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An Addition to pestalotioid fungi in China: *Neopestalotiopsis fragariae* sp. nov. causing leaf spots on *Fragaria* × *ananassa*

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Abstract

Pestalotioid fungi are widely distributed and cause diseases on a myriad of crops, including strawberries. In this study, we introduce a new species, *Neopestalotiopsis fragariae,* that has been isolated from a leaf spot of strawberry in Guangdong Province, China. *Neopestalotiopsis fragariae* is classified as a new taxon based on the morphology and phylogenetic analysis of the combined ITS, *β-TUB* and *TEF-1α* sequence data, together with pairwise homoplasy index results. To our knowledge, this is the first report of *N. fragariae* that causes leaf spots on strawberries in China. This study provides novel data about the plant-pathogenic species associated with strawberries.

Keywords – Ascomycota – Plant pathogens – Sordariomycetes – Strawberry

Introduction

Pestalotioid taxa comprise *Seiridium*, *Neopestalotiopsis*, *Pestalotiopsis* and *Pseudopestalotiopsis* (Amphisphaeriales, Sordariomycetes) (Maharachchikumbura et al. 2011, 2014b, Bonthond et al. 2018, Hyde et al. 2020). These taxa have previously been reported from various hosts as pathogens, saprobes and endophytes in tropical, subtropical and temperate regions worldwide (Nag Raj 1993, Maharachchikumbura et al. 2012, Gualberto et al. 2021, Tsai et al. 2021). There are at least 62 pestalotioid species that are considered pathogenic (Maharachchikumbura et al. 2014b, Shu et al. 2020). Pestalotioid taxa cannot be solely identified based on morphological characters due to overlapping morphologies and conidial sizes (Maharachchikumbura et al. 2011, Maharachchikumbura et al. 2012, Prasannath et al. 2021). The species in the pestalotioid group are delimited based on conidial median cell colour and indistinct conidiophores (Maharachchikumbura et al. 2014b, Jayawardena et al. 2016, Jayawardena et al. 2020). *Neopestalotiopsis* species are characterized by versicoloured conidial median cells and indistinct conidiophores that are generally reduced to conidiogenous cells. Therefore, along with morphological features, molecular data are required (Maharachchikumbura et al. 2014b, Jayawardena et al. 2016, Chethana et al. 2021). Similar to other pestalotioid taxa, *Neopestalotiopsis*

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exhibit endophytic, saprobic and pathogenic lifestyles (Maharachchikumbura et al. 2014b, Maharachchikumbura et al. 2016a, Gerardo-Lugo et al. 2020b, Hyde et al. 2020). *Neopestalotiopsis* was established, with *N. protearum* as the type species (Maharachchikumbura et al. 2014a, Maharachchikumbura et al. 2014b). There are 72 species in Index Fungorum (2022) and 48 species in Species Fungorum (2022).

Many *Neopestalotiopsis* taxa are known as pathogens that cause diseases on a wide range of economically important crops, including strawberries (Jayawardena et al. 2016, Zhao et al. 2016, Norphanphoun et al. 2019b, Rebollar-Alviter et al. 2020, Diogo et al. 2021, Jiang et al. 2021). Strawberries (*Fragaria* × *ananassa*) are widely cultivated for local consumption, foreign trade and industrial market products (jams, syrups, yogurt, and ice cream) (Kafkas 2016, Samtani et al. 2019, Nishizawa 2021). China is known as one of the largest strawberry producers and an exporter in the world. At present, China's total annual production and plantings exceed 3.72 million tons and 113,989 ha, respectively (Zhang et al. 2016). The consumption of strawberries progressively expanded due to the improving standard of living and the people's concerns about their diverse and nutritious diet.

Fungi are the most common microorganisms that are pathogenic on strawberries. They cause damage at different developmental stages of strawberry growth (Rebollar-Alviter et al. 2020, Sun et al. 2021). One of the major fungal diseases associated with strawberry production is fruit rot (Ayoubi & Soleimani 2016). Therefore, control and management strategies are required to prevent diseases or mitigate their effects (Fernández et al. 2021, Traversari et al. 2021). In addition to *Neopestalotiopsis* spp., other taxa causing strawberry fruit rot are *Alternaria* spp., *Botrytis cinerea*, *Colletotrichum* spp., *Fusarium* spp., *Phytophthora cactorum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Ayoubi et al. 2016, Al-Rahbi et al. 2021, Khan et al. 2021, Li et al. 2021, Vanti et al. 2021). Disease symptoms caused by *Neospestalotiopsis* spp. include leaf spots, blights, scabby canker, and post-harvest fruit and stem rot (Yasuda et al. 2003, Feng et al. 2014, Maharachchikumbura et al. 2014b, Maharachchikumbura et al. 2016b).

Herein, we provide a complete morphological and molecular characterization of *N. fragariae*, a new species causing strawberry leaf spots in China. This species has been established based on multi-gene phylogenetic analyses of the combined internal transcribed spacer (ITS), β-tubulin (*β-TUB*), and translation elongation factor (*TEF-1α*). The pathogenicity of *N. fragariae* was confirmed by fulfilling Koch's postulate.

Materials & Methods

Sample Collection

Fresh leaves with leaf spots were collected from a living *Fragaria* × *ananassa* (*Rosaceae*) in the Guangdong Province, China (E 110°3', N 21°2') in 2021. Specimens were collected and brought to the laboratory in paper bags to prevent warm and humid conditions**.**

Fungal Isolation and Purification

The indirect isolation method was used following Senanayake et al. (2020). Firstly, the leaf samples were washed with water. Using a sterile scissor, each necrotic leaf spot was cut into four sections (5 mm^2) consisting of both symptomatic and healthy tissue. All pieces were surface-sterilized in 75% ethyl alcohol for ten seconds, 2.5% sodium hypochlorite for 15 seconds, rinsed thrice in double distilled water and dried on sterilized tissue paper (Dumin et al. 2020, Dong et al. 2021). Each dried piece was transferred to fresh PDA plates and incubated at 25 ± 1 °C. Pure cultures were obtained following hyphal tip isolation.

Micro-morphological observation

Colony characteristics and conidial shape, size and colour were observed from culture, following the formation of pycnidia. Photographs were captured using a Nikon ECLIPSE 80i compound microscope. The scale was measured using NIS-Elements BR 3.2 software. Adobe

Photoshop 2020 was used to prepare the photoplate. Ex-type living culture is deposited in the Culture Collection of Zhongkai University of Agriculture and Engineering (ZHKUCC 22-0113) and the herbarium specimens were deposited in the Herbarium of Zhongkai University of Agriculture and Engineering (ZHKU 22-0061). Faces of Fungi (FoF) number was obtained following Jayasiri et al. (2015).

