Mycosphere 8(1): 121–130(2017) www.mycosphere.org ISSN 2077 7019
Article
Doi 10.5943/mycosphere/8/1/11

Two new records in *Pestalotiopsidaceae* associated with Orchidaceae disease in Guangxi Province, China

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Ran SF, Maharachchikumbura SSN, Ren YL, Liu H, Chen KR, Wang YX, Wang Y – 2017 Two new records in *Pestalotiopsidaceae* associated with Orchidaceae disease in Guangxi Province, China. Mycosphere 8(1), 121–130, Doi 10.5943/mycosphere/8/1/11

Abstract

Two coelomycetous taxa belonging to *Pestalotiopsidaceae* were collected from dried stems and disease leaves of *Orchidaceae*, collected from Guangxi Province, China. After morphological observation, these two taxa were found to belong to *Pestalotiopsis* and *Neopestalotiopsis*, respectively. Analysis of combined ITS, β-tubulin and tef1 gene regions indicated that these two fungal strains are *Neopestalotiopsis protearum* and *Pestalotiopsis chamaeropsis*. Based on morphological evidence and phylogenetic analysis, *Neopestalotiopsis protearum* and *Pestalotiopsis chamaeropsis* are reported from China for the first time. The taxa are described and illustrated for ease in future disease identifications.

Key words – morphology – orchid – phylogeny – taxonomy

Introduction

The genus *Pestalotiopsis* Steyaert was established by Steyaert (1949) and is placed in *Pestalotiopsidaceae* Amphisphaeriales or Xylariales (Senanayake et al. 2015). Pestalotioid fungi are commonly distributed in tropical and temperate ecosystems (Maharachchikumbura et al. 2011, 2012, Ariyawansa et al. 2015, Hyde et al. 2016) and are plant pathogens (Maharachchikumbura et al. 2013a, b), endophytes that produce a variety of bioactive secondary metabolites (Hu et al. 2007, Xu et al. 2010, 2014, Debbab et al. 2012, Heinig et al. 2013) and saprobes (Wu et al. 1982, Yanna et al. 2002, Hu et al. 2007, Maharachchikumbura et al. 2014a). Maharachchikumbura et al. (2014b) contributed to a major revision of *Pestalotiopsis*-like taxa and established two novel genera, *Neopestalotiopsis* and *Pseudopestalotiopsis* based on morphology and sequence data. *Neopestalotiopsis* is easily distinguished from *Pseudopestalotiopsis* and *Pestalotiopsis* by its versicoloured median cells (Maharachchikumbura et al. 2014b). Species with dark concolourous median cells with knobbed apical appendages were defined as *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014b). Since the monograph of Maharachchikumbura et al. (2014b),

Submitted 19 January 2017, Accepted 30 January 2017, Published 10 February 2017
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several new pestaloid species have also been introduced (Ariyawansa et al. 2015, Hyde et al. 2016, Jayawardera et al. 2016, Maharachchikumbura et al. 2016a, b, c).

In 2015, we surveyed the orchid fungal diseases in Yachang National Nature Reserve, in Guangxi Province, China. More than 90 plant samples were collected, and after isolation in the laboratory, more than 200 strains were obtained, which included species of *Alternaria*, *Colletotrichum*, *Diaporthe*, *Neopestalotiopsis*, *Pestalotiopsis* and *Phoma* based on NCBI-BLASTs of ITS sequence data. In this paper, we report on one strain of each *Pestalotiopsis* and *Neopestalotiopsis* which are important as they cause disease of leaves of *Vandopsis gigantea* and diseased stems of *Bulbophyllum thouars*. Their taxonomic placement was evaluated by morphological comparison and combined multi-gene analyses (ITS+β–tubulin+tef1).

