.7-5.5 μm long ($\bar{x}$=3.6 μm), conic to acute, hyaline, thin and smooth-walled; with 1-3 tubular appendages on the apical cell (often 1-2), arising from the apex of the apical cell, unbranched, strairht to flexuous, 9-20.8 μm long ($\bar{x}$=15.4 μm); single or no basal appendage, unbranched, tubular, centric, 0.8-3.8 μm long ($\bar{x}$=1.9 μm). Sexual morph not observed.

Culture characteristics: The colonies reached 70 mm diameter after 12 days on PDA at room temperature, edge circular, white, medium dense, aerial hyphae on the surface flat, with filiform margin; fruit bodies black. And its reverse was lemon-yellow.

Material examined: China, Hainan Province, Wuzhishan city, Shuiman Town, Yongxun village, from leaf spot of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (YX116-0708).

Notes:

Neopestalotiopsis wuzhishanensis clusters a sister group to *Neopestalotiopsis cubana* (CBS 600.96). While, *N. wuzhishanensis* is different from *N. cubana* depending on *ITS*, *tef-1α* and *tub2* sequences (3/481 in *ITS*, 4/446 in *tef-1α*, 3/720 in *tub2*). Additionally, there are remarkably discrepancies in morphological characteristics, *N. wuzhishanensis* is thinner (*N. wuzhishanensis*: 4.5-6.3 μm, $\bar{x}$=5.2 μm *vs. N. cubana* 8-9.5 μm, $\bar{x}$=8.8 μm), shorter in apical appendages (*N. wuzhishanensis*: 9-20.8 μm, $\bar{x}$=15.4 μm vs. *N. cubana*: 21-27 μm, $\bar{x}$=24 μm) and base appendage (*N. wuzhishanensis*: 0.8-3.8 μm, $\bar{x}$=1.9 μm vs. *N. cubana*: 4-7 μm); additionally, three media cells of *N. wuzhishanensis* are paler than *N. cubana*; furthermore, *N. cubana* having 1-3 apical appendages, while *N. cubana* carrying 2-4 appendages. The result of PHI test showed that *N. wuzhishanensis* has no significant recombination with their closely taxa. Therefore, *N. wuzhishanensis* is classified as a new species at present study.

Neopestalotiopsis yongxunensis X.F. Cui and Z.G. Hao, sp. nov. (Figure 10)

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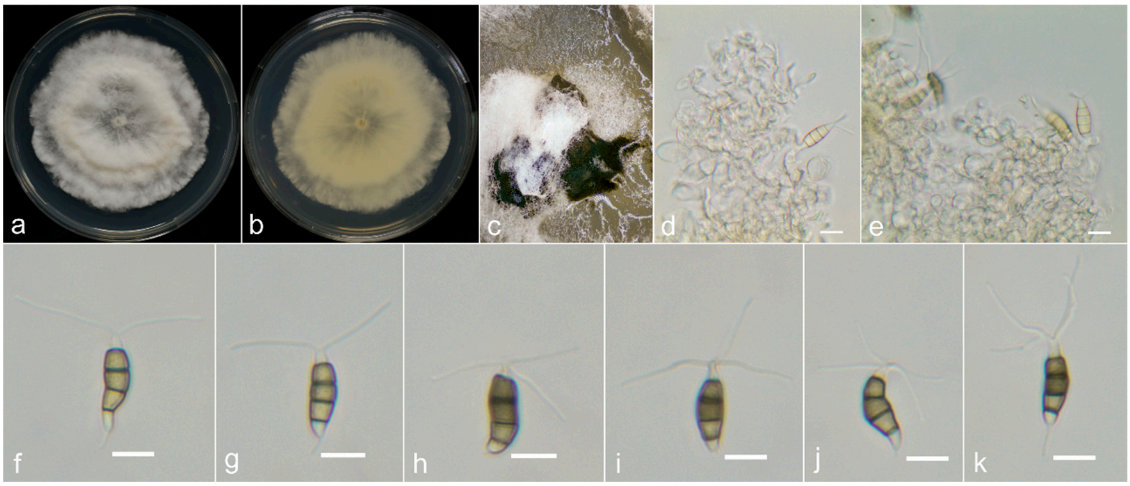

Figure 10. *Neopestalotiopsis yongxunensis* (YX101-0708, holotype). (**a-b**) Colony on PDA (above and reverse), (**c**) Conidiomata on PDA, (**d-e**) Conidiogenous cells, (**f-k**) Conidia. Scale bars = 10 µm.

