



A new species of *Stephanonectria* (*Bionectriaceae*) from southwestern China

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Abstract

A new species, *Stephanonectria ellipsoidea*, was isolated from decaying fruit in Kunming City, Yunnan Province, China. It is characterized by pale orange colonies, densely clustered, irregularly branched conidiophores, cylindrical phialides, and hyaline, ellipsoidal conidia. Its morphology fits well with the generic concept of *Stephanonectria*. This species is closely related to *S. keithii* based on phylogenetic analysis of concatenated LSU and ITS sequences. The combination of morphological observation and molecular data supports the erection of the new species.

Keywords – Asexual morph – Fruits – Hypocreales – New species

Introduction

Bionectriaceae (*Hypocreales*) was introduced by Rossman et al. (1999) and typified with *Bionectria*. The sexual-aseexual connection between *Bionectria* and *Clonostachys* has been monographed by Schroers (2001). *Bionectriaceae* species were commonly encountered in soil or associated with bryophytes as symbionts (Döbbeler 2004), with plants as endophytes (White et al. 1993), epiphytes, saprotrophs (Torcato et al. 2020), and with lichen as parasites (Farkas & Flakus 2016). The sexual genus *Bionectria* subsequently was considered a synonym of the asexual genus *Clonostachys* (Rossman et al. 2013). Wijayawardene et al. (2022) listed 47 genera in *Bionectriaceae*, of which 14 genera (*Acremonium*, *Bryocentria*, *Clonostachys*, *Fusariella*, *Geosmithia*, *Gliomastix*, *Hydropisphaera*, *Ijuhya*, *Lasionectria*, *Nectriella*, *Nectriopsis*, *Pronectria*, *Protocreopsis*, *Trichonectria*) have more than ten species. Most genera in *Bionectriaceae* contain species with insufficient molecular data (Hyde et al. 2020a). Before the molecular era, the identification of nectriaceous fungi was based on the nature of stroma, perithecial surface characteristics and ascospore morphology (Forin et al. 2020). *Bionectriaceae* has typical features of nectriaceous fungi, but it can be separated from *Nectriaceae* by having white to orange or brown perithecia, which do not change colour in 3% potassium hydroxide (KOH) or 100% lactic acid (LA) (Rossman et al. 1999), while that of the latter family are orange to red and turning dark red or purple in KOH and yellow in LA (Hirooka et al. 2010). *Bionectriaceae* has been described as

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having ascomata that are perithecial or cleistothecial, superficial on the substrate or embedded in poorly or well-developed stroma, clavate to cylindrical asci, and ascospores ranging from aseptate to multi-septate, globose, fusiform to ellipsoidal (Hyde et al. 2020b). Several conidiophore structures, including acromonium-like, gliocladium-like or penicillium-like, have been recorded from *Bionectriaceae* (Hyde et al. 2020a). Generally, the establishment of the new genus and species in *Bionectriaceae* was based on molecular data: *act-rpb1*-LSU (Hirooka et al. 2010), ITS-*tub-rpb2-tefla* (Grum-Grzhimaylo et al. 2013), LSU, *rpb1*, *rpb2*, *tefla* (Voglmayr & Jaklitsch 2019), individual ITS (Forin et al. 2020), LSU-ITS (Trovão et al. 2022).

Stephanonectria was introduced by Schroers (1999) to accommodate *S. keithii* which was initially recognized as a member of *Nectria*, but this species was excluded from *Nectria* because of its crown-like structure around the ostiole (Schroers 1999). *Stephanonectria keithii* has been phylogenetically confirmed as a member of *Bionectriaceae* by several studies (Castlebury et al. 2004, Supaphon et al. 2017, Zeng & Zhuang 2018). *Stephanonectria* is characterised by forming brown, smooth, solitary to crown perithecia on flat superficial to erumpent stroma (Schroers et al. 1999). Perithecia are subglobose with slightly flat apex where the crowns are produced surrounding the ostioles. The colour of perithecia does not change in KOH. Asci are clavate and contain uniseriate to biseriate ascospores that are hyaline, ellipsoidal, 1–2-celled, and longitudinally oriented striae (Schroers et al. 1999).

In this study, dried fruits colonized by white to pale orange mycelia were collected from a forest in Yunnan Province, China. This isolate was identified as a new species of *Stephanonectria* on the basis of molecular and morphological evidence.