DNA Extraction, PCR Amplification and Sequencing

DNA extraction, PCR amplification and sequencing were carried out following the methods described by Maharachchikumbura et al. (2014b). Total genomic DNA was extracted from mycelia (Ye et al. 2021). Polymerase chain reaction amplification was done for internal transcribed spacer (ITS), β-tubulin (*β-TUB*) and translation elongation factor 1-α (*TEF-1α*) genes. Each gene was amplified using the primer pairs ITS5/ ITS4 (White et al. 1990), Bt2A/ Bt2B (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997), and EF1-526F/ EF1-1567R (O'Donnell et al. 1998) to amplify ITS, *β-TUB* and *TEF-1α* regions, respectively. The total volume at which PCR was performed was 25 µl. The PCR mixture contained 1.0 µl of genomic DNA template, 0.5 µl each of forward and reverse primer (10 µM), 2.5 µl of 10× Taq buffer with $MgCl_2$, 0.5 µl of Taq DNA polymerase (5 U per 1 µl), 0.5 µl of dNTP (10 mM each) and 19.5 µl of double-distilled water. The thermal cycling program was carried out following Maharachchikumbura et al. (2012) (Table 1). Agarose gel electrophoresis was performed to check the quality of PCR products. Purification of PCR products and sequencing were performed by Guangzhou Tianyi Science and Technology Co. Ltd (Guangzhou, China). The newly generated sequences have been deposited in GenBank (Table 2).

Table 1 Gene regions and the respective thermal cycling program used in the study.

Amplified GENE	Optimized PCR protocols
ITS	95 ºC for 3 min; 95 ºC for 30 s; 52 ºC for 45 s; 72 ºC for 90 s and 72 ºC for 10 min Amplification cycles: 35
β-TUB	95 ºC for 3 min; 94 ºC for 60 s; 55 ºC for 50 s; 72 ºC for 60 s and 72 ºC for 10 min Amplification cycles: 35
TEF-1α	94 ºC for 5 min; 94 ºC for 30 s; 53-56 ºC for 55 s; 72 ºC for 90 s and 72 ºC for 7 min Amplification cycles: 36

Phylogenetic Analyses

Forward and reverse sequences were assembled using SeqMan v. 7.0.0 and consensus sequences were subjected to BLAST search in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare our sequence to existing sequences. The Taxa table was constructed by referring to recent publications (Diogo et al. 2021, Liu et al. 2021, Li et al. 2022, Peng et al. 2022, Zhang et al. 2022). Sequence data were retrieved from the GenBank database. The sequences (ITS, *β-TUB*, *TEF-1α*) were aligned using MAFFT v.7.036 (Katoh & Standley 2013) and trimmed using Trimal to eliminate uneven ends (Capella-Gutiérrez et al. 2009). Internal transcribed spacer (ITS), *β-TUB* and *TEF-1α* were combined using BioEdit (v.7.0). The combined alignment in FASTA format was converted to PHYLIP and NEXUS formats using the Alignment Transformation Environment (ALTER) website (Glez-Peña et al. 2010). Phylogenetic trees [Maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP)] were performed separately for single genes and combined sequence data (Dissanayake et al. 2020).

The ML analysis was performed using RAxML-HPC2 on XSEDE (8.2.12) (Stamatakis 2014) in the CIPRES portal (Miller et al. 2010). The GTR + GAMMA substitution model was run with 1000 non-parametric bootstrapping iterations. The final tree was designated among suboptimal trees from each run.

Maximum parsimony (MP) was conducted using Phylogenetic Analysis Using Parsimony (PAUP) v4.0 (Swofford 2002). Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. Set up of 1,000 maxtrees and zero-length branches were collapsed in MP which resulted in multiple parsimonious trees. The following parameters were calculated for generated trees under various optimization criteria, including retention index (RI), homoplasy index (HI), Tree length (TL) and consistency index (CI). The stability and robustness of the most parsimonious clade were assessed using the analysis of 1000 bootstrap replications with 10 replicates of random step-by-step addition of taxa (Felsenstein 1985).

MrBayes (v.3.1.2) was used to analyse Bayesian inference (BI) (Huelsenbeck & Ronquist 2001). MrModeltest was conducted to determine the appropriate models of nucleotide substitution for each gene region (Nylander 2004). The Akaike information criterion (AIC) (Akaike 1974) gave relevant data for implementing the analysis in PAUP* v. 4.0b10. The HKY+G model was used for ITS and *β-TUB*, and the GTR+I+G model for *TEF-1α*. Bayesian inference (BI) was conducted using Markov Chain Monte Carlo (MCMC) method with MrBayes 3.2.7a (Ronquist et al. 2012) in the CIPRES Science Gateway portal. Markov chain Monte Carlo chains were run simultaneously, starting from 1,000,000 generations of random trees until it reached the average standard deviation of split frequencies of < 0.01. The first 0.25 of trees were excluded as the burning fraction of the analysis. Posterior probabilities (Rannala & Yang 1996) were calculated from a majority rule consensus tree. FigTree v1.4.0 was used to visualize and edit phylograms.

Furthermore, base pair differences in protein-coding genes were calculated for our new taxon and its sister taxa by comparing pairwise distances using MEGA version 11.0.11 (Kumar et al. 2004).

Genealogical Concordance Phylogenetic Species Recognition (GCPSR) analysis

Phylogenetic and morphologic networks of closely related species were analyzed to examine significant recombinant events using the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) model (Taylor et al. 2000), which comprised a pairwise homoplasy index (Φw) test (PHI). The concatenated (ITS, *β-TUB* and *TEF-1α*) dataset with closely related sister type species were used for this analysis. The PHI test was conducted in Split Tree4 (www.splitstree.org) (Huson & Bryant 2006) to determine this recombinant level. The results were shown in a split graph.

Pathogenicity Test

As per Koch's postulates, pathogenicity tests were carried out on healthy potted strawberry seedlings (attached method) using the three isolates obtained in this study (ZHKUCC 22-0113, ZHKUCC 22-0114 and ZHKUCC 22-0115). Five healthy leaves of the same development stages were selected. Leaves were surface-sterilized with 75% ethanol, followed by 5% sodium hypochlorite and sterilized distilled water. Pathogenicity tests were performed on both wounded and non-wounded leaves. Wounds were generated using a sterilized needle. Pathogenicity tests were performed by using both mycelial plug and spore suspension methods. For the mycelial plug method, 5 mm diameter mycelial plugs were placed on both wounded and non-wounded leaves and covered with parafilm. Plain PDA plugs were used as control. For the spore suspension method, a spore suspension of 1×10^6, diluted in sterilized distilled water was used. Twenty-five µl of spore suspension was placed on both wounded and non-wounded leaves and was allowed to air-dry. Sterilised water was used as a control. Experimental plants were kept in the greenhouse (25 °C), with five replicates. Experiments were monitored to observe the formation of disease symptoms. Following disease development, the pathogen was re-isolated to confirm its pathogenicity.

Table 2 Strains and GenBank accession numbers used in this study.