Etymology: named referring to the first collection village of Yongxun in Hainan province.

Holotype: YX101-0708.

Description:

Conidiomata on PDA solitary or aggregated, globose, dark, embedded or semi-immersed. Conidiophores often degenerated to conidiogenous cells. Conidiogenous cells unclear. Conidia, fusiform, straight to curved, 18.2-25.5×5.8-7.5 µm ($\bar{x}$=21.6×6.6 µm), 4 septate; basal cell conical, hyaline, thin and smooth-walled, 3.0-5.2 µm long ($\bar{x}$=4.1 µm); three median cells, 12-15.2 µm ($\bar{x}$=13.7 µm), versicolor, pale brown to brown, septa and periclinal walls darker than the rest of the cell; second cell from base pale brown, paler than the other two cells, 3.5-5.3 µm long ($\bar{x}$=4.3 µm), third cell brown, darker than the other two, 3.8-5.3 µm long ($\bar{x}$=4.6 µm), fourth cell brown, 4.0-5.2 µm long ($\bar{x}$=4.6 µm); apical cell 2.5-5.0 µm long ($\bar{x}$=3.7 µm), conic to subcylindrical, hyaline, thin and smooth-walled; with 2-4 tubular appendages on the apical cell, arising from the apex of the apical cell, filiform, unbranched, strairht to flexuous, 10.5-24.7 µm long ($\bar{x}$=18.2 µm); single basal appendage, unbranched, tubular, centric, 1.7-7 µm long ($\bar{x}$=4.2 µm). Sexual morph not observed.

Culture characteristics: The colonies reached 70 mm diameter after 4 days on PDA at room temperature, edge circular, white, dense aerial mycelium on the surface; reverse similar in color. Fruit bodies black, mostly under the hyphae, visible on the back.

Material examined: China, Hainan Province, Wuzhishan city, Shuiman Town, Yongxun village, from leaf spot of *A. oxyphylla*, 8 July 2022, X.F. Cui and Z.G. Hao (YX101-0708);

Notes:

Neopestalotiopsis yongxunensis is related to *N. dendrobii* (MFLUCC 14-0106) and *N. paeonia-suffruticosa* (HKAS 123212) in the phylogenetic analysis. While, *N. yongxunensis* can be differed from *N. dendrobii* and *N. paeonia-suffruticosa* depending on *ITS*, *tef1-α* and *tub2* sequences, 7 bp difference (2/284 in *tef1-α*, 5/441 in *tub2*) with *N. dendrobii* and 10 bp difference (9/440 in *tef1-α*, 1/740 in *tub2*) with *N. paeonia-suffruticosa*. In addition, there are remarkably discrepancies in morphological characteristics, *N. yongxunensis* is thinner in conidia (*N. yongxunensis*: 5.8-7.5 µm, $\bar{x}$=6.6 µm *vs. N. paeonia-suffruticosa*: 9-11 µm, $\bar{x}$=9.5 µm), has different numbers of apical appendages (*N. yongxunensis* 2-4 *vs. N. paeonia-suffruticosa* 3-4) and shorter apical appendages (*N. yongxunensis*: 10.5-24.7 µm *vs. N. paeonia-suffruticosa* 22.5-34 µm). While *N. yongxunensis* differs from *N. dendrobii* in having longer apical appendages (*N. yongxunensis*: 10.5-24.7 µm *vs. N. dendrobii* 5-6.5 µm) with different numbers (*N. yongxunensis* 2-4 *vs. N. dendrobii* 2-3). Furthermore, the PHI test indicates that there is no significant recombination between *N. yongxunensis* and its closely species. Therefore, *N. yongxunensis* is classified as a new species at present study.