Materials & Methods

Isolates and morphology

All samples were collected from Guangxi in China. The Guangxi Yachang Orchid Germplasm Gene Park, located in the Leye county, Baise City, Guangxi Zhuang Automomous Region. The Gene Park, located under the subtropical evergreen broad-leaved forest near the wind cave in the Guangxi Yachang Orchid National Nature Reserve, covers an area of over 133.3 acres, at the altitude of 985−1000 m, with the annual average temperature of 16 ºC. Strains were isolated by single spore culture technique to obtain pure colonies (Maharachchikumbura et al. 2013c). They were then transferred to potato-dextrose agar (PDA) and incubated at room temperature (25 ºC). The morphological characters of colonies were recorded according to the reference of Hu et al. (2007). Conidia and conidiophores were observed using a compound microscope (Nikon Eclipse E600 DIC microscope and a Nikon DS-U2 camera or a Nikon 80i compound microscope fitted with a Canon 450D digital camera). The specimens and living culture are also deposited in the herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP).

DNA extraction and sequencing

Fresh fungal mycelia were scraped from the cultures grown on PDA medium at 18 °C until nearly covering the Petri dish. A BIOMIGA Fungus Genomic DNA Extraction Kit (GD2416) was used to rapidly extract their genome DNA. DNA amplification was performed in three regions of the internal transcribed spacers (primers ITS4 and ITS5) (White et al. 1990), partial β-tubulin gene region (primers BT2A and BT2B) (Glass & Donaldson et al. 1995, O'Donnell & Cigelnik et al. 1997), *tef*1 (primers EF1-526 F and EF1-1567R) (Rehner et al. 2001). PCR was performed with the 25 μL reaction system containing 9.5 μL of double distilled water, 12.5 μL of 2 × Taq buffer with $MgCl_2$, 1.0 μL of each primer, and 1.0 μL of DNA template. The thermal cycling protocols follow (Maharachchikumbura et al. 2012). Amplified PCR fragments were checked on 1% agarose electrophoresis gels stained with ethidium bromide (EB). Purified PCR products were sent to SinoGenoMax Co., Beijing, China for sequencing. Sequences generated in this study were deposited at GenBank (Table 1).

Phylogenetic analyses

Two phylogenies were used to show the placement of new isolates in *Pestalotiopsis* and *Neopestalotiopsis* with data from Maharachchikumbura et al. (2014b, 2016a, b, c), Ariyawansa et al. (2015), Hyde et al. (2016) and Jayawardera et al. (2016). Sequences were optimized manually to allow maximum alignment and maximum sequence similarity, as detailed in (Maharachchikumbura et al. 2012). A maximum parsimony analysis (MP) was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees were set up to 5,000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) were calculated for trees generated

under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 1,000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stemwise addition of taxa (Felsenstein 1985). Trees were viewed in Treeview (Page 1996). Sequences were generated from forward and reverse primers and these were subsequently lodged with GenBank (Table 1).

Table 1 Sequences used for phylogenetic analysis. Most sequences are from Maharachchikumbura et al. (2014) and Jayawardera et al. (2016).