Species name	Isolate number	GenBank Accessions			Reference
		ITS	*β-TUB*	*TEF-1α*	
Neopestalotiopsis acrostichi*	**MFLUCC 17-1754**	MK764272	MK764338	MK764316	Norphanphoun et al. (2019a)
N. acrostichi	MFLUCC 17-1755	MK764273	MK764339	MK764317	Norphanphoun et al. (2019a)
N. alpapicalis*	**MFLUCC 17-2544**	MK357772	MK463545	MK463547	Kumar et al. (2019)
N. alpapicalis	MFLUCC 17-2545	MK357773	MK463546	MK463548	Kumar et al. (2019)
N. aotearoa*	**CBS 367.54**	KM199369	KM199454	KM199526	Maharachchikumbura et al. (2014b)
N. asiatica*	**MFLUCC 12-0286**	JX398983	JX399018	JX399049	Maharachchikumbura et al. (2014b)
N. asiatica	SM11	MW166227	MW218520	MW199746	Maharachchikumbura et al. (2014b)
N. australis*	**CBS 114159**	KM199348	KM199432	KM199537	Maharachchikumbura et al. (2014b)
N. brachiata*	**MFLUCC 17-1555**	MK764274	MK764340	MK764318	Norphanphoun et al. (2019a)
N. brasiliensis*	**COAD 2166**	MG686469	MG692400	MG692402	Bezerra et al. (2018)
N. cavernicola*	**KUMCC 20-0269**	MW545802	MW557596	MW550735	Liu et al. (2021)
N. cavernicola	KUMCC 20-0332	MW581238	MW590328	MW590327	Liu et al. (2021)
N. chiangmaiensis	MFLUCC 19-0048	MW248391	–	MW259070	Tibpromma et al. (2018)
N. chiangmaiensis *	**MFLUCC 18-0113**	–	MH412725	MH388404	Tibpromma et al. (2018)
N. chrysea*	**MFLUCC 12-0261**	JX398985	JX399020	JX399051	Maharachchikumbura et al. (2012)
N. chrysea	MFLUCC 12–0262	JX398986	JX399021	JX399052	Maharachchikumbura et al. (2012)
N. clavispora*	**MFLUCC 12-0281**	JX398979	JX399014	JX399045	Maharachchikumbura et al. (2012)
N. clavispora	MFLUCC 12-0280	JX398978	JX399013	JX399044	Maharachchikumbura et al. (2012)
N. cocoes*	**MFLUCC 15-0152**	KX789687	–	KX789689	Hyde et al. (2016)
N. coffeae-arabicae	HGUP4015	KF412647	KF412641	KF412644	Song et al. (2013)
N. coffeae-arabicae*	**HGUP4019**	KF412649	KF412643	KF412646	Song et al. (2013)
N. cubana*	**CBS 600.96**	KM199347	KM199438	KM199521	Maharachchikumbura et al. (2014b)
N. dendrobii*	**MFLUCC 14-0106**	MK993571	MK975835	MK975829	Ma et al. (2019)
N. dendrobii	MFLUCC 14-0099	MK993570	MK975834	MK975828	Ma et al. (2019)
N. drenthii	BRIP 72263a	MZ303786	MZ312679	MZ344171	Prasannath et al. (2021)
N. drenthii*	**BRIP 72264a**	MZ303787	MZ312680	MZ344172	Prasannath et al. (2021)
N. egyptiaca*	**CBS 140162**	KP943747	KP943746	KP943748	Crous et al. (2015)
N. ellipsospora	MFLUCC12-0284	JX398981	JX399015	JX399046	Maharachchikumbura et al. (2012)
N. ellipsospora*	**MFLUCC12-0283**	JX398980	JX399016	JX399047	Maharachchikumbura et al. (2012)
N. eucalypticola*	**CBS 264.37**	KM199376	KM199431	KM199551	Maharachchikumbura et al. (2014b)
N. eucalyptorum*	**MEAN_1308**	MW794108	MW802841	MW805397	Diogo et al. (2021)
N. eucalyptorum	MEAN_1309	MW794098	MW802831	MW805398	Diogo et al. (2021)
N. foedans*	**CGMCC3.9123**	JX398987	JX399022	JX399053	Maharachchikumbura et al. (2012)

Table 2 Continued.

Species name	Isolate number	GenBank Accessions			Reference
		ITS	*β-TUB*	*TEF-1α*	
N. foedans	CGMCC3.9178	JX398989	JX399024	JX399055	Maharachchikumbura et al. (2012)
N. formicarum*	**CBS 362.72**	KM199358	KM199455	KM199517	Maharachchikumbura et al. (2014a)
N. formicarum	CBS 115.83	KM199344	KM199444	KM199519	Maharachchikumbura et al. (2014a)
N. fragariae*	**ZHKUCC 22-0113**	ON553410	ON569075	ON569076	**This study**
N. fragariae	**ZHKUCC 22-0114**	ON651145	ON685198	ON685196	**This study**
N. fragariae	**ZHKUCC 22-0115**	ON651146	ON685199	ON685197	**This study**
N. hadrolaeliae*	**COAD2637**	MK454709	MK465120	MK465122	Freitas et al. (2019)
N. haikouensis*	**SAUCC212271**	OK087294	OK104870	OK104877	Zhang et al. (2022)
N. haikouensis	SAUCC212272	OK087295	OK104871	OK104878	Zhang et al. (2022)
N. hispanica*	**MEAN_1310**	MW794107	MW802840	MW805399	Diogo et al. (2021)
N. hispanica	MEAN_1311	MW794106	MW802839	MW805400	Diogo et al. (2021)
N. honoluluana*	**CBS 114495**	KM199364	KM199457	KM199548	Maharachchikumbura et al. (2014b)
N. honoluluana	CBS 111535	KM199363	KM199461	KM199546	Maharachchikumbura et al. (2014b)
N. hydeana*	**MFLUCC 20-0132**	MW266069	MW251119	MW251129	(Huanaluek et al. 2021)
N. hydeana	MFLUCC 20–0130	MW266064	MW251114	MW251124	(Huanaluek et al. 2021)
N. iberica*	**MEAN_1313**	MW794111	MW802844	MW805402	Diogo et al. (2021)
N. iberica	MEAN_1314	MW794114	MW802847	MW805403	Diogo et al. (2021)
N. iranensis*	**CBS 137768**	KM074048	KM074057	KM074051	Ayoubi & Soleimani (2016)
N. iranensis	CBS 137767	KM074045	KM074056	KM074053	Ayoubi & Soleimani (2016)
N. javaensis*	**CBS 257.31**	KM199357	KM199437	KM199543	Maharachchikumbura et al. (2014b)
N. javaensis	MFLUCC 12-0594	KX816905	KX816933	KX816874	Maharachchikumbura et al. (2014b)
N. keteleeria*	**MFLUCC 13-0915**	KJ023087	KJ023088	KJ023089	Song et al. (2014)
N. longiappendiculata*	**MEAN_1315**	MW794112	MW802845	MW805404	Diogo et al. (2021)
N. longiappendiculata	MEAN_1316	MW794103	MW802836	MW805405	Diogo et al. (2021)
N. lusitanica*	**MEAN_1317**	MW794110	MW802843	MW805406	Diogo et al. (2021)
N. lusitanica	MEAN_1318	MW794093	MW802826	MW805407	Diogo et al. (2021)
N. macadamiae	BRIP 63736a	KX186601	KX186651	KX186623	Akinsanmi et al. (2017)
N. macadamiae*	**BRIP 63737c**	KX186604	KX186654	KX186627	Akinsanmi et al. (2017)
N. maddoxii*	**BRIP 72266a**	MZ303782	MZ312675	MZ344167	Prasannath et al. (2021)
N. maddoxii	BRIP 72262a	MZ303781	MZ312674	MZ344166	Prasannath et al. (2021)
N. magna*	**MFLUCC 12-0652**	KF582795	KF582793	KF582791	Maharachchikumbura et al. (2014b)
N. mesopotamica*	**CBS 336.86**	KM199362	KM199441	KM199555	Maharachchikumbura et al. (2014b)
N. mesopotamica	CBS 299.74	KM199361	KM199435	KM199541	Maharachchikumbura et al. (2014b)
N. musae*	**MFLUCC 15-0776**	KX789683	KX789686	KX789685	Hyde et al. (2016)
N. natalensis*	**CBS 138.41**	KM199377	KM199466	KM199552	Maharachchikumbura et al. (2014b)
N. nebuloides*	**BRIP 66617**	MK966338	MK977632	MK977633	Crous et al. (2020)
N. olumideae*	**BRIP 72273a**	MZ303790	MZ312683	MZ344175	Prasannath et al. (2021)
N. olumideae	BRIP 72283a	MZ303791	MZ312684	MZ344176	Prasannath et al. (2021)
N. paeoniae	CBS 318.74	MH554031	MH554707	–	Steyaert (1949)
N. pandanicola*	**KUMCC 17-0175**	–	MH412720	–	Tibpromma et al. (2018)