3.4. Pathogenicity Assay

Sixteen of the 20 tested Pestalotioid isolates were able to cause typical brown lesions after inoculation, while the other 4 isolates did not, including *Neopestalotiopsis* sp.4 MH133-0708, *N. coffeae-arabicae* NM42-0706, *N. oblatespora* YJ11-0708 and *Neopestalotiopsis* sp.3 SX11-0706. The lesion areas measured 5 days after inoculation were 54.02, 13.86, 15.57, 4.65, 11.08, 117.40, 100.63, 82.31, 8.55, 80.25, 32.03, 7.02, 104.86, 84.48, 102.04 and 16.17 mm^2 for isolates of *P. hydei* BA11-0708, *Ps. myanmarina* JR34-0709, *Ps. avicenniae* LF48-0709, *N. coffeae-arabicae* BL32-0708, *N. coffeae-arabicae* LF51-0709, *N. cubana* MH51-0708, *N. cubana* YX112-0708, *N. wuzhishanensis* YX116-0708, *N. yongxunensis* YX101-0708, *N. baotingensis* SX41-0706, *N. vaccinii* JR31-0709, *N. rosicola* NM47-0708, *N. oxyphylla* LF55-0709, *N. brachiata* SX31-0706, *Neopestalotiopsis* sp.5 XC11-0709 and *N. olivaceous* LF25-0709, respectively (Figure 11). The morphology of purified fungi re-isolated from the lesion of inoculation was identical with those of the isolateds used for inoculation, which were also confirmed by PCR and gene sequences. The results of pathogenicity and phylogenetic analysis showed that the strains close to *N. cubana* and *N. brachiata* had stronger pathogenicity (Figure 1 and Figure 11B).