Taxon	***Isolate number***	***GenBank accession Number***		
		ITS	**β-tubulin**	***tef1***
Neopestalotiopsis aotearoa	CBS 367.54	KM199369	KM199454	KM199526
N. asiatica	MFLUCC12-0286	JX398983	JX399018	JX399049
N. australis	CBS 114159	KM199348	KM199432	KM199537
N. chrysea	MFLUCC12-0261	JX398985	JX399020	JX399051
N. chrysea	MFLUCC 12-0262	JX398986	JX399021	JX399052
N. clavispora	MFLUCC 12-0280	JX398978	JX399013	JX399044
N. clavispora	MFLUCC 12-0281	JX398979	JX399014	JX399045
N. cubana	CBS 600.96	KM199347	KM199438	KM199521
N. ellipsospora	CBS 115113	KM199343	KM199450	KM199544
N. ellipsospora	MFLUCC 12-0283	JX398980	JX399016	JX399047
N. eucalypticola	CBS 264.37	KM199376	KM199431	KM199551
N. formicarum	CBS 115.83	KM199344	KM199444	KM199519
N. formicarum	CBS 362.72	KM199358	KM199455	KM199517
N. honoluluana	CBS 111535	KM199363	KM199461	KM199546
N. honoluluana	CBS 114495	KM199364	KM199457	KM199548
N. javaensis	CBS 257.31	KM199357	KM199437	KM199543
N. protearum	CBS 114178	JN712498	KM199463	KM199542
N. protearum	**HGUP7003**	**KX196813**	**KX673488**	**KX673490**
N. rosae	CBS 101057	KM199359	KM199429	KM199523
N. rosae	CBS 124745	KM199360	KM199430	KM199524
N. samarangensis	CBS 115451	KM199365	KM199447	KM199556
N. samarangensis	SS010	JQ968609	JQ968610	JQ968611
N. saprophytica	CBS 115452	KM199345	KM199433	KM199538
N. saprophyta	MFLUCC 12-0282	JX398982	JX399017	JX399048
N. surinamensis	CBS 111494	-	KM199462	KM199530
N. surinamensis	CBS 450.74	KM199351	KM199465	KM199518
N. umbrinospora	MFLUCC 12-0285	JX398984	JX399019	JX399050
N. vitis	CBS 110.20	KM199342	KM199442	KM199540
N. zimbabwana	CBS 111495	-	KM199456	KM199545
Neopestalotiopsis sp.	CBS 233.79	KM199373	KM199464	KM199528
Neopestalotiopsis sp.	CBS 322.76	KM199366	KM199446	KM199536
Neopestalotiopsis sp.	CBS 664.94	KM199354	KM199449	KM199525
Neopestalotiopsis sp.	CBS 164.42	KM199367	KM199434	KM199520
Neopestalotiopsis sp.	CBS 360.61	KM199346	KM199440	KM199522
Neopestalotiopsis sp.	CBS 119.75	KM199356	KM199439	KM199531
Neopestalotiopsis sp.	CBS 266.80	KM199352	-	KM199532
Neopestalotiopsis sp.	CBS 266.37	KM199349	KM199459	KM199547
Neopestalotiopsis sp.	CBS 323.76	KM199350	KM199458	KM199550
Neopestalotiopsis sp.	CBS 361.61	KM199355	KM199460	KM199549
Pestalotiopsis adusta	ICMP 6088	JX399006	JX399037	JX399070
P. australis	CBS 111503	KM199331	KM199382	KM199557
P. australis	CBS 114193	KM199332	KM199383	KM199475
P. australis	CBS 114474	KM199334	KM199385	KM199477
P. australis	CBS 119350	KM199333	KM199384	KM199476
P. biciliata	CBS 124463	KM199308	KM199399	KM199505

P. chamaeropis	CBS 113604	KM199323	KM199389	KM199471
P. chamaeropis	CBS 113607	KM199325	KM199390	KM199472
P. chamaeropis	CBS 186.71	KM199326	KM199391	KM199473
P. chamaeropis	CBS 237.38	KM199324	KM199392	KM199474
P. chamaeropis	**HGUP7002**	**KX196814**	**KX673487**	**KX673489**
P. clavata	MFLUCC 12-0268	JX398990	JX399025	JX399056
P. furcata	MFLUCC 12-0054	JQ683724	JQ683708	JQ683740
P. hollandica	CBS 265.33	KM199328	KM199388	KM199481
P. humus	CBS 336.97	KM199317	KM199420	KM199484
P. inflexa	MFLUCC 12-0270	JX399008	JX399039	JX399072
P. intermedia	MFLUCC 12-0259	JX398993	JX399028	JX399059
P. knightiae	CBS 114138	KM199310	KM199408	KM199497
P. linearis	MFLUCC 12-0271	JX398992	JX399027	JX399058
P. monochaeta	CBS 144.97	KM199327	KM199386	KM199479
P. monochaeta	CBS 440.83	KM199329	KM199387	KM199480
P. oryzae	CBS 353.69	KM199299	KM199398	KM199496
P. papuana	CBS 887.96	KM199318	KM199415	KM199492
P. portugalica	CBS 393.48	KM199335	KM199422	KM199510
P. rhododendri	OP086	KC537804	KC537818	KC537811
P. rosea	MFLUCC 12-0258	JX399005	JX399036	JX399069
P. scoparia	CBS 176.25	KM199330	KM199393	KM199478
P. unicolor	MFLUCC 12-0275	JX398998	JX399029	JX399063
P. unicolor	MFLUCC 12-0276	JX398999	JX399030	-