Materials & Methods

Collection, isolation and morphological study

Samples were surveyed from a deciduous forest near Songhuaba Reservoir in Kunming City, Yunnan Province, China. The specimens were collected into zip-lock bags and transported to the laboratory for further examination. The fungal colonies on dried fruit were observed using a Nikon SMZ 745T dissecting microscope. A small mass of mycelia was mounted in water on a slide using a sterile needle. A NIKON ECLIPSE Ni-U compound microscope was used to observe conidiophores and conidia. The micro-morphological images were photographed using a DS-Ri2 camera fitted onto the compound microscope. The images used for the figure were processed with Adobe Photoshop. Pure cultures were obtained via single spore isolation using potato dextrose agar (PDA) as described in Senanayake et al. (2020) and incubated at 25 °C for one week. The living cultures were deposited in the Kunming Institute of Botany Culture Collection (KUNCC). The dried specimens were deposited in the Herbarium of Cryptogamic Kunming Institute of Botany Academia Sinica (KUN) at the Chinese Academy of Sciences, Kunming, China. Facesoffungi and Index Fungorum numbers were registered following the protocol described in Jayasiri et al. (2015) and Index Fungorum (2023), respectively.

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelia growing on a PDA plate using TriliefTM Plant Genomic DNA Kit (Tsingke biological technology Co., LTD, Beijing, China), following the instructions of the manufacturer. Primers ITS5/ITS4 (White et al. 1990) and LROR/LR7 (Vilgalys & Hester 1990) were used for the amplification of the ITS and LSU sequences, respectively. Polymerase chain reaction (PCR) was performed in a 25 µl reaction volume containing 21 µl Taq PCR Master Mix (TSINGKE TSE101, Tsingke Biotechnology Co., Ltd., Beijing, China), 1 µl of each primer, and 2 µl of DNA template. PCR conditions of ITS and LSU were as follows: initialization for 5 min at 98 °C, followed by 40 cycles of denaturation for 30 s at 98 °C, annealing for 40 s at 53 °C and elongation for 30 s at 72 °C, and final extension for 10 min at 72 °C. The PCR products were visualized using agarose gel electrophoresis, and those with the targeted bands were sent to Tsingke

Biotech Co. Ltd., Kunming, China, for sequencing. The newly generated sequences were submitted to GenBank for assignment of accession numbers.

Sequence alignment and phylogenetic analyses

The raw sequences were assembled with Sequencing Project Management (SeqMan) (Clewley 1995). The assembled sequences were compared with the data in GenBank to determine their close relatives. The blast result shows that our specimens have a close affinity with species of *Bionectriaceae*. Reference sequences representing 11 genera of *Bionectriaceae* were sampled following Perera et al. (2023) and are presented in Table 1. Each gene matrix was independently aligned with MAFFT v 6.8 (Katoh et al. 2019). The aligned datasets were manually edited using BioEdit v. 7.0.9 (Hall 1999) and combined with SequenceMatrix v. 1.7.8 (Vaidya et al. 2011). The combined alignment was used for maximum likelihood (ML) and Bayesian inference (BI) analyses.

Maximum likelihood analysis was performed using RAxML-HPC2 on XSEDE (8.2.10) in CIPRES Science Gateway V. 3.3 (Miller et al. 2010), employing default parameters but with the following adjustments: bootstrap iterations were set to 1000, and the substitution model was set to GTR+GAMMA+I. Mrmodeltest v. 2.3 was used to select the best model for each gene. Bayesian Inference analysis was carried out in MrBayes v3.2.6 (Ronquist et al. 2012). Six simultaneous Markov Chain Monte Carlo (MCMC) chains were run for 500,000 generations, and trees were sampled at every 1000th generation until the standard deviation of the split frequencies fell below 0.01. The phylogenetic trees were summarized, and posterior probabilities (PP) were calculated by discarding the first 25% of generations as the burn-in phase (Huelsenbeck & Ronquist 2001). The phylogenetic tree was visualized using FigTree v.1.4.0 and edited with Adobe Illustrator 2020 (Adobe Systems Incorporated, America).