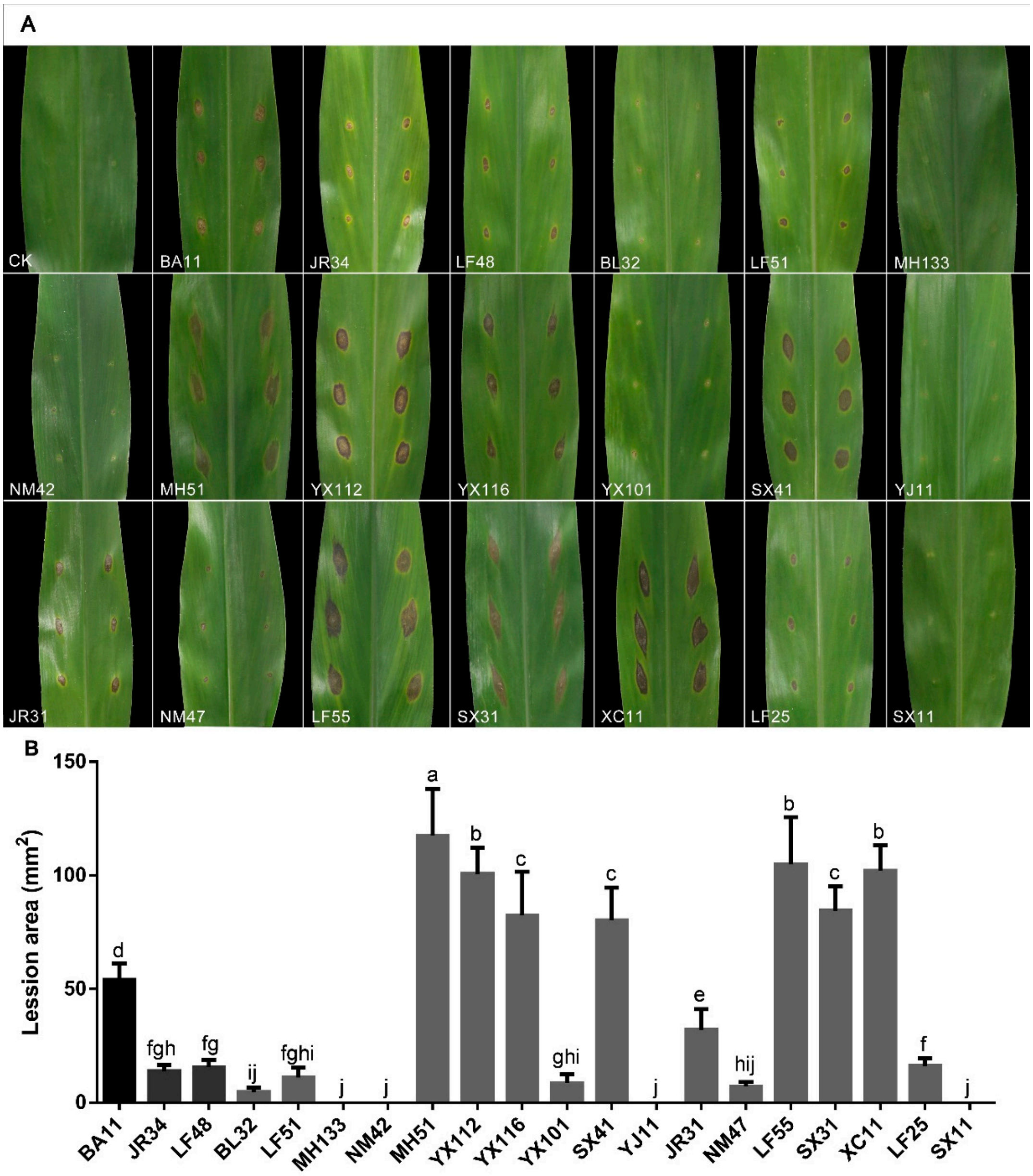

Figure 11. Pathogenicity test results of 20 pestalotioid species on *Alpinia oxyphylla* leaves. (**A**) symptoms on leaves after 5 days. Icons in figures in sequence are CK, *P. hydei* BA11-0708, *Ps.*

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myanmarina JR34-0709, *Ps. avicenniae* LF48-0709, *N. coffeae-arabicae* BL32-0708, *N. coffeae-arabicae* LF51-0709, *Neopestalotiopsis* sp.4 MH133-0708, *N. coffeae-arabicae* NM42-0706, *N. cubana* MH51-0708, *N. cubana* YX112-0708, *N. wuzhishanensis* YX116-0708, *N. yongxunensis* YX101-0708, *N. baotingensis* SX41-0706, *N. oblatespora* YJ11-0708, *N. vaccinii* JR31-0709, *N. rosicola* NM47-0708, *N. oxyphylla* LF55-0709, *N. brachiata*, SX31-0706, *Neopestalotiopsis* sp.5 XC11-0709, *N. olivaceous* LF25-0709, *Neopestalotiopsis* sp.3 SX11-0706. (**B**) Pathogenicity of the isolates was evaluated by measuring area of the necrotic lesions after 5 days. Error bars indicate the standard deviation of the mean. Significant differences ($p < 0.05$) are indicated with different letters according to Duncan's multiple range test. The abscissa designation corresponds sequentially to (A), excluding "CK".

4. Discussion

In this study, 36 *pestalotioid* strains were isolated. According to multi-locus phylogeny (*ITS*, *tef-1α* and *tub2*) and morphological characteristics analyses, one *Pestalotiopsis* sp, two *Pseudopestalotiopsis* spp., and 14 *Neopestalotiopsis* spp. were identified. Six new species (*N. baotingensis, N. oblatespora, N. olivaceous*, *N. oxyphylla*, *N. wuzhishanensis* and *N. yongxunensis*) were described. Among 36 strains, the isolation frequency of *N. coffeae-arabicae* and *N. cubana* was both 16.67%, higher than the others; additionally, *N. coffeae-arabicae*, *N. olivaceous* and *N. oxyphylla* were isolated from 5, 3 and 3 cities separately, with more widely distribution in Hainan than others. This is the first systematic report of *Neopestalotiopsis*, *Pestalotiopsis* and *Pseudopestalotiopsis* fungi related with *A. oxyphylla* in its main planted area.