Table 2 Continued**.**

Species name	Isolate number	GenBank Accessions			Reference
		ITS	***β-TUB***	***TEF-1α***	
N. pernambucana*	**URM7148-01**	KJ792466	–	KU306739	Silvério et al**.** **(**2016**)**
N. pernambucana	URM 7148-02	KJ792467	–	KU306740	Silvério et al. (2016)
N. petila*	**MFLUCC 17-1737**	MK764275	MK764341	MK764319	Norphanphoun et al**.** **(**2019a**)**
N. petila	MFLUCC 17–1738	MK764276	MK764342	MK764320	Norphanphoun et al. (2019a)
N. piceana*	**CBS 394.48**	KM199368	KM199453	KM199527	Maharachchikumbura et al**.** **(**2014b**)**
N. piceana	CBS 254.32	KM199372	KM199452	KM199529	Maharachchikumbura et al. (2014b)
N. protearum	CMM 1357	KY549597	KY549632	KY549594	Maharachchikumbura et al. (2014b)
N. protearum*	**CBS 114178**	LT853103	LT853251	KM199542	Maharachchikumbura et al**.** **(**2014b**)**
N. rhapidis*	**GUCC 21501**	MW931620	MW980441	MW980442	Yang et al**.** **(**2021**)**
N. rhizophorae	MFLUCC 17–1550	MK764278	MK764344	MK764322	Norphanphoun et al. (2019a)
N. rhizophorae*	**MFLUCC 17-1551**	MK764277	–	MK764321	Norphanphoun et al**.** **(**2019a**)**
N. rhododendri*	**GUCC 21504**	MW979577	MW980443	MW980444	Yang et al**.** **(**2021**)**
N. rhododendri	GUCC 21505	MW979576	MW980445	MW980446	Yang et al. (2021)
N. rosae*	**CBS 101057**	KM199359	KM199429	KM199523	Maharachchikumbura et al**.** **(**2014b**)**
N. rosae	CBS 124745	KM199360	KM199430	KM199524	Maharachchikumbura et al. (2014b)
N. rosicola*	**CFCC 51992**	KY885239	KY885245	KY885243	Jiang et al**.** **(**2018**)**
N. rosicola	CFCC 51993	KY885240	KY885246	KY885244	Jiang et al. (2018)
N. samarangensis*	**MFLUCC 12-0233**	NR_120125	–	–	Maharachchikumbura et al**.** **(**2013**)**
N. saprophytica*	**MFLUCC 12-0282**	JX398982	JX399017	JX399048	Maharachchikumbura et al**.** **(**2012**)**
N. scalabiensis*	**CAA1029**	MW969748	MW934611	MW959100	Santos et al**.** **(**2022**)**
N. sichuanensis*	**CFCC 54338**	MW166231	MW218524	MW199750	Jiang et al**.** **(**2021**)**
N. sichuanensis	SM15-1C	MW166232	MW218525	MW199751	Jiang et al**.** **(**2021**)**
N. sonneratae*	**MFLUCC 17-1744**	MK764279	MK764345	MK764323	Norphanphoun et al**.** **(**2019a**)**
N. sonneratae	MFLUCC 17–1745	MK764280	MK764346	MK764324	Norphanphoun et al. (2019a)
N. steyaertii*	**IMI 192475**	KF582796	KF582794	KF582792	Maharachchikumbura et al**.** **(**2014a, b**)**
N. surinamensis*	**CBS 450.74**	KM199351	KM199465	KM199518	Maharachchikumbura et al**.** **(**2014b**)**
N. thailandica*	**MFLUCC 17-1730**	MK764281	MK764347	MK764325	Norphanphoun et al**.** **(**2019a**)**
N. thailandica	MFLUCC 17–1731	MK764282	MK764348	MK764326	Norphanphoun et al. (2019a)
N. umbrinospora*	**MFLUCC 12-0285**	JX398984	JX399019	JX399050	Maharachchikumbura et al**.** **(**2012**)**
N. vaccinii	CAA1027	MW969746	MW934609	MW959098	Santos et al. (2022)
N. vaccinii*	**CAA1059**	MW969747	MW934610	MW959099	Santos et al**.** **(**2022**)**
N. vacciniicola*	**CAA1055**	MW969751	MW934614	MW959103	Santos et al**.** **(**2022**)**
N. vacciniicola	CAA1053	MW969749	MW934612	MW959101	Santos et al. (2022)
N. vheenae*	**BRIP 72293a**	MZ303792	MZ312685	MZ344177	Prasannath et al**.** **(**2021**)**
N. vitis*	**MFLUCC 15-1265**	KU140694	KU140685	KU140676	Jayawardena et al**.** **(**2016**)**
N. vitis	MFLUCC 15-1266	KU140695	KU140686	KU140677	Jayawardena et al. (2016)
N. zakeelii	BRIP 72271a	MZ303788	MZ312681	MZ344173	Prasannath et al. (2021)

Table 2 Continued**.**

Species name	**Isolate number**	**GenBank Accessions**			**Reference**
		ITS	***β-TUB***	***TEF-1α***	
N. zakeelii*	**BRIP 72282a**	MZ303789	MZ312682	MZ344174	Prasannath et al**. (**2021**)**
N. zimbabwana*	**CBS 111495**	–	KM199456	KM199545	Maharachchikumbura et al**. (**2014b**)**
N. zimbabwana	MEAN 1329	MW794095	MW802828	MW805418	Maharachchikumbura et al. (2014b)
Pestalotiopsis diploclisia*	**CBS 115587**	KM199320	KM199419	KM199486	Maharachchikumbura et al**. (**2014b**)**
P. diploclisia	CBS 115585	KM199315	KM199417	KM199483	Maharachchikumbura et al. (2014b)
P. knightiae*	**CBS 114138**	KM199310	KM199408	KM199497	Maharachchikumbura et al**. (**2014b**)**
P. knightiae	CBS 111963	KM199311	KM199406	KM199495	Maharachchikumbura et al. (2014b)

CAA: Personal Culture Collection Artur Alves; BRIP: Queensland Plant Pathology Herbarium, Brisbane; CBS: Culture Collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Research Institute of Forest Ecology, Environment and Protection, Beijing, China ; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; CMM – Culture Collection of Phytopathogenic fungi “Prof. Maria Menezes”; COAD: Coleção Octávio Almeida Drummond, Universidade Federal de Viçosa, Brazil; GUCC: Department of Plant Pathology, Agriculture College, Guizhou University, China; HGUP: Herbarium of Department of Plant Pathology, Agricultural College, Guizhou University, China; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; KUMCC: Kunming Institute of Botany Culture Collection, Yunnan, China; MEAN: Instituto Nacional de Investigação Agrária e Veterinária I. P. Fungal Culture Collection, Oeiras, Portugal; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; SAUCC: Shandong Agricultural University Culture Collection, China; URM: Culture Collection of the Universidade Federal de Pernambuco, Brazil.