Results

Phylogenetic analyses

The alignment for *Neopestalotiopsis* comprised 1845 characters including gaps (ITS: 1−547, β-tubulin: 548−1308 and *tef*1: 1309−1845). Among these characters, 1348 were constant, 223 variable characters were parsimony-uninformative and 274 are parsimony-informative. Tree Length=887, CI=0.71, RI =0.79, RC=0.60 and HI=0.29. The parsimony analysis resulted in 150 equally parsimonious trees and the first tree represent in Fig. 1. In the *Neopestalotiopsis* tree (Fig. 1), all *Neopestalotiopsis* isolates and *Pseudopestalotiopsis* isolates grouped into two clades, respectively. our strain HGUP 7003 clustered together with *Neopestalotiopsis protearum* (CBS 114178), *Neopestalotiopsis* sp. (CBS 233.79) and *N. surinamensi*s (CBS 450.74).

The alignment for *Pestalotiopsis* consisted of 77 taxa, including *Neopestalotiopsis saprophyta* (MFLUCC 12-0282) as the outgroup taxon. The alignment comprised with 2007 characters including gaps (ITS: 1−557, β-tubulin: 558−1446 and *tef*1: 2447−2007). Among them, 1334 characters are constant, 297 variable characters are parsimony-uninformative and 376 are parsimony-informative. The parsimony analysis resulted in 21 equally parsimonious trees and the first tree (TL=1507, CI=0.62, RI =0.81, RC=0.50 and HI=0.38) was selected to represented as Fig. 2. In Fig. 2, our isolate HGUP 7002 clusters with four *P. chamaeropis* isolate (CBS 113604, CBS 113607, CBS 186.71 and CBS 237.38) with 68% bootstrap support.