The development of molecular biology has greatly facilitated the identification of microorganisms, and the phylogeny analyses of combined *ITS*, *tef-1α* and *tub2* can better distinguish *Neopestalotiopsis*, *Pestalotiopsis* and *Pseudopestalotiopsis*. For example, *N. olivaceous* and *N. wuzhishanensis* in this study do not conform to the morphological characteristics of versicolorous median cells depicted in *Neopestalotiopsis*. This phenomenon was also mentioned by Sun et al [16], so the phylogeny analyses can overcome the discrimination of the three genera only by intermediate color cells. While the three gene sequences of *N. oxyphylla*, *N. aotearoa* and *N. brachiata* are closely similar only with 0-2 bp difference of the combined sequence, also between *N. oblatespora* and *N. guajavicola* with 2 bp difference, while they have obvious discrepancy in morphological characteristics. Similar phenomenon was observed between *N. alpapicalis* MFLUCC 17-2544^T and *N. rhizophorae* MFLUCC 17-1551^T [41]. Therefore, more gene fragments need to be introduced in order to further differentiate closly related species of pestalotioid fungi.

RLB disease is an important disease in the cultivation process of *A. oxyphylla* according to previous reports. Its pathogen was reported as *Pestalotia palmarum* in 1986[11], now classified as *Pestalotiopsis palmarum.* While the ring brown spot (RBS) disease with similar symptom to RLB disease was caused by *Pestalosphaeria alpinia,* the sexual morph of Pestalotioid, reported in 1994[25]. Perhaps due to the differences in classification method and limitation on sample size, *P. palmarum* and *P. alpinia* were not isolated in this study, which explained the potential diversity of pestalotioid fungi in this host needed to be further explored. In addition, the symptoms of RLB and RBS disease are similar both caused by pestalotioid fungi with different morph, so it is recommended to merge the two diseases into one for future research and disease management.

Through the pathogenicity tests of 20 pestalotioid strains, most species can cause obvious symptoms on the leaves indicating the diversity pathogen of RLB disease, and the *Neopestalotiopsis* species (the lesion area over 75 mm^2 of 7 species) were more tended to infect *A. oxyphylla* and caused more serious disease than *Pestalotiopsis* (the lesion area about 50 mm^2) and *Pseudopestalotiopsis* (less than 50 mm^2 both of the two). The reports that the disease caused by *Neopestalotiopsis* fungi were more in recent years [16]. In addition, all pathogenicity tests were carried out with a single cultivar of *A. oxyphylla* and constant environmental conditions. As we all know, differences in varieties and changes in environmental conditions can both affect the occurrence of diseases. Therefore, more attempts need to be performed on different varieties under different environmental conditions.

What is worth noting is that most of pestalotioid species have a broad range of hosts, and one species of pestalotioid species can infect several economic plants, while a plant can be harmed by several pestalotioid fungi. For example, *N. cubana* can infect rubber trees [96], *Camellia oleifera* [17] and *Ixora chinensis* [97], and a new leaf fall disease of rubber trees was caused by *N. aotearoa*, *N. cubana* and *N. formicarum* [96]. *A. oxyphylla* is a semi-shade plant mainly planted under rubber forests. In this study, 6 strains of *N. cubana*, one strain of *Neopestalotiopsis* sp.3 SX11-0706 clustered with *N. formicarum* and 5 strains (*N. oxyphylla*, *N. brachiata* and *Neopestalotiopsis* sp.5 XC11-0709) closely related to *N. aotearoa* were isolated. It refers that some pestalotioid species may infect both rubber tree and *A. oxyphylla*. The promotion of cultivation medicinal plant under the forest should also pay attention to the occurrence of cross-infection diseases, in order to prevent them in advance.

A comprehensive understanding of the species and genetic diversity of pathogens is the foundation for sustainable disease management. Since there is no research about the resistance varieties of *A. oxyphylla* to RLB disease. The strains with different characteristics and pathogenicity isolated in this study may provide a material basis for subsequent screening of resistant varieties, including highly active biological and chemical agents friendly to the environment.

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