



Article New Species of Nectriaceae (Hypocreales) from China

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Abstract: Species of *Nectriaceae* commonly occur on living and decaying woody substrates, soil, fruitbodies of other fungi, and insects. Some of them are reported as endophytes, opportunistic pathogens of crops and humans, or producers of mycotoxins. To explore the species diversity of the family, specimens from different regions of China were collected and examined. Four novel taxa of *Penicillifer, Pseudocosmospora*, and *Thelonectria* were introduced on the basis of morphological characteristics and DNA sequence analyses of combined datasets of the act, ITS, LSU, rpb1, rpb2, tef1, and tub2 regions. Differences between the new species and their close relatives were compared and discussed.

Keywords: Ascomycota; morphology; multigene analyses; taxonomy

1. Introduction

The family *Nectriaceae* was established in 1865 to accommodate those fungi producing uniloculate perithecia that are yellow, orange-red to purple, or brown; often change color in 3% potassium hydroxide (KOH) and 100% lactic acid (LA); and have a tropical and subtropical distribution [1]. Approximately 69 genera are currently accepted [1–3], including genera such as *Penicillifer* Emden, *Pseudocosmospora* C.S. Herrera & P. Chaverri, and *Thelonectria* P. Chaverri & C. Salgado. The generic concepts and phylogenetic relationships of the family were comprehensively stated by Lombard et al. [2].

The genus *Penicillifer*, typified by *Penicillifer pulcher* Emden., was introduced by Emden [4]. It was treated as the asexual stage of *Viridispora* Samuels & Rossman [1] and recommended as the correct name for this group of fungi [2]. Seven species are currently known in the genus [1,2,5]. The genus *Pseudocosmospora*, typified by *P. eutypellae* C.S. Herrera & P. Chaverri, was established by Herrera et al. [6] to accommodate *Cosmospora vilior* (Starbäck) Rossman & Samuels and related species that are usually fungicolous. Sixteen species are recognized [6–9]. The genus *Thelonectria*, typified by *T. discophora* (Mont.) P. Chaverri & C. Salgado, was established by Chaverri et al. [10] to include the species formerly placed in the *Nectria mammoidea* and *N. veuillotiana* groups with a cosmopolitan distribution [11]. Forty-seven species are accepted in the genus [10–18].

In our study of the hypocrealean specimens from different regions of China, four unusual fungi were encountered. Judging by their perithecial gross morphology, anatomy, and culture characteristics, they represent four undescribed species of *Penicillifer*, *Pseudocosmospora*, and *Thelonectria*. Their taxonomic placements were further confirmed by multigene phylogenetic analyses of α -actin (act), nuclear ribosomal DNA ITS1-5.8S-ITS2 (ITS), large subunit of nuclear ribosomal DNA (LSU), the largest subunit of RNA polymerase II (rpb1), the second largest subunit of RNA polymerase II (rpb2), translation elongation factor 1- α (tef1), and β -tubulin (tub2). The differences between the novel taxa and their close relatives were compared.



Citation: Zeng, Z.-Q.; Zhuang, W.-Y. New Species of *Nectriaceae* (*Hypocreales*) from China. J. Fungi 2022, 8, 1075. https://doi.org/ 10.3390/jof8101075

Academic Editor: Jian Kui Liu

Received: 14 September 2022 Accepted: 10 October 2022 Published: 13 October 2022

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2. Materials and Methods

2.1. Sampling and Morphological Studies

Specimens were collected from Beijing and the Guangxi Zhuang Autonomous Region, and they are preserved in Herbarium Mycologicum Academiae Sinicae (HMAS). Cultures were obtained by single ascospore isolation from the fresh perithecium and deposited in the China General Microbiological Culture Collection Center (CGMCC). The method of Lombard et al. [2] was followed for morphological observations. The ascomatal wall reactions to 3% KOH and 100% LA were tested. Sections were prepared with a freezing microtome (YD-1508-III, Jinhua, China) at a thickness of 6–8 µm for anatomic examination. Lactophenol cotton blue solution was used as a mounting medium for the measurements of the perithecia, asci, ascospores, conidiophores, and conidia. Photographs were taken with a Leica DFC450 digital camera (Wetzlar, Germany) attached to a Leica M125 stereomicroscope (Milton Keynes, UK) for gross morphology and a Zeiss AxioCam MRc 5 digital camera (Jena, Germany) attached to a Zeiss Axio Imager A2 microscope (Göttingen, Germany) for microscopic features. For colony morphology and growth rates, strains were grown on potato dextrose agar (PDA, 20% w/v potato + 2% w/v dextrose + 2% w/v agar) and synthetic nutrient-poor agar (SNA) [19] in 90 mm plastic Petri dishes at 25 °C for 14 d with alternating periods of light and darkness (12 h/12 h).

2.2. DNA Extraction, PCR Amplification, Sequencing, and Phylogenetic Analyses

Genomic DNA was extracted from fresh mycelium following the method of Lombard et al. [2]. Seven primer pairs, act1/act2 [20], ITS5/ITS4 [21], LR0R/LR5 [22,23], rpb1a/rpb1c [24], RPB2-5f/RPB2-7cR [25], 728F/EF2 [26,27], and T1/T22 [28], were used to amplify the sequences of the act, ITS, LSU, rpb1, rpb2, tef1, and tub2 regions, respectively. PCR reactions were performed using an ABI 2720 Thermal Cycler (Applied Biosciences, Foster City, CA, USA), and DNA sequencing was carried out in both directions on an ABI 3730XL DNA Sequencer (Applied Biosciences, Foster City, CA, USA).