Results

Phylogenetic analyses

The combined ITS and LSU dataset comprised 43 taxa, with *Calcarisporium arbuscula* (CBS:900.68), *C. arbuscula* (CBS:552.66) and *C. xylariicola* (IT 1264) as the outgroup taxa. The dataset consisted of 1307 total characters, including gaps (LSU: 1–805 bp and ITS: 806–1307). The matrix had 438 distinct alignment patterns, with 22.21% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.239146, C = 0.244522, G = 0.291527, T = 0.224805; substitution rates: AC = 2.292484, AG = 3.067293, AT = 2.505902, CG = 1.061138, CT = 8.264153, GT = 1.000000; gamma distribution shape parameter $\alpha = 0.621905$. The best-scoring RAxML tree with a final likelihood value of -7395.876990 is presented in Fig. 1. The tree topology obtained from ML analysis is similar to the one inferred from BI analysis. Our isolates formed a distinct clade sister to *Stephanonectria keithii* with 99% ML bootstrap support and 1.00 posterior probability support (Fig. 1).

Table 1. GenBank accession numbers of the taxa used in the phylogenetic analyses in this study.

Taxon name	Culture collection number	GenBank accession numbers	
		LSU	ITS
<i>Calcarisporium arbuscula</i>	CBS:900.68	MH870978	MH859249
<i>C. arbuscula</i>	CBS:552.66	MH870538	MH858880
<i>C. xylariicola</i>	IT 1264	KX442601	KX442603
<i>Clonostachys agarwalii</i>	CBS:533.81	N/A	MH861375
<i>C. agarwalii</i>	MFLU:19-0976	OM276821	OM276726
<i>C. aureofulvella</i>	CBS 195.93	N/A	AF358226
<i>C. araneorum</i>	GZAC-QLS0625	N/A	KT895417
<i>C. buxi</i>	CBS 696.93	KM231721	KM231840

Table 1. Continued.

Taxon name	Culture collection number	GenBank accession numbers	
		LSU	ITS
<i>C. candelabrum</i>	P6634	MN308617	MN310304
<i>C. candelabrum</i>	P6636	MN308618	MN310305
<i>Heleococcum aurantiacum</i>	CBS:201.35	MH867154	MH855645
<i>H. japonense</i>	CBS 397.67	JX158442	JX158420
<i>Lasionectria antillana</i>	CBS 122797	KY607552	KY607537
<i>L. krabiense</i>	MFLUCC 15-0673	MH376725	MH388352
<i>L. lecanodes</i>	NE322	N/A	MH393445
<i>L. lecanodes</i>	NE321	N/A	MH393446
<i>L. oenanthicola</i>	CBS 129747	KY607557	KY607542
<i>Lasionectriella herbicola</i>	CBS 140156	KU593582	N/A
<i>L. rubioi</i>	CBS 140157	KU593581	N/A
<i>Nectriopsis exigua</i>	CBS:126110	MH875481	MH864022
<i>N. exigua</i>	CBS:400.66	MH870474	MH858837
<i>N. fuliginicola</i>	CBS 400.82	N/A	KU382175
<i>Ochronectria calami</i>	CBS125.87	AY489717	N/A
<i>O. thailandica</i>	MFLUCC 15-0140	KU564069	KU564071
<i>Paracylindrocarpon aloicola</i>	CPC 27362	KX228328	KX228277
<i>P. nabanheensis</i>	KUMCC 16-0147	MH376730	MH388356
<i>P. pandanicola</i>	KUMCC 17-0272	MH376731	MH388357
<i>Protocreopsis caricicola</i>	CLL15081	KU198184	N/A
<i>P. euphorbiae</i>	CPC 38896	OK663739	OK664700
<i>P. korfii</i>	CBS 138733	KT852955	N/A
<i>P. pertusa</i>	C.T.R. 72-184	GQ506002	N/A
<i>P. phormiicola</i>	CBS:567.76	MH872774	MH861001
<i>Roumegueriella rufula</i>	GJS 91-164	EF469082	N/A
<i>R. rufula</i>	CBS 346.85	DQ518776	N/A
<i>Stephanonectria chromolaenae</i>	MFLUCC 18-0589	ON230059	ON230051
<i>S. ellipsoidea</i>	HKAS 124022	OP205363	OP205375
<i>S. ellipsoidea</i>	HKAS 124024	OP205362	OP205374
<i>S. keithii</i>	CBS:943.72	MH878469	N/A
<i>S. keithii</i>	CBS:434.70	MH871546	MH859783
<i>S. keithii</i>	GJS92-133	AY489727	N/A
<i>Synnemellisia acaciae</i>	BRIP 71652	N/A	OK342123
<i>S. aurantia</i>	COAD:2070	KX866396	KX866395
<i>S. urenae</i>	BRIP 71675	OK342135	N/A

Notes: The newly generated sequences are shown in red, while the type strains are in bold font. N/A means the data are not available in GenBank.