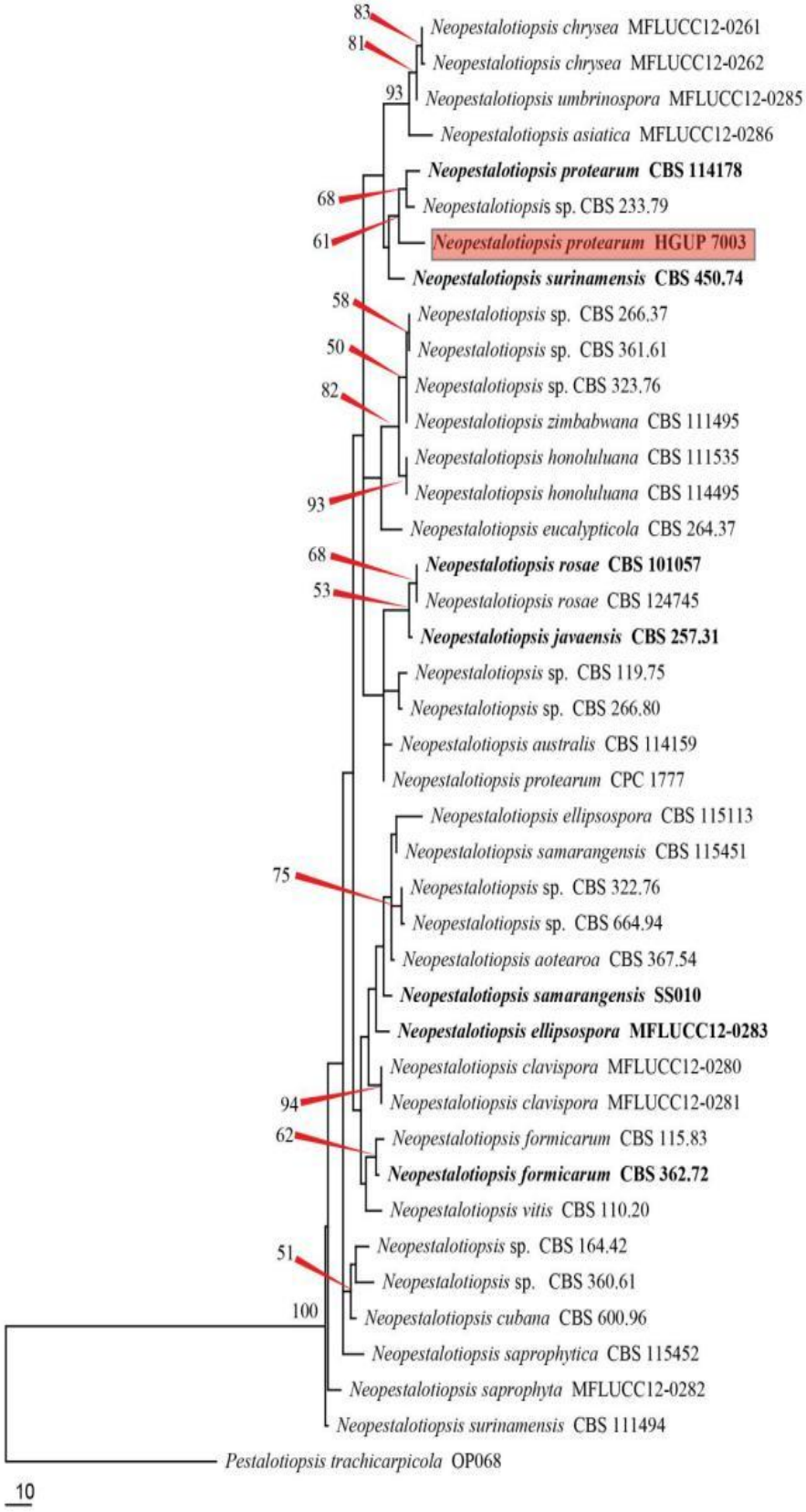

Figure 1 – Phylogenetic tree for *Neopestalotiopsis* based on maximum parsimony (MP) generated from combination of ITS, β-tubulin and *tef*1 sequences. *Pestalotiopsis trachicarpicola* (OP068) was used as the outgroup taxon. New strains are in bold and red and MP bootstrap values higher than 50% are shown at nodes. Type or voucher specimens are in bold.