Newly obtained sequences and those retrieved from GenBank are listed in Tables 1–3. The sequences were assembled and aligned, and the primer sequences were trimmed using BioEdit 7.0.5 [29] and converted to nexus files by ClustalX 1.83 [30]. A partition homogeneity test (PHT) was performed with 1000 replicates in PAUP*4.0b10 [31] to evaluate statistical congruence amongst these loci. The aligned sequences were combined in BioEdit and analyzed with Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP) methods to determine the phylogenetic positions of the new species. The BI analysis was conducted by MrBayes 3.1.2 [32] using a Markov chain Monte Carlo algorithm. Nucleotide substitution models were determined by MrModeltest 2.3 [33]. Four Markov chains were run simultaneously for 1,000,000 generations, with the trees sampled every 100 generations. A 50% majority rule consensus tree was computed after excluding the first 2500 trees as 'burn-in'. The Bayesian inference posterior probability (BIPP) was determined from the remaining trees. Branch support measures were calculated with 1000 bootstrap replicates. The ML analysis was performed via IQ-Tree 1.6.12 [34] using the best model for each locus chosen by ModelFinder [35]. The MP analysis was performed with PAUP 4.0b10 [31] using heuristic searches with 1000 replicates of random addition of sequences and subsequent TBR (tree bisection and reconnection) branch swapping. The topological confidence of the resulting trees and the statistical supports of the branches were tested by maximum parsimony bootstrap proportion (MPBP) with 1000 replications, each with 10 replicates of random addition of taxa. Trees were examined by TreeView 1.6.6 [36]. Maximum likelihood bootstrap proportion (MLBP) and MPBP greater than 70% and BIPP greater than 90% were shown at the nodes.

Species	Herbarium/Strain Numbers	GenBank Accession Numbers					
Species		ITS	LSU	rpb2	tef1		
Corallonectria jatrophae	CBS 91396 ^T	NR153873	KM231611	KM232298	KM231863		
Dematiocladium celtidis	CBS 115994 ^T	AY793430	AY793438	-	KM231864		
P. bipapillatus	CBS 42088 ^T	KM231740	KM231608	KM232295	KM231860		
P. diparietisporus	CBS 37659 ^T	NR154310	MH869437	KM232296	KM231861		
P. macrosporus	CBS 42388 ^T	MH862133	KM231607	KM232294	KM231859		
P. martinii	BRIP 59225 ^T	NR168155	NG068753	-	KJ869241		
P. pulcher	CBS 56067 ^T	NR154311	NG058093	KM232297	KM231862		
P. sinicus	CGMCC 3.24130 ^T	OP223439 ^a	OP223435	OP272863	OP272864		
Stachybotrys chartarum	CBS 12913	MH854622	MH866145	KM232434	KM231994		

Table 1. List of *Penicillifer* species, herbarium/strain numbers, and GenBank accession numbers of materials used in this study.

^T indicates the ex-type culture. ^a Numbers in bold indicate the newly provided sequences.

Table 2. List of *Pseudocosmospora* species, herbarium/strain numbers, and GenBank accession numbers of materials used in this study.

S manian	Herbarium/Strain	GenBank Accession Numbers				
Species	Number	ITS	LSU	tub2		
Corallomycetella repens	orallomycetella repens AR 4547		JF832679	JF832838		
Microcera larvarum	AR 4580	KC291751	KC291759	KC291935		
P. beijingensis	CGMCC 3.24131 ^T	OP223438 ^a	OP223434	OP272862		
P. curvispora	CGMCC 3.20176 ^T	MT592897	MT592879	MT606156		
P. eutypae	CH 1101 ^T IMI 73016 AR 4527	KC291735 KC291736 KC291720	KC291766 KC291786 KC291756	KC291925 - KC291909		
P. eutypellae	AR 4562 ^T GJS 10248	KC291721 KC291722	KC291757 KC291772	KC291912 KC291911		
P. henanensis	HMAS 183528 ^T	GU075856	GU075863	HM054103		
P. hypoxylicola	cLL 19020 ^T	MN886606	MN886608	-		
P. joca	AR 4779 ^T	KC291746	KC291762	KC291924		
P. metajoca	AR 4576 ^T	KC291745	KC291758	KC291923		
P. rogersonii	GJS 9056 ^T GJS 10296 GJS 091384	KC291729 KC291727 KC291726	KC291780 KC291774 KC291770	KC291915 KC291917 KC291914		
P. shennongjiana	CGMCC 3.20177 ^T	MT592898	MT592880	MT606157		
P. vilior	AR 4810 ^T AR 4771 PC 1246	KC291737 KC291734 KC291738	KC291763 KC291761 KC291791	KC291928 KC291926 KC291927		

^T indicates the ex-type culture. ^a Numbers in bold indicate the newly provided sequences.

Table 3. List of *Thelonectria* species, herbarium/strain numbers, and GenBank accession numbers of materials used in this study.

Spacios	Herbarium/Strain _ Numbers	GenBank Accession Numbers					
Species		act	ITS	LSU	rpb1	tub2	
Cosmospora coccinea	CBS 114050	GQ505967	FJ474072	GQ505990	GQ506020	DQ522501	
Nectria cinnabarina	AR 4477/AR 4302	HM484627	HM484548	HM484562	M484577	HM484820	
T. acrotyla	GJS 90171	JQ365047	JQ403329	JQ403368	JQ403407	JQ394720	
T. amamiensis	MAFF 239819	JQ365054	JQ403337	JQ403375	KJ022408	JQ394727	

Species	Herbarium/Strain _ Numbers	GenBank Accession Numbers					
		act	ITS	LSU	rpb1	tub2	
T. chlamydospora	ST 121 ^T	LC519560	LC509450	LC509452	-	-	
T. globulosa	CGMCC 3.24132 ^T	OP272865 ^a	OP223436	OP223432	OP272867	OP586762	
T. gongylodes	GJS 04171 ^T	JQ365038	JQ4033	JQ403358	JQ403395	JQ394710	
T. nodosa	GJS 04155 ^T	JQ365037	JQ403317	JQ403357	JQ403394	-	
T. olida	CBS 21567 ^T	HM352884	KJ021982	KJ022058	HM364334	KM232024	
T. rubrococca	IMI 324475 ^T	KJ022275	KJ022008	KJ022061	KJ022439	KJ022329	
T. spinulospora	CGMCC 3.24133 ^T	OP272866	OP223437	OP223433	OP272868	OP586764	
T. torulosa	AR 4768A	JQ365031	JQ403310	JQ403350	JQ403386	JQ394702	
T. trachosa	GJS 9245 ^T	KF569832	KF569842	KF569851	KF569879	KF569869	
T. truncata	GJS 04357 ^T	JQ365039	JQ403319	JQ403359	JQ403396	KJ022324	
T. veuillotiana	AR 1751	KJ022273	JQ403305	JQ403345	JQ403382	JQ394698	

Table 3. Cont.