Isolates obtained in this study are in bold. Ex-type strains are in bold and denoted with *.

Results

Phylogenetic analyses

The combined ITS, *β-TUB* and *TEF-1α* dataset comprises 113 strains of *Neopestalotiopsis*, including our new taxon and excluding outgroups. The topology of the phylogenetic tree generated by RAxML was similar to those of BI and MP analyses. The best-scoring RAxML tree was constructed with a final optimization likelihood value of -9973.473077. The matrix had 747 distinct alignment patterns, with 27.83% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.234275, C = 0.273315, G = 0.216839, T = 0.275571; substitution rates AC = 0.946147, AG = 2.926305, AT = 1.167644, CG = 0.701018, CT = 3.846275, GT = 1.000000; gamma distribution shape parameter alpha = 0.327869. Out of the total 2155 characters, 1535 are constant, 171 are variable and the remaining 449 parsimony informative characters resulted in 1000 equally parsimonious trees, with 1000 replicates (CI = 0.660, RI = 0.828, RC = 0.547 and HI = 0.340).

The Maximum likelihood (ML) and MP bootstrap support and BI posterior probability values are given at each respective node of the phylogram (Fig. 1). From our phylogram, we infer that *N. fragariae* is a new taxon, isolated from *Fragaria* × *ananassa* (*Rosaceae*)*.* Our isolate is sister to *N. asiatica*, *N. chrysea*, *N. samarangensis, N. umbrinospora* and forms a monophyletic clade with strong support [88% ML, 83% MP, 0.95 posterior probability values (PP)].

The pairwise homoplasy index (PHI) test on *N. fragariae* revealed that there is no significant recombination ($\Phi w < 1.0$), among the *N. fragariae* and sister taxa (*N. asiatica, N. chrysea, N. macadamiae, N. samarangensis, and N. umbrinospora*) (Fig. 2).

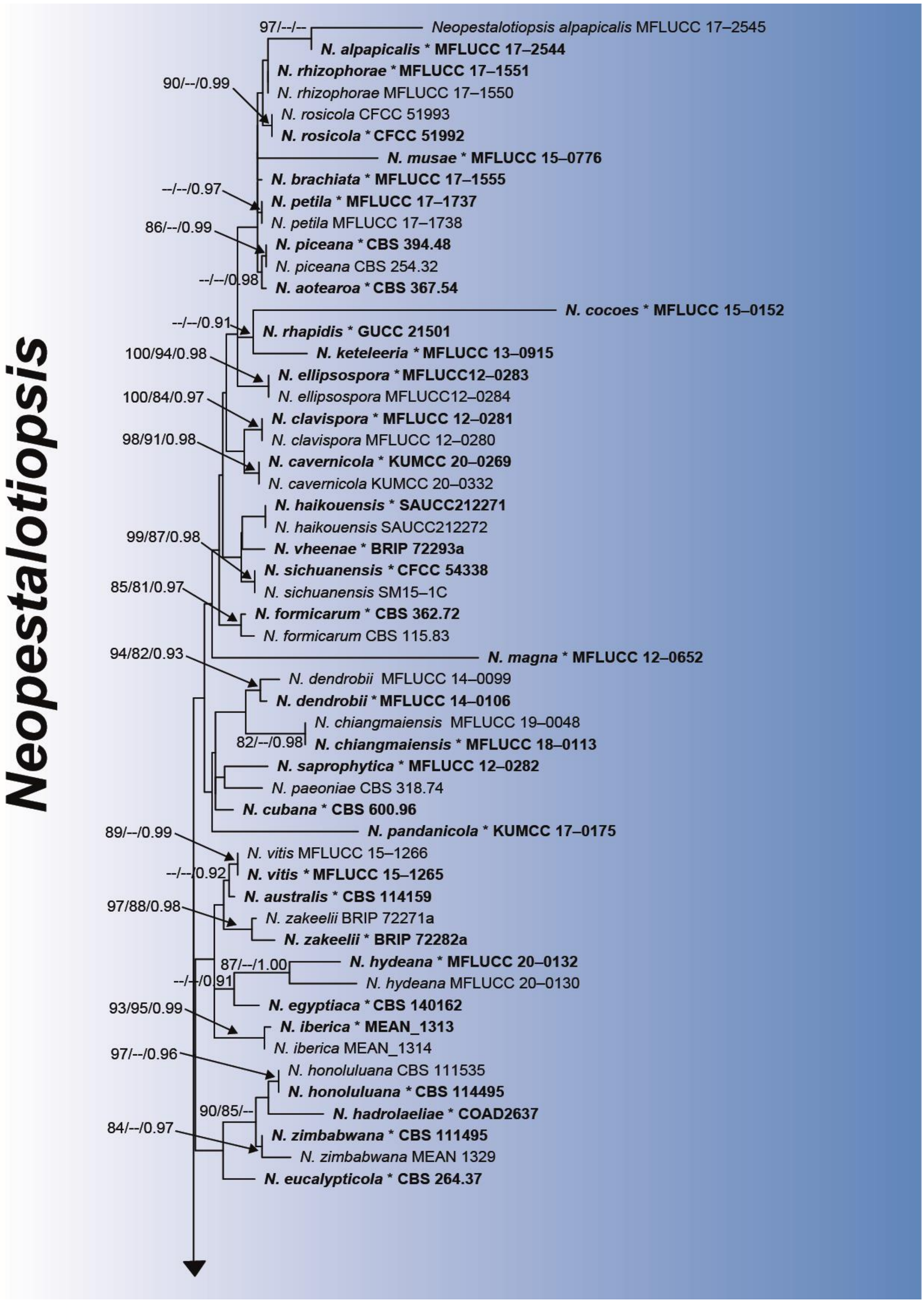

Fig. 1 – Best scoring RAxML tree of the combined alignment of ITS, *β-TUB* and *TEF-1α*. The tree was rooted with *Pestalotiopsis diploclisia* and *P. knightiae* (CBS115587, CBS115585, CBS 114138 and CBS 111963). Bootstrap support values equal to or greater than 80% for ML and MP and PP values equal or greater than 0.90 are shown as ML/MP/PP at the respective node. The isolates obtained in this study are in blue. Ex-type strains are in bold and denoted with*. The scale bar represents the expected number of nucleotide substitutions per site.