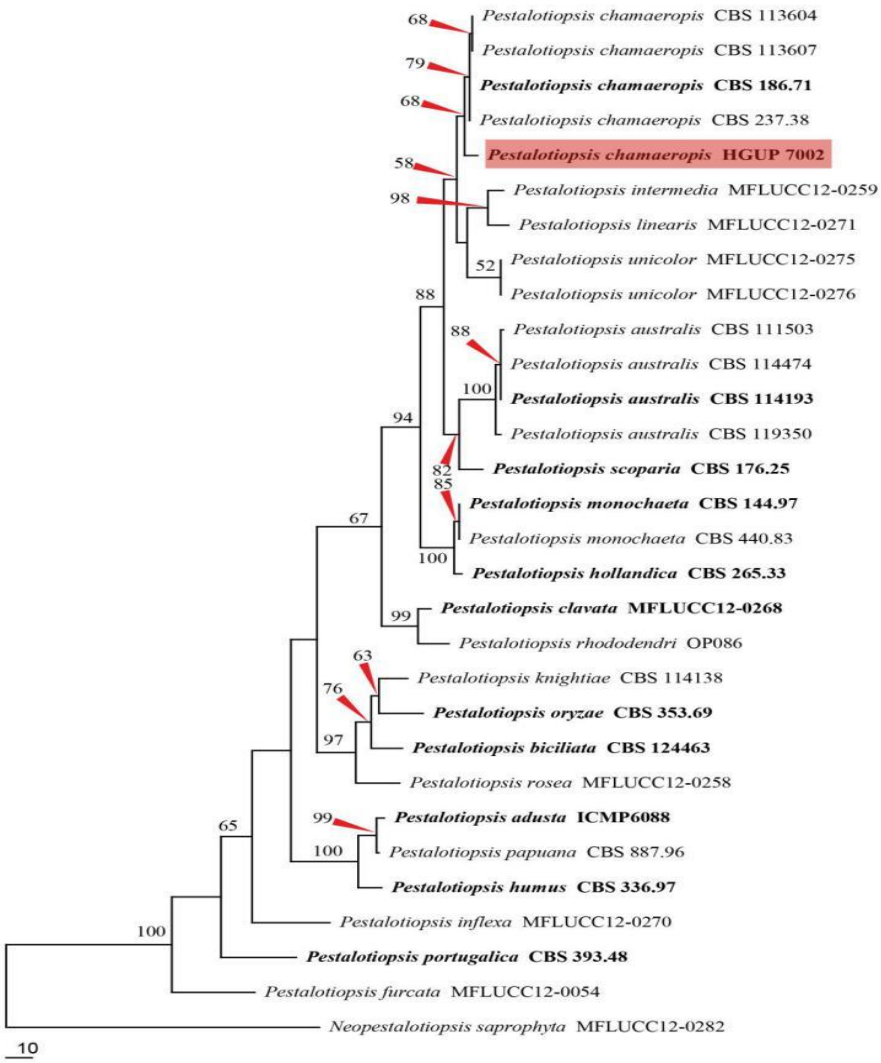

Figure 2 – Phylogenetic tree for *Pestalotiopsis* based on maximum parsimony generated from combination of ITS, β-tubulin and tef1 sequences. *Neopestalotiopsis saprophyta* (MFLUCC 12-0282) was used as the outgroup taxon. New strains are in bold and red and MP bootstrap values higher than 50% are shown at nodes. Type or voucher specimens are in bold

Taxonomy

Neopestalotiopsis protearum (Crous & L. Swart) Maharachch., K.D. Hyde & Crous, Studies in Mycology 79: 147 (2014b) Fig. 3

≡ *Pestalotiopsis protearum* Crous & L. Swart, Persoonia 27: 34 (2011)

Material examined – China, Guangxi, Baise City, Yachang Orchid National Nature Reserve, on dead stem of *Bulbophyllum thouars* (*Orchidaceae*), 9 November 2015, S.F. Ran (HGUP 7003), living culture YC5502.

On dead stem of orchid. Sexual morph: Undetermined. Asexual morph: Coelomycetous. *Conidiomata* pycnidial, globose, solitary, black. *Conidiophores* 3.8−16.3 × 1.7−2.5 μm ($\bar{x}$ = 8.1 × 2.2 μm, n = 40) long, cylindrical, hyaline to subhyaline, smooth-walled. *Conidiogenous cells* simple, integrated, hyaline. *Conidia* fusiform, straight to slightly curved, 4 septate, 18.3−25.5 × 5.7−8.1 μm ($\bar{x}$ = 22.0 × 7.0 μm, n = 40), three median cells versicolourous, doliiform to cylindrical, 14.1−17.3 × 5.7−8.1 μm ($\bar{x}$ = 15.5 × 7.0 μm, n = 40), constricted at the septa; apical cell hyaline, obconic to cylindrical, 2.0−4.5 μm ($\bar{x}$ = 3.1 μm, n = 40) long, 2.6−4.5 μm ($\bar{x}$ = 3.6 μm, n = 40) width, with 1 to 3 (often 3) tubulars apical appendage, arising from the apical crest, 12−22 μm, ($\bar{x}$ = 17.7 μm, n = 40); basal cell obconic to cylindrical, hyaline, 3.2−5.2 μm ($\bar{x}$ = 3.85 μm, n = 40)

length, 3−4.3 µm ($\bar{x}$ = 3.7 µm, n = 40) width, basal appendages filiform, unbranched, 1.6−3.8 µm ($\bar{x}$ = 2.7 µm, n = 40) long.