^T indicates the ex-type culture. ^a Numbers in bold indicate the newly provided sequences.

3. Results

3.1. Phylogeny

The sequences of the ITS, LSU, rpb2, and tef1 regions from six *Penicillifer* species were analyzed. *Stachybotrys chartarum* (Ehrenb.) S. Hughes was used as the outgroup taxon. The partition homogeneity test (p = 0.01) indicated that the individual partitions were not highly incongruent [37]; thus, these four loci were combined for the phylogenetic analyses. The ML tree is shown in Figure 1. The topologies of the BI and MP trees were similar to that of the ML tree. The isolate CGMCC 3.24130 grouped with other members of *Penicillifer* and received high statistical support (MLBP/MLBP/BIPP = 96%/100%/100%).



Figure 1. The maximum likelihood tree inferred from combined ITS, LSU, rpb2, and tef1 sequences of representative species of *Penicillifer*. MLBP (**left**) and MPBP (**middle**) values greater than 70% and BIPP (**right**) values greater than 90% are shown at the nodes.

The sequences of ITS, LSU, and tub2 regions from 11 *Pseudocosmospora* species were analyzed. *Corallomycetella repens* (Berk. & Broome) Rossman & Samuels and *Microcera larvarum* (Fuckel) Gräfenhan, Seifert & Schroers were used as outgroup taxa. The partition homogeneity test (p = 0.01) indicated that the individual partitions were not highly incongruent [37]; thus, these three loci were combined for the phylogenetic analyses. The ML tree is shown in Figure 2. The topologies of the BI and MP trees were similar to that of the ML tree. The isolate CGMCC 3.24131 grouped with other species of *Pseudocosmospora* and received high statistical support (MLBP/MLBP/BIPP = 92%/98%/100%).



Figure 2. The maximum likelihood tree inferred from combined ITS, LSU, and tub2 sequences of representative species of *Pseudocosmospora*. MLBP (**left**) and MPBP (**middle**) values greater than 70% and BIPP (**right**) values greater than 90% were shown at the nodes.

The sequences of the act, ITS, LSU, rpb1, and tub2 regions from 13 *Thelonectria* species were analyzed. *Cosmospora coccinea* Rabenh. and *Nectria cinnabarina* (Tode) Fr. were used as outgroup taxa. The partition homogeneity test (p = 0.01) indicated that the individual partitions were not highly incongruent [37]; thus, these five loci were combined for the phylogenetic analyses. The ML tree is shown in Figure 3. The topologies of the BI and MP trees were similar to that of ML tree. The isolates CGMCC 3.24132 and CGMCC 3.24133 were well-located among other *Thelonectria* species and received high supporting values (MLBP/MLBP/BIPP = 100%/100%/100%). The isolate CGMCC 3.24133 was related to *T. rubrococca* (Brayford & Samuels) Salgado & P. Chaverri, receiving high statistic values (MLBP/MLBP/BIPP = 100%/100%/100%), and the strain CGMCC 3.24132 formed an independent lineage and was related to the *T. veuillotiana* complex (MLBP/MLBP/BIPP = 100%/72%/100%).



Figure 3. The maximum likelihood tree inferred from combined act, ITS, LSU, rpb1, and tub2 sequences of representative species of *Thelonectria*. MLBP (**left**) and MPBP (**middle**) values greater than 70% and BIPP (**right**) values greater than 90% were shown at the nodes.

3.2. Taxonomy

Penicillifer sinicus Z.Q. Zeng & W.Y. Zhuang, sp. nov. Figures 4 and 5. **Fungal Names:** FN571297.

Etymology: The epithet refers to the country where the fungus was collected.

Typification: CHINA, Guangxi Zhuang Autonomous Region, Guilin City, Mao'er Mountain, on rotten twigs, 7 December 2019, Z.Q. Zeng & H.D. Zheng 12496 (holotype HMAS 247865, ex-type strain CGMCC 3.24130).

GenBank accession numbers: ITS OP223439, LSU OP223435, rpb2 OP272863, tef1 OP272864, rpb1 OP586759, tub2 OP586763.



Figure 4. Macroscopic and microscopic morphology of *Penicillifer sinicus* (HMAS 247865). (**a**–**c**) Ascomata on natural substratum; (**d**) median section of perithecium in lactophenol cotton blue; (**e**–**i**) asci with ascospores in lactophenol cotton blue; (**j**–**n**) ascospore in lactophenol cotton blue. Bars: (**a**–**c**) = 1 mm; (**d**) = 50 μ m; (**e**–**n**) = 10 μ m.



Figure 5. Colonial and microscopic morphology of *Penicillifer sinicus* (CGMCC 3.24130). (a) Colony after 1 week at 25 °C on PDA; (b) colony after 1 week at 25 °C on SNA; (c–k) conidiophores, phialides, and macroconidia in lactophenol cotton blue; (l) macroconidia in lactophenol cotton blue. Bars: $(c-l) = 10 \ \mu m$.

Mycelium was not visible on the natural substratum. Perithecia were superficial, solitary, non-stromatic or with a basal stroma, and subglobose to globose with an acute to blunt papilla; the surface was warted; they did not collapse upon drying; they were yellowish brown to brown and did not change color in 3% KOH or 100% LA, and the size was 235–314 × 176–274 μ m. The perithecial surface had warts 15–55 μ m high. Perithecial walls were two-layered, 23–35 μ m thick; the outer layer was of the textura angularis, 16–25 μ m thick, with cell 5–9 × 4–8 μ m, and cell walls 1–1.2 μ m thick; the inner layer was of the textura prismatica, 7–10 μ m thick, with cell 5–13 × 2–3 μ m, and cell walls 0.8–1 μ m thick. Asci were unitunicate, cylindrical, and eight-spored with an apical ring, and 60–85 × 4.5–8 μ m. Ascospores were ellipsoidal to fusiform, (0–)1-septate, constricted or not at septum, hyaline to light brown, smooth-walled, uniseriate, overlapping obliquely, and 10–15 × 4.5–5.3 μ m.