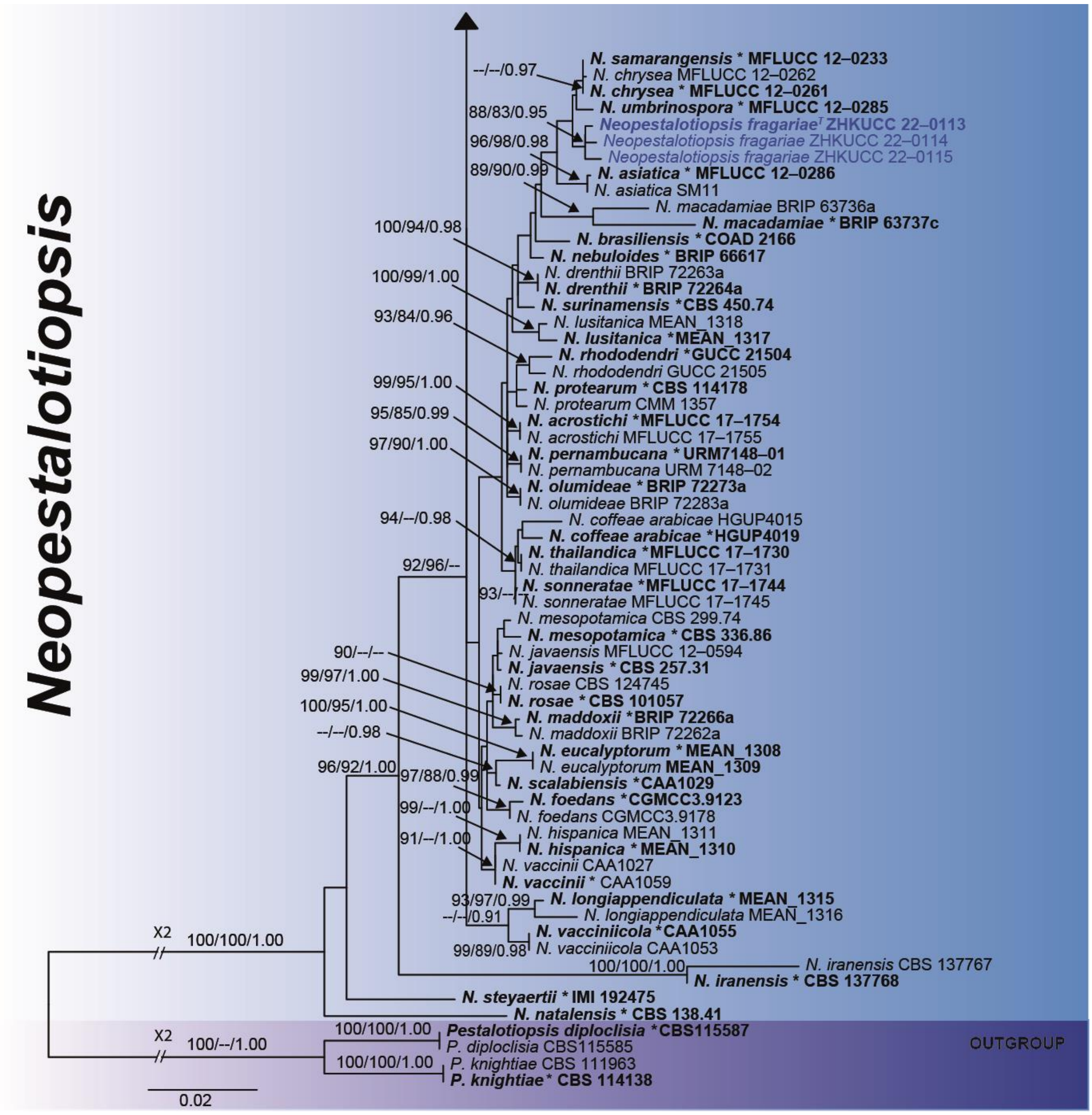

Fig. 1 – Continued.

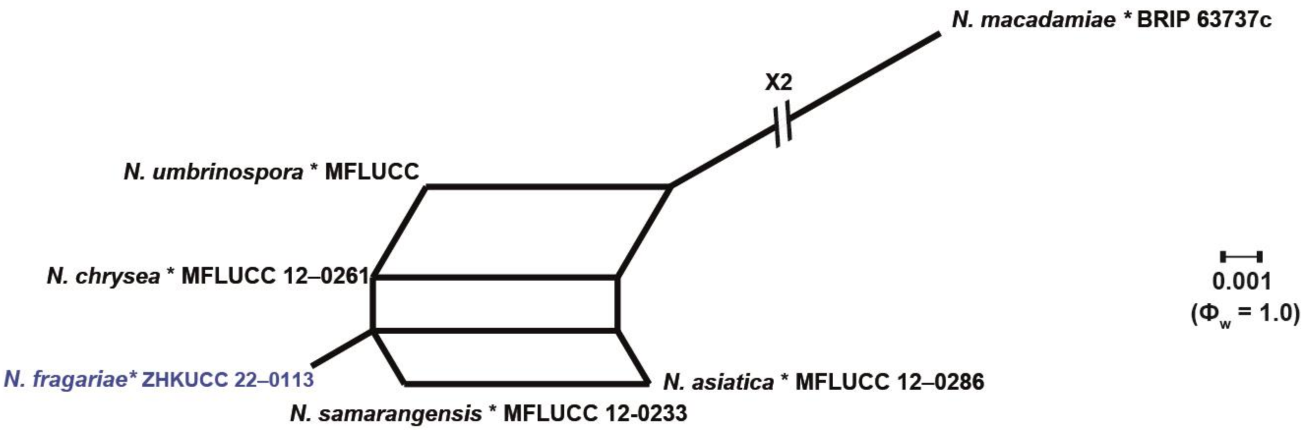

Fig. 2 – Results of the PHI test using both LogDet transformation and splits decomposition. The new taxon described in this study is indicated in blue.

Taxonomy

Neopestalotiopsis fragariae Prematunga & Jayaward, **sp. nov.** Fig. 3

Index Fungorum number: IF555038; Facesoffungi number: FoF11702; MycoBank: MB844495

Etymology – The specific epithet *fragariae* refers to the host from which the fungus was collected.

Pathogenic on *Fragaria* × *ananassa* leaves. There are many irregular spots on strawberry leaves, initially small spots with dark purple colour. Disease spots gradually expand, with dark red edges, reddish brown centers, with dark red edges of leaves. Sexual morph: Not observed. Asexual morph: *Conidiomata* (on PDA) pycnidial, globose to oval, aggregated or solitary, semi-immersed, black, 960–1600 µm diam; exuding globose, black, glistening, conidial masses. *Conidiophores* indistinct, often reduced to conidiogenous cells. *Conidiogenous cells* 8–13 µm high × 1–2 µm wide ($\bar{x}$ = 10 × 1 µm, n = 30), discrete, simple, short, hyaline, ampulliform to filiform or lageniform. *Conidia* fusoid to ellipsoid, straight to slightly curved, constricted at septa, 4-septate, 17–26 µm high × 5–9 µm wide ($\bar{x}$ = 22 × 7 µm, n = 40), L/W ratio = 3.1, basal cell conic to acute with a truncate base, hyaline, rugose, thin-walled, 3–6 µm long, three-median cells, doliform, 12–17 µm long, $\bar{x}$± SD = 14 ± 1.3 µm, wall rugose, versicoloured, septa darker than the rest of the cell (second cell from base pale brown to olivaceous, 3.4–6.1 µm long; third cell olivaceous to darker brown, 3.3–6 µm long; fourth cell olivaceous to darker brown, 3.7–5.8 µm long); apical cell 2.8–4.7 µm long, hyaline, cylindrical to sub cylindrical, thin and smooth walled, with 2–4 tubular apical appendages (mostly 3), arising from the apical crest, unbranched, filiform, flexuous 10.4–32.8 µm long, $\bar{x}$± SD = 19.3 ± 5.1 µm, basal appendage single, unbranched, tubular, centric, 3.0–11.5 µm long.