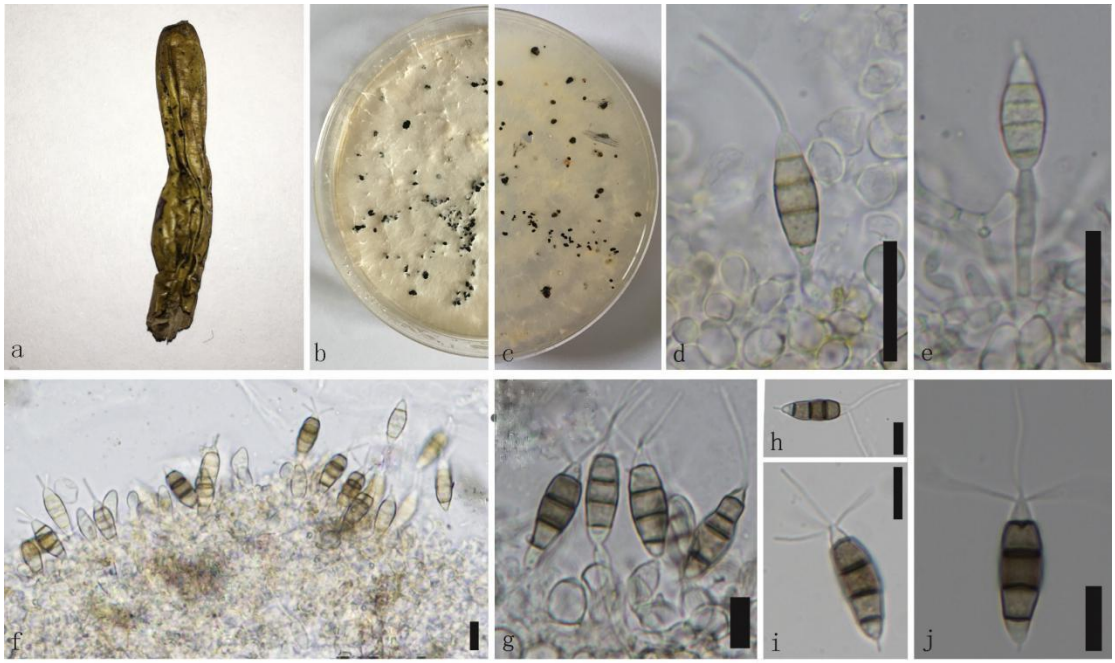

Figure 3 – *Neopestalotiopsis protearum* (HGUP 7003). **a** Host material. **b** Colony on PDA from above. **c.** from below. **d**–**g** Conidioma and conidiophores. **h**–**j** Conidia. Scale bars: **d**–**e** = 20 µm, **f**–**h** =5 µm, **i**—**j** =10 µm.

Pestalotiopsis chamaeropis Maharachch., K.D. Hyde & Crous, Studies in Mycology 79: 158 (2014) Fig. 4