Colony characteristics: On PDA, the colony was 20 mm in diam. after 1 week at 25 °C, the surface was cottony with dense, whitish aerial mycelium producing yellowish-brown pigments. On SNA, the colony was 26 mm in diam. after 1 week at 25 °C, the surface was velvet with sparse, whitish aerial mycelium. Conidiophores were verticillium-like, septate, and hyaline, with 1–2 whorls and a terminal whorl of 2–8 phialides, 30–120 μ m long, and 2–3.5 μ m wide at the base. Phialides were subulate, tapering toward the apex, 15–45 μ m long, 1.5–2.5 μ m wide at the base, and 0.2–0.3 μ m wide at the apex. Macroconidia were ellipsoidal to fusiform or cylindrical, slightly curved, (0–)1(–3)-septate, smooth-walled, hyaline, and 10–28 × 3–5.5 μ m.

Notes: Amongst the known species of the genus, *P. sinicus* is morphologically most similar to *P. macrosporus* Samuels in having superficial, solitary, non-stromatic, globose perithecia with warted surfaces; smooth-walled, 1-septate ascospores; and cylindrical, bicellular conidia [1]. However, *P. macrosporus* has a thicker perithecial wall (ca. 65 μ m thick), clavate asci without apical rings, wider ascospores (5–7 μ m wide), and longer conidia (33–47 μ m long) [1]. In addition, there were 34 bp, 19 bp, 23 bp, 31 bp, and 23 bp divergences in the ITS, LSU, rpb1, rpb2, and tef1 regions between the ex-type cultures of

the two species (CGMCC 3.24130 and CBS 423.88). Thus, both the morphological and the molecular evidence support their separation at the species level.

Pseudocosmospora beijingensis Z.Q. Zeng & W.Y. Zhuang, sp. nov. Figures 6 and 7.



Figure 6. Macroscopic and microscopic morphology of *Pseudocosmospora beijingensis* (HMAS 290896). (**a–d**) Ascomata on natural substratum; (**e**,**f**) median section of perithecia in lactic acid; (**g**,**h**) asci with ascospores in lactophenol cotton blue; (**i–k**) ascospore in lactophenol cotton blue. Bars: (**a–d**) = 1 mm; (**e**,**f**) = 50 μ m; (**g–k**) = 10 μ m.



Figure 7. Colonial and microscopic morphology of *Pseudocosmospora beijingensis* (CGMCC 3.24131). (a) Colony after 1 week at 25 °C on PDA; (b) colony after 1 week at 25 °C on SNA; (c–i) conidiophores, phialides, and microconidia in lactophenol cotton blue. Bars: $(c-i) = 10 \mu m$.

Fungal Names: FN571298.

Etymology: The epithet refers to the type locality of the fungus.

Typification: CHINA, Beijing, Beidagou forest, on rotten bark associated with other fungi, 10 August 2017, H.D. Zheng, X.C. Wang, Y.B. Zhang, C. Wang & P. Li 11339 (holotype HMAS 290896, ex-type strain CGMCC 3.24131).

GenBank accession numbers: ITS OP223438, LSU OP223434, tub2 OP272862.

Mycelium was not visible on the natural substratum. Perithecia were superficial and gregarious with a well-developed stroma, subglobose to globose, slightly roughened surface, laterally collapsed upon drying, orange-red to bright red, turning dark red in 3% KOH and light yellow in 100% LA, and 147–196 \times 118–176 µm. Perithecial walls were two-layered, and 20–42 µm thick; the outer layer was of the textura globulosa to textura

angularis, 15–25 μ m thick, with cell 4–13 × 2.5–4.5 μ m, and cell walls 1–1.2 μ m thick; the inner layer was of the textura prismatica, 5–8 μ m thick, with cell 6–10 × 2.5–3.5 μ m, and cell walls 0.8–1 μ m thick. Asci were unitunicate, cylindrical, with a simple apex, eight-spored, and 38–58 × 2.5–5 μ m. Ascospores were ellipsoidal, 1-septate, not constricted at the septum, light yellow-brown, smooth-walled, uniseriate, and 8–10 × 2.5–4 μ m.

Colony characteristics: On PDA, the colony was 25 mm in diam. after 1 week at 25 °C, surface crustose, producing yellowish-white pigments. On SNA, the colony was 15 mm in diam. after 1 week at 25 °C, surface velvet, with sparse, whitish aerial mycelium. Conidiophores were acremonium- to verticillium-like, septate, of indefinite length, and hyaline, with 1–2 whorls and a terminal whorl of 2–6 phialides. Phialides were subulate, tapering toward the apex, 10–55 μ m long, 0.9–1.2 μ m wide at the base, and 0.2–0.3 μ m wide at the tip. Conidia were allantoid, curved, unicellular, smooth-walled, hyaline, and 2.6–4.5 \times 0.9–1.8 μ m.

Notes: Among the known species of *Pseudocosmospora*, *P. beijingensis* most resembles *P. curvispora* Z.Q. Zeng & W.Y. Zhuang in having subglobose to globose perithecia that are laterally collapsed upon drying; having asci without an apical ring; having ellipsoidal, 1-septate, smooth-walled, and light yellow-brown ascospores; and producing acremonium-to verticillium-like conidiophores, and allantoid, unicellular, curved conidia [8]. However, *P. curvispora* differs in clavate and somewhat longer asci (53–68 μm long) and narrower conidia (0.8–1.2 μm wide) [8]. Sequence comparisons revealed that there were 30 bp, 22 bp, and 90 bp divergences detected for the ITS, LSU, and tub2 regions. Obviously, they are not conspecific.