Culture characteristics – Colonies on PDA reaching 7 cm diameter after 6 days at 25°C, entire edge, whitish to olivaceous, aerial mycelium on surface, fruiting bodies black, gregarious.

Material examined – China, Guangdong Province, on living leaves of *Fragaria* × *ananassa* (*Rosaceae*) leaves, January 2021, Xiangying Zou (Holotype ZHKU 22-0061), ex-type culture ZHKUCC 22-0113; *ibid* living cultures ZHKUCC 22-0114, 22-0114).

GenBank numbers – ON553410 (ITS); ON569075 (*β-TUB*); ON569076 (*TEF-1α*).

Notes: Our isolate (ZHKUCC 22-0113) forms a monophyletic clade with 88% maximum likelihood bootstrap, 83% maximum parsimony and 0.95 posterior probability values. Thus, based on our phylogenetic analyses, we introduce our isolate (ZHKUCC 22-0113) as a new species of *Neopestalotiopsis*, causing leaf spots in *Fragaria* × *ananassa*. Our isolate is morphologically similar to *N. asiatica*, *N. chrysea* and *N. umbrinispora*. Versicoloured, 4-celled conidia with tubular apical appendages and indistinct conidiophores are common in these taxa (Maharachchikumbura et al. 2014b). However, the number of appendages can vary among these taxa. Similar to *N. chrysea*, our isolate *N. fragariae* has four appendages. On the other hand, *N. asiatica* and *N. umbrinispora* have 3 appendages each (Maharachchikumbura et al. 2014b). Nevertheless, there are differences in culture characteristics. The colony of *N. asiatica* has a crenate edge, while that of *N. chrysea* is irregular. The *N. umbrinispora* and *N. fragariae* display entire colony edge (Maharachchikumbura *et al.* 2014b). While the obverse colour of the colonies of *N. asiatica*, *N. umbrinispora* and *N. fragariae* are whitish, *N. chrysea* exhibits a yellowish colour. Therefore many of these taxa are morphologically indistinguishable, and molecular data are required to delineate between the cryptic *Neopestalotiopsis* species (Gerardo-Lugo et al. 2020a, Chethana et al. 2021, Ul Haq et al. 2021, Xavier et al. 2021).

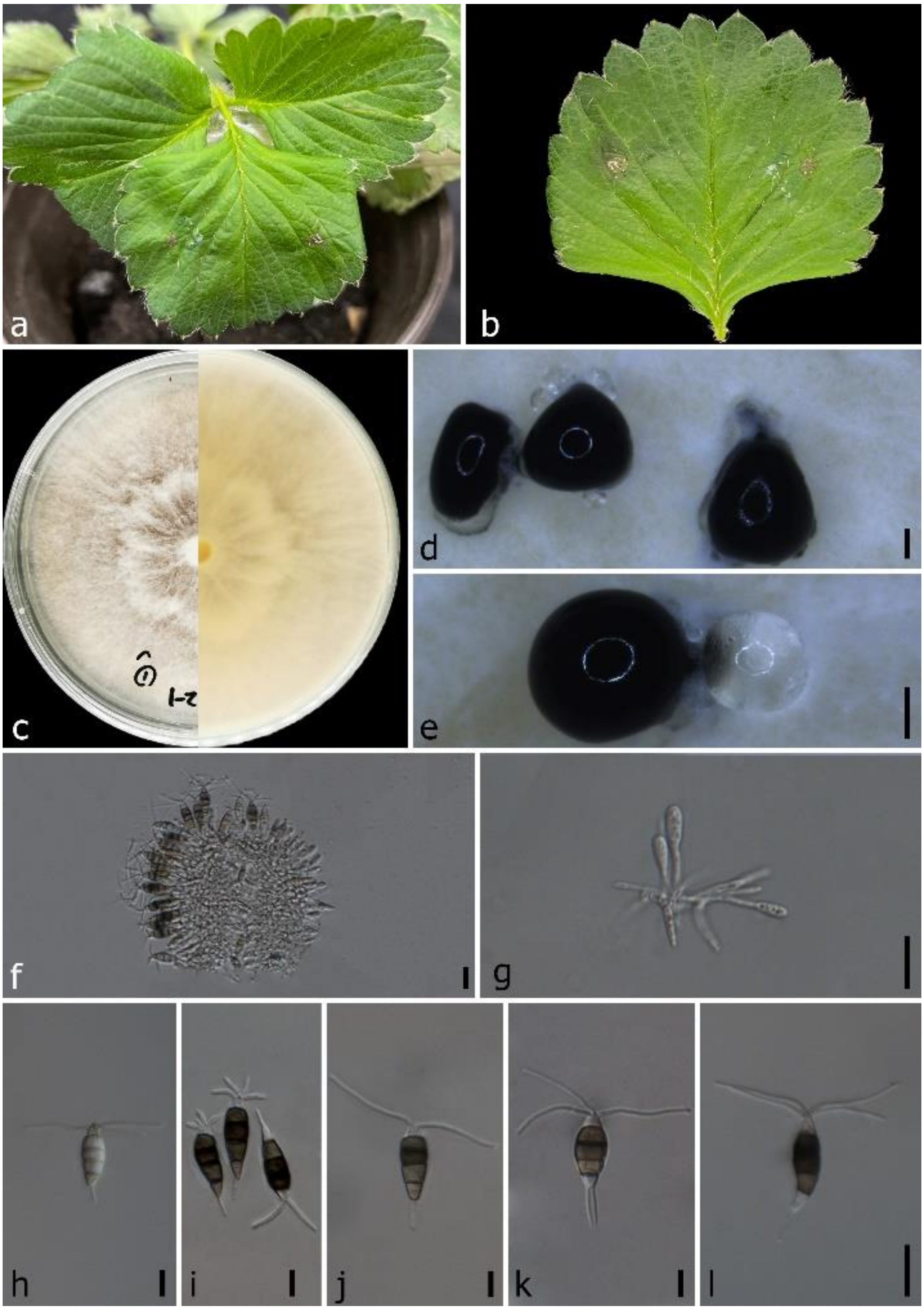

Fig. 3 – *Neopestalotiopsis fragariae* ZHKUCC 22-0113. a Infected leaf sample. b Leaf spot on *Fragaria* × *ananassa*. c Colony on PDA (obverse, reverse). d–e Conidiomata on PDA. f–g Conidiophores and conidiogenous cells. h–l Conidia. Scale bars: d = 200 µm, e = 300 µm, f = 7 µm, g = 15 µm, h–k = 8 µm, l = 10 µm

Pathogenicity

Inoculated leaves of *Fragaria* × *ananassa* initially displayed necrotic lesions after seven days in the greenhouse. Initial small dark brown disease spots expanded gradually with time. Light yellow halos were produced around the margins of each leaf spot, with a greyish white centre in the region where wounds were generated (Fig. 4). The lower surface of the wounds gradually turned to light brown. In addition, the lesions were observed in the inter-veinal regions and leaf lobes. None of these symptoms was observed in control plants (Fig. 4). The pathogen was re-isolated from the diseased leaves, and the isolates were confirmed as *N. fragariae*, based on morphology and culture characteristics, as shown in Fig. 3, thus, fulfilling the requirements of Koch's postulates.