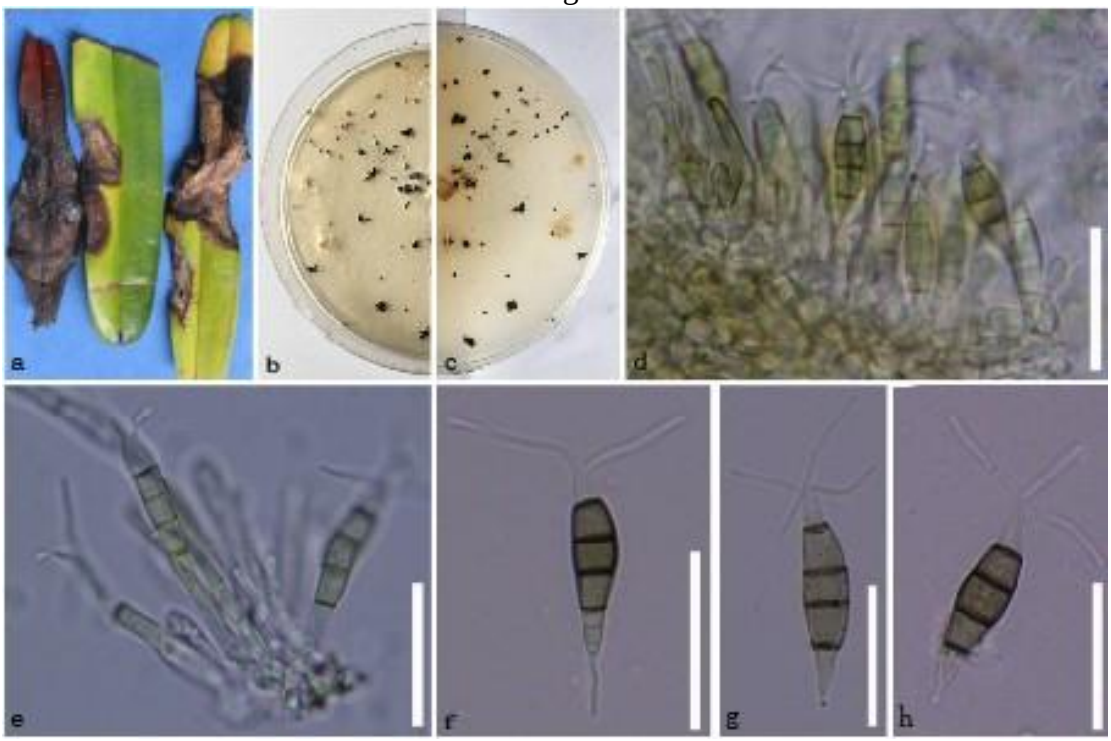

Fig 4 – *Pestalotiopsis chamaeropis* (HGUP 7002). **a** Diseased host material. **b** Colony on PDA from above. **c.** Colony on PDA from below. **d**–**e** Conidioma and conidiophores. **f**–**h** Conidia. Scale bars: **a**–**h** = 10 µm.

Material examined – China, Guangxi, Baise City, Yachang Orchid National Nature Reserve, on dead stem of *Vandopsis gigantea* (*Orchidaceae*), 9 November 2015, S.F. Ran (HGUP 7002), living culture YC5582.

Habit Vandopsis gigantea. Conidiomata pycnidial, globose, solitary, black. *Conidiophores* 4.7−23.2 × 2.7−5.1 μm ($\bar{x}$ = 12.6 × 4.0 μm, n = 40) long, subcylindrical, hyaline, verruculose. *Conidiogenous cells* simple, integrated, hyaline. *Conidia* fusiform, straight to slightly curved, 4 septate, 21.2−26.3 × 5.4−6.7 μm ($\bar{x}$ = 23.9 × 6.1 μm, n = 40); three median cell concolourous, , smooth-walled, doliiform to cylindrical, brown, 13.3−16.8 × 5.4−6.7 μm ($\bar{x}$ = 15.2 × 6.1 μm, n = 40); apical cell hyaline, subcylindrical, 4.3−5.3 μm ($\bar{x}$ = 4.6 μm, n = 40), with 1 to 4 tubulars apical appendage, arising from the apical crest, 11−24 μm ($\bar{x}$ = 16.3 μm, n = 40); basal cell obconic to cylindrical, hyaline, 3−5.9 × 2.8−4.6 μm ($\bar{x}$ = 4.4 × 3.5 μm, n = 40), basal appendage filiform, unbranched, 2.6−7.8 μm ($\bar{x}$ = 5.1 μm, n = 40) long.

Discussion

Neopestalotiopsis protearum collected in this study have conidia (26−28.5 × 7−7.5 μm) which are similar in size to those of *N. surinamensis* (24−28 × 7.5−9 μm) (Crous et al. 2011, Maharachchikumbura et al. 2014b) and overlap with those of the type of *N. protearum*. The molecular analysis (Fig. 1) however, indicates that our collection is more similar to *N. protearum.* The collection of *Pestalotiopsis chamaeropis* is similar to the type of *P. chamaeropis* and this is confirmed by the sequence data. The MP bootstrap support for naming our new collections as *Neopestalotiopsis protearum* (61%) and *Pestalotiopsis chamaeropis* (68%) is relatively low. This is probably because of the short β-tubulin and *tef*1 sequences of the isolates introduced in present study. In view of the phylogenetic analysis and morphological comparison, we conclude that our two isolates represent two Chinese new records in *Pestalotiopsidaceae*, *Neopestalotiopsis protearum* and *Pestalotiopsis*

Acknowledgements

The research is supported by the projects of National Natural Science Foundation of China (No. 31560489), Fundamental Research on Science and Technology, Ministry of Science and Technology of China (2014FY120100), Qiandongnan of Guizhou Province, Blueberry industry science of Technology cooperation (No. [2015]06), Guizhou Province, medicinal plant breeding and cultivation of talent base (No. [2013]15) and postgraduate education innovation program of Guizhou Province (ZYRC [2014]004).

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