Thelonectria globulosa Z.Q. Zeng & W.Y. Zhuang, sp. nov. Figures 8 and 9.

Fungal Names: FN571299.

Etymology: The epithet refers to the globose microconidia.

Typification: CHINA, Guangxi Zhuang Autonomous Region, Guilin City, Mao'er Mountain, on rotten roots, 5 December 2019, Z.Q. Zeng & H.D. Zheng 12434 (holotype HMAS 255835, ex-type strain CGMCC 3.24132).

GenBank accession numbers: act OP272865, ITS OP223436, LSU OP223432, rpb1 OP272867, rpb2 OP586760, tub2 OP586762.



Figure 8. Macroscopic and microscopic morphology of *Thelonectria globulosa* (HMAS 255835). (**a**–**d**) Ascomata on natural substratum; (**e**,**f**) median section of perithecium in lactophenol cotton blue; (**g**,**h**) asci with ascospores in lactophenol cotton blue; (**i**–**k**) ascospore in lactophenol cotton blue. Bars: (**a**–**d**) = 1 mm; (**e**,**f**) = 50 μ m; (**g**–**k**) = 10 μ m.



Figure 9. Colonial and microscopic morphology of *Thelonectria globulosa* (CGMCC 3.24132). (a) Colony after 2 week at 25 °C on PDA; (b) colony after 2 week at 25 °C on SNA; (c) conidiophores in lactophenol cotton blue; (\mathbf{d} - \mathbf{i}) conidiophores and macroconidia in lactophenol cotton blue; (\mathbf{j} - \mathbf{l}) microconidia in lactophenol cotton blue; (\mathbf{m}) chlamydospores in lactophenol cotton blue. Bars: (\mathbf{c} - \mathbf{m}) = 10 µm.

Mycelium was not visible on the natural substratum. Perithecia were superficial, solitary to gregarious, with a basal or well-developed stroma, subglobose to globose, slightly roughened surface, with blunt papilla of 32–65 μ m high and 52–75 μ m wide at the base, did not collapse upon drying, orange-red to red, turning dark red in 3% KOH and light yellow in 100% LA, and 235–323 × 148–245 μ m. Perithecial walls were two-layered, 20–40 μ m thick; the outer layer was of the textura globulosa to textura angularis, 15–30 μ m thick, with cell 5–15 × 4–12 μ m, and cell walls 0.8–1 μ m thick; the inner layer was of the textura prismatica, 5–10 μ m thick, with cell 6–15 × 3–10 μ m, and cell walls 1–1.2 μ m thick. Asci were unitunicate, cylindrical to clavate, eight-spored, with a simple apex, and 53–75 × 8–13 μ m. Ascospores were ellipsoidal to fusiform, 1-septate, constricted at the septum, hyaline, smooth to spinulose, uniseriate or irregular biseriate in asci, and 13–20 × 5.5–8 μ m.

Colony characteristics: The colony on PDA was 22 mm in diam. after 1 week at 25 °C and had a cottony surface with dense, whitish aerial mycelium producing yellowish brown pigments. The colony on SNA was 35 mm in diam. after 1 week at 25 °C and had a cottony surface with sparse, whitish aerial mycelium. Conidiophores were mostly unbranched; rarely had simple branches; and were septate, hyaline, 25–89 µm long, and 1.5–2.5 µm wide at the base. Macroconidia were cylindrical to rod-shaped, slightly curved, 1–3(–4)-septate, smooth-walled, hyaline, and 20–58 × 3.2–5.8 µm. Microconidia were globose, smooth-walled, hyaline, and 3–4.5 µm in diam. Chlamydospores were globose to subglobose, and $4–10 \times 3–8$ µm.

Notes: Among the known species of *Thelonectria*, *T. globulosa* is distinct because of its globose microconidia. Morphologically, *T. globulosa* resembles *T. nodosa* C.G. Salgado & P. Chaverri in having solitary to gregarious, globose perithecia that do not collapse upon drying; cylindrical to clavate asci; ellipsoidal to fusiform ascospores; and cylindrical macroconidia. However, *T. nodosa* differs because of its larger asci (68–115 × 10–17 µm) with an apical ring, macroconidia possessing more septa (up to six septa), and lack of microconidia formation [12]. Moreover, there are 38 bp, 96 bp, 37 bp, and 65 bp divergences

in the act, ITS, LSU, and rpb1 regions between the ex-type cultures of the two taxa (CGMCC 3.24132 and GJS 04155). Both the morphology and DNA sequence data distinguish them as different species.

Thelonectria spinulospora Z.Q. Zeng & W.Y. Zhuang, sp. nov. Figures 10 and 11.



Figure 10. Macroscopic and microscopic morphology of *Thelonectria spinulospora* (HMAS 290897). (**a**,**b**) Ascomata on natural substratum; (**c**) median section of perithecium in lactophenol cotton blue; (**d**–**i**) ascospore in lactophenol cotton blue. Bars: (**a**,**b**) = 1 mm; (**c**) = 50 μ m; (**d**–**i**) = 10 μ m.



Figure 11. Colonial and microscopic morphology of *Thelonectria spinulospora* (CGMCC 3.24133). (a) Colony after 2 week at 25 °C on PDA; (b) colony after 2 week at 25 °C on SNA; (**c**–**e**) conidiophores in lactophenol cotton blue; (**f**–**j**) conidiophores and macroconidia in lactophenol cotton blue; (**k**–**o**) macroconidia in lactophenol cotton blue; (**p**) chlamydospore in lactophenol cotton blue. Bars: (**c**–**p**) = 10 µm.

Fungal Names: FN571300.

Etymology: The specific epithet refers to the spinulose ascospores.

Typification: CHINA, Guangxi Zhuang Autonomous Region, Guilin City, Mao'er Mountain, on rotten twigs, 7 December 2019, Z.Q. Zeng & H.D. Zheng 12499 (holotype HMAS 290897, ex-type strain CGMCC 3.24133).