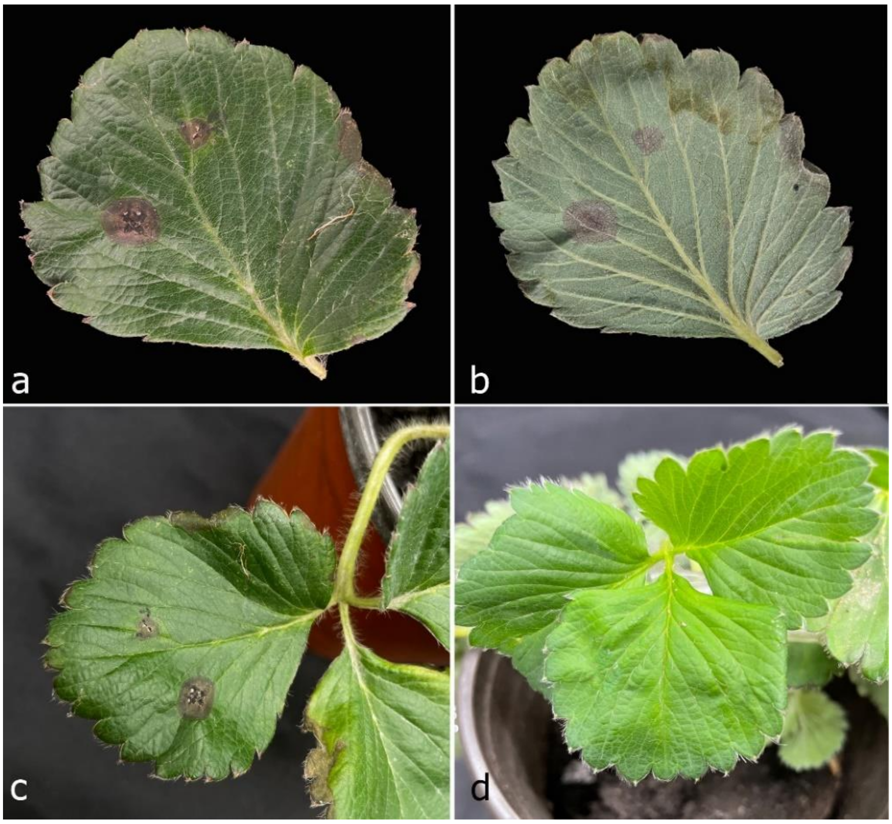

Fig. 4 – Pathogenicity test results. a Leaf spots on the upper surface of the strawberry leaf. b Leaf spots on the lower surface of the strawberry leaf. c Treated plant. d Control plant (*N. fragariae*)

Discussion

In this study, we introduce a new species, *Neopestalotiopsis fragariae*, causing leaf spots on strawberries in China. Based on morphological characters and multi-gene phylogenetic analyses of the concatenated ITS, *β-TUB* and *TEF-1α* alignment of *Neopestalotiopsis*, we accept 68 species in this genus, comprising 2155 characters (ITS: 1–483, *β-TUB:* 484–901, *TEF-1α:* 902–2155). We also performed single gene trees, which revealed the same phylogenetic relationships as in multi-gene phylogram. The following taxa, *N. phangngaensis* (MFLUCC 18-0119), *N. psidii* (FMBCC 11.2), *N. perukae* (FMBCC 11.3), *N. guajavae* (FMBCC 11.1), *N. guajavicola* (FMBCC 11.4), *N. camelliae-oleiferae* (CSUFTCC81) and *N. siciliana* (CBS 149117) have been excluded from the analysis due to the formation of ambiguous branch lengths. The inclusion of the above-mentioned taxa reduced the average length of internal branches and made phylogenetic relationship estimation problematic. Therfore, the data set had to be trimmed to get rid of as many ambiguous characters as feasible.

Furthermore, we compared the base pair differences of our new taxon with *N. asiatica*, *N. chrysea* and *N. umbrinispora*. There are no significant differences in the base pair comparison of the 481 nucleotides across the ITS gene region of *N. fragariae* compared to the type species of *N. asiatica* (MFLUCC 12–0286), *N. chrysea* (MFLUCC 12–0261) and *N. umbrinospora* (MFLUCC 12–0285). The following graph (Figure 5) illustrates the base pair differences without gaps across 794 nucleotides in *β-TUB* and 292 nucleotides in the *TEF-1α* gene regions (Figure 5). To delimit species of the pestalotioid group, the ITS alone is not sufficient (Maharachchikumbura et al. 2014b). Based on our multi-gene phylogenetic analyses, we confirm *N. fragariae* as a new taxon.

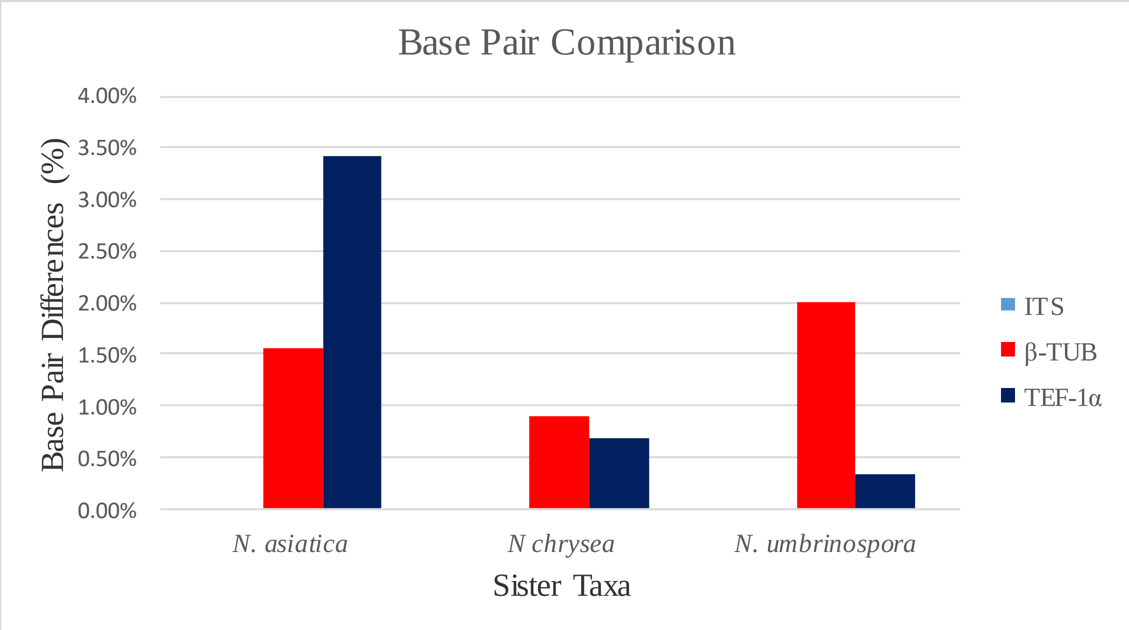

Fig. 5 – Base pair comparison of *N.* fragariae with three sister taxa.

Our isolate is a confirmed pathogen of *Fragaria* × *ananassa* following Koch's postulates. In pathogenicity testing, Koch's postulates determine the causative relationship between a microorganism and a disease (Ross & Woodward 2016, Bhunjun et al. 2021). Incorrect identification of plant pathogens always has a negative impact on control measures. Therefore, correct taxonomic identification of plant pathogens plays an essential role in the control strategies designed by plant pathologists (Jayawardena et al. 2015, Jayawardena et al. 2021). Future studies are necessary to document data regarding host specificity and geographic distribution of this new species.

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