GenBank accession numbers: act OP272866, ITS OP223437, LSU OP223433, rpb1 OP272868, rpb2 OP586761, tub2 OP586764.

Mycelium was not visible on the natural substratum. Perithecia were superficial, solitary, with a basal stroma, subglobose to broad-pyriform, surface slightly roughened, sometimes collapsed laterally upon drying, orange-red to red, turning dark red in 3% KOH and light yellow in 100% LA, and 123–195 × 143–212 μ m. Perithecial walls were two-layered, 8–18 μ m thick; the outer layer was of the textura globulosa to textura angularis, 5–13 μ m thick, with cell 6–10 × 4–9 μ m, and cell walls 1–1.2 μ m thick; the inner layer was of the textura prismatica, 3–5 μ m thick, with cell 2–8 × 3–10 μ m, and cell walls 0.8–1 μ m thick. Asci were not observed. Ascospores were ellipsoidal, 1-septate, not constricted or slightly constricted at the septum, hyaline, spinulose, and 12–18 × 5.6–8 μ m.

Colony characteristics: On PDA, the colony was 40 mm in diam. after 2 weeks at 25 °C, the surface was cottony with dense, whitish aerial mycelium producing light yellow pigments. On SNA, the colony was 28 mm in diam. after 2 weeks at 25 °C, surface velvet, with sparse, whitish aerial mycelium. Conidiophores were acremonium-like, rarely with simple branches, septate, hyaline, and 45–102 × 2.2–4 µm. Macroconidia were cylindrical, slightly curved, (1–2–)3-septate, smooth-walled, hyaline, and 28–62 × 2.8–4.5 µm. Chlamydospores were globose to subglobose, smooth-walled, hyaline, and 6–8 µm in diam.

Notes: The morphological features, such as the superficial, globose to subglobose, broad-pyriform perithecia that do not collapse when dry; ellipsoidal, two-celled, and hyaline ascospores; and curved macroconidia with rounded ends, indicate the placement of *T. spinulospora* in *Thelonectria*, which was confirmed by sequence analyses of the act, ITS, LSU, rpb1, and tub2 regions (Figure 3). Amongst the known species of the genus, the new species is morphologically similar and phylogenetically related to *T. rubrococca* (Brayford & Samuels) C.G. Salgado & P. Chaverri in having solitary to gregarious, globose perithecia that do not collapse upon drying, ellipsoidal ascospores, and cylindrical macroconidia. However, the latter differs in its larger perithecia (200–450 µm in diam.), smaller ascospores (8–14.5 × 3.6–6.6 µm), and macroconidia with more septa (up to five septa) [38]. Sequence comparisons between the ex-type cultures of the two species revealed that 24 bp, 8 bp, 0 bp, 22 bp, and 28 divergences were detected for the act, ITS, LSU, rpb1, and tub2 regions. Both the morphology and DNA sequence data support their distinction at the species level.

4. Discussion

The genus *Penicillifer* is proposed as the preferable name over *Viridispora* [2], following the International Code of Nomenclature for algae, fungi, and plants [39]. Our analyses, inferred from sequences of ITS, LSU, rpb2, and tef1 and including the new taxon, revealed a tree topology (Figure 1) similar to that given by Lombard et al. [2]. The phylogenetic tree shows that *Penicillifer* species forms a well-supported monophyletic clade (MLBS/MPBP/BIPP/ = 96%/100%/100%) (Figure 1). *Penicillifer sinicus* is closely related to *P. macrosporus* (MLBS/MPBP/BIPP/ = 100%/99%/100%). The sequence comparisons revealed that there were 34 bp, 19 bp, 23 bp, 31 bp, and 23 bp differences detected for the ITS, LSU, rpb1, rpb2, and tef1 regions. Therefore, both the molecular and the morphological evidence supports the separation of the two fungi at a specific level. Among the known species of *Penicillifer*, *P. martinii* P. Wong, Y.P. Tan & R.G. Shivas is known solely by its sexual stage [5], and only asexual stages of *P. japonicus* Matsush. and *P. pulcher* have been discovered [4,40]; the rest species of the genus are holomorphic, including the newly added one.

Historically, nectriaceous species producing small, reddish, smooth, thin-walled perithecia were categorized as *Cosmospora* Rabenh. *sensu lato* [1]. The accumulated morpho-

logical and phylogenetic information indicated that the genus was not monophyletic [41,42]. Herrera et al. [6] established *Pseudocosmospora* to accommodate ten cosmospora-like fungi on *Eutypa* and *Eutypella* and with acremonium- to verticillium-like asexual stages. Since then, six additional taxa have joined the group [7–9]. The genus has become distributed worldwide and displays high species diversity in warm temperate and tropical regions [6]. Species of the genus have the following features in common: they are superficial perithecia, gregarious, KOH+, LA+, laterally collapsed upon drying, and usually less than 250 µm in height; and they have asci containing eight 1-septate ascospores, acremonium- to verticillium-like conidiophores, and non-septate conidia. *Pseudocosmospora beijingensis* fits well the generic concept. The multigene analyses indicated its distinctions from any other species of the genus (Figure 2).

Members of *Thelonectria* are often found on twigs and branches, trunks of recently killed or dying trees, and rotting roots; they occasionally cause small cankers and are mainly distributed in tropical, subtropical, and temperate regions [10,11,17]. Among the species of the genus, *T. coronata* (Penz. & Sacc.) P. Chaverri & C. Salgado, *T. discophora, T. lucida* (Höhn.) P. Chaverri & C. Salgado, and *T. veuillotiana* (Roum. & Sacc.) P. Chaverri & Salgado are cosmopolitan and are treated as species complexes [38,43,44]. Salgado-Salazar et al. [11–13] carried out a revisionary work on the above species complexes and described 30 cryptic species on the basis of genealogical concordance phylogenetic species recognition. Our phylogenetic results indicated that *T. globulosa* was associated with but clearly separated from members of the *T. veuillotiana* complex. *Thelonectria aurea*, known only by the asexual stage, can be easily distinguished in the absence of microconidia and chlamydospores in culture [17]. Moreover, there were 93 bp and 64 bp divergences in the ITS and tub2 regions between the ex-type culture of the two species.

There are 47 species currently known in this genus, of which 20 species have been reported in China [11,13,15]. Large-scale surveys of fungal resources in various regions with different climates, vegetation, geographic structures, and multiple niches will improve our understanding of the species diversity of nectriaceous fungi in the country.

5. Conclusions

The species diversity of the family *Nectriaceae* was investigated, and four novel taxa were discovered. With the joining of the new species, the phylogenetic relationships among species of these three genera were updated.

Author Contributions: Conceptualization, W.-Y.Z.; methodology, Z.-Q.Z.; software, Z.-Q.Z.; validation, Z.-Q.Z.; formal analysis, Z.-Q.Z.; investigation, Z.-Q.Z.; resources, W.-Y.Z. and Z.-Q.Z.; data curation, Z.-Q.Z.; writing—original draft preparation, Z.-Q.Z.; writing—review and editing, W.-Y.Z. and Z.-Q.Z.; visualization, Z.-Q.Z.; supervision, Z.-Q.Z. and W.-Y.Z.; project administration, W.-Y.Z.; funding acquisition, W.-Y.Z. and Z.-Q.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (31750001, 31870012) and the Frontier Key Program of the Chinese Academy of Sciences (QYZDY-SSW-SMC029).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Names of the new species were formally registered in the database Fungal Names (https://nmdc.cn/fungalnames (accessed on 22 August 2022)). Specimens were deposited in the Herbarium Mycologicum Academiae Sinicae (HMAS). The newly generated sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank (accessed on 5 October 2022)).

Acknowledgments: The authors would like to thank Huan-Di Zheng, Xin-Cun Wang, Yu-Bo Zhang, Chao Wang, and Peng Li for collecting specimens jointly and Ting Huang for technique help with this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

- 1. Rossman, A.Y.; Samuels, G.J.; Rogerson, C.T.; Lowen, R. Genera of *Bionectriaceae*, *Hypocreaceae*, and *Nectriaceae* (*Hypocreales*, *Ascomycetes*). *Stud. Mycol.* **1999**, 42, 1–260.
- Lombard, L.; van der Merwe, N.A.; Groenewald, J.Z.; Crous, P.W. Generic concepts in *Nectriaceae*. *Stud. Mycol.* 2015, *80*, 189–245. [CrossRef] [PubMed]
- 3. Hyde, K.D.; Norphanphoun, C.; Maharachchikumbura, S.S.N.; Bhat, D.J.; Jones, E.B.G.; Bundhun, D.; Chen, Y.J.; Bao, D.F.; Boonmee, S.; Calabon, M.S.; et al. Refined families of Sordariomycetes. *Mycosphere* **2020**, *11*, 305–1059. [CrossRef]
- 4. van Emden, J.H. Penicillifer, a new genus of Hyphomycetes from soil. Acta Bot. Neerl. 1968, 17, 54–58. [CrossRef]
- Crous, P.W.; Shivas, R.G.; Quaedvlieg, W.; van der Bank, M.; Zhang, Y.; Summerell, B.A.; Guarro, J.; Wingfield, M.J.; Wood, A.R.; Alfenas, A.C.; et al. Fungal Planet description sheets: 214–280. *Persoonia* 2014, 32, 184–306. [CrossRef] [PubMed]
- 6. Herrera, C.S.; Rossman, A.Y.; Samuels, G.J.; Chaverri, P. *Pseudocosmospora*, a new genus to accommodate *Cosmospora vilior* and related species. *Mycologia* **2013**, *105*, 1287–1305. [CrossRef] [PubMed]
- 7. Zeng, Z.Q.; Zhuang, W.Y. Eight new combinations of *Bionectriaceae* and *Nectriaceae*. Mycosystema 2017, 36, 278–281.
- Zeng, Z.Q.; Zhuang, W.Y. Our current understanding of the genus *Pseudocosmospora* (*Hypocreales*, *Nectriaceae*) in China. *Mycol.* Prog. 2021, 20, 419–429. [CrossRef]
- Lechat, C.; Fournier, J. Pseudocosmospora hypoxylicola (Nectriaceae), a new species from the French Alps. Ascomycete.org 2020, 12, 6–10.
- Chaverri, P.; Salgado, C.; Hirooka, Y.; Rossman, A.Y.; Samuels, G.J. Delimitation of *Neonectria* and *Cylindrocarpon* (*Nectriaceae*, *Hypocreales*, *Ascomycota*) and related genera with *Cylindrocarpon*-like anamorphs. *Stud. Mycol.* 2011, *68*, 57–68. [CrossRef] [PubMed]
- 11. Salgado-Salazar, C.; Rossman, A.Y.; Chaverri, P. The genus *Thelonectria* (*Nectriaceae, Hypocreales, Ascomycota*) and closely related species with *Cylindrocarpon*-like asexual states. *Fungal Divers.* **2016**, *80*, 411–455. [CrossRef]
- 12. Salgado-Salazar, C.; Rossman, A.; Samuels, G.J.; Capdet, M.; Chaverri, P. Multigene phylogenetic analyses of the *Thelonectria* coronata and *T. veuillotiana* species complexes. *Mycologia* **2012**, *104*, 1325–1350. [CrossRef] [PubMed]
- Salgado-Salazar, C.; Rossman, A.Y.; Samuels, G.J.; Hirooka, Y.; Sanchez, R.M.; Chaverri, P. Phylogeny and taxonomic revision of Thelonectria discophora (Ascomycota, Hypocreales, Nectriaceae) species complex. Fungal Divers. 2015, 70, 1–29. [CrossRef]
- Zeng, Z.Q.; Zhuang, W.Y. Four new taxa of *Ilyonectria* and *Thelonectria* (*Nectriaceae*) revealed by morphology and combined ITS and β-tubulin sequence data. *Phytotaxa* 2013, 85, 15–25. [CrossRef]
- 15. Zeng, Z.Q.; Zhuang, W.Y. The genera *Rugonectria* and *Thelonectria* (*Hypocreales*, *Nectriaceae*) in China. *MycoKeys* **2019**, *55*, 101–120. [CrossRef]
- 16. Crous, P.W.; Luangsa-ard, J.J.; Wingfield, M.J.; Carnegie, A.J.; Hernández-Restrepo, M.; Lombard, L.; Roux, J.; Barreto, R.W.; Baseia, I.G.; Cano-Lira, J.F.; et al. Fungal Planet description sheets: 785–867. *Persoonia* **2018**, *41*, 238–417. [CrossRef]
- 17. Lawrence, D.P.; Nouri, M.T.; Trouillas, F.P. Taxonomy and multi-locus phylogeny of cylindrocarpon-like species associated with diseased roots of grapevine and other fruit and nut crops in California. *Fungal Syst. Evol.* **2019**, *4*, 59–75. [CrossRef]
- Braun, U.; Bensch, K. Annotated list of taxonomic novelties published in "Fungi Rhenani Exsiccati" Supplementi Fasc. 1 to 5, issued by K. W. G. L. Fuckel between 1865 and 1867. *Schlechtendalia* 2020, 37, 80–133.
- 19. Nirenberg, H.I. Studies on the morphologic and biologic differentiation in *Fusarium* section *Liseola*. *Mitt. Biol. Bundesanst. Land-Forstw.* **1976**, *169*, 1–117.
- 20. Samuels, G.J.; Dodd, S.; Lu, B.S.; Petrini, O.; Schroers, H.J.; Druzhinina, I.S. The *Trichoderma koningii* aggregate species. *Stud. Mycol.* **2006**, *56*, 67–133. [CrossRef]
- White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications; Innis, M.A., Gelfland, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
- 22. Vilgalys, R.; Hester, M. Rapid genetic identification and mapping enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* **1990**, 172, 4238–4246. [CrossRef] [PubMed]
- Rehner, S.A.; Samuels, G.J. Taxonomy and phylogeny of *Gliocladium* analyzed from nuclear large subunit ribosomal DNA sequences. *Mycol. Res.* 1994, 98, 625–634. [CrossRef]
- Matheny, P.B.; Liu, Y.J.; Ammirati, J.F.; Hall, B.D. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). Am. J. Bot. 2002, 89, 688–698. [CrossRef] [PubMed]
- Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic relationships among ascomycetes: Evidence from an RNA Polymerase II Subunit. *Mol. Biol. Evol.* 1999, 16, 1799–1808. [CrossRef] [PubMed]
- Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 1999, 91, 553–556. [CrossRef]
- O'Donnell, K.; Kistler, H.C.; Cigelnik, E.; Ploetz, R.C. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 1998, 95, 2044–2049. [CrossRef]
- 28. O'Donnell, K.; Cigelnik, E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* **1997**, *7*, 103–116. [CrossRef]

- Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 1999, 41, 95–98.
- Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgin, D.G. The ClustalX windows interface: Flexible strategies for multiple sequences alignment aided by quality analysis tools. *Nucleic Acids Res.* 1997, 25, 4876–4883. [CrossRef]
- 31. Swofford, D.L. PAUP 4.0b10: Phylogenetic Analysis Using Parsimony (* and Other Methods); Sinauer Associates: Sunderland, MA, USA, 2002.
- 32. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003, *19*, 1572–1574. [CrossRef]
- Nylander, J.A.A. MrModeltest v2. Program Distributed by the Author; Evolutionary Biology Centre, Uppsala University: Uppsala, Sweden, 2004.
- Nguyen, L.T.; Schmidt, H.A.; von Haeseler, A.; Minh, B.Q. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Mol. Biol. Evol.* 2015, 32, 268–274. [CrossRef] [PubMed]
- 35. Chernomor, O.; von Haeseler, A.; Minh, B.Q. Terrace aware data structure for phylogenomic inference from supermatrices. *Mol. Syst. Biol.* **2016**, *65*, 997–1008. [CrossRef] [PubMed]
- 36. Page, R.D. TreeView: An application to display phylogenetic trees on personal computers. *Comp. Appl. Biosci.* **1996**, *12*, 357–358.
- 37. Cunningham, C.W. Can three incongruence tests predict when data should be combined? *Mol. Biol. Evol.* **1997**, *14*, 733–740. [CrossRef]
- Brayford, D.; Samuels, G.J. Some didymosporous species of *Nectria* with nonmicroconidial *Cylindrocarpon* anamorphs. *Mycologia* 1993, 85, 612–637. [CrossRef]
- Turland, N.J.; Wiersema, J.H.; Barrie, F.R.; Greuter, W.; Hawksworth, D.L.; Herendeen, P.S.; Knapp, S.; Kusber, W.H.; Li, D.Z.; Marhold, K.; et al. *International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code)*; Koeltz Scientific Books: Berlin/Heidelberg, Germany, 2018; pp. 1–254.
- 40. Matsushima, T. Matsushima Mycological Memoirs No 4; Published by the author; Kobe, Japan, 1985; pp. 1–68.
- Zhang, X.M.; Zhuang, W.Y. Phylogeny of some genera in the *Nectriaceae* (Hypocreales, Ascomycetes) inferred from 28S nrDNA partial sequences. *Mycosystema* 2006, 25, 15–22.
- 42. Luo, J.; Zhuang, W.Y. *Chaetopsinectria* (*Nectriaceae*, *Hypocreales*), a new genus with *Chaetopsina* anamorphs. *Mycologia* **2010**, 102, 976–984. [CrossRef]
- 43. Samuels, G.J.; Doi, Y.; Rogerson, C.T. Contributions toward a mycobiota of Indonesia: *Hypocreales. Mem. N. Y. Bot. Gard.* **1990**, *59*, 6–108.
- 44. Samuels, G.J.; Brayford, D. Species of Nectria (sensu lato) with red perithecia and striate ascospores. Sydowia 1994, 46, 75–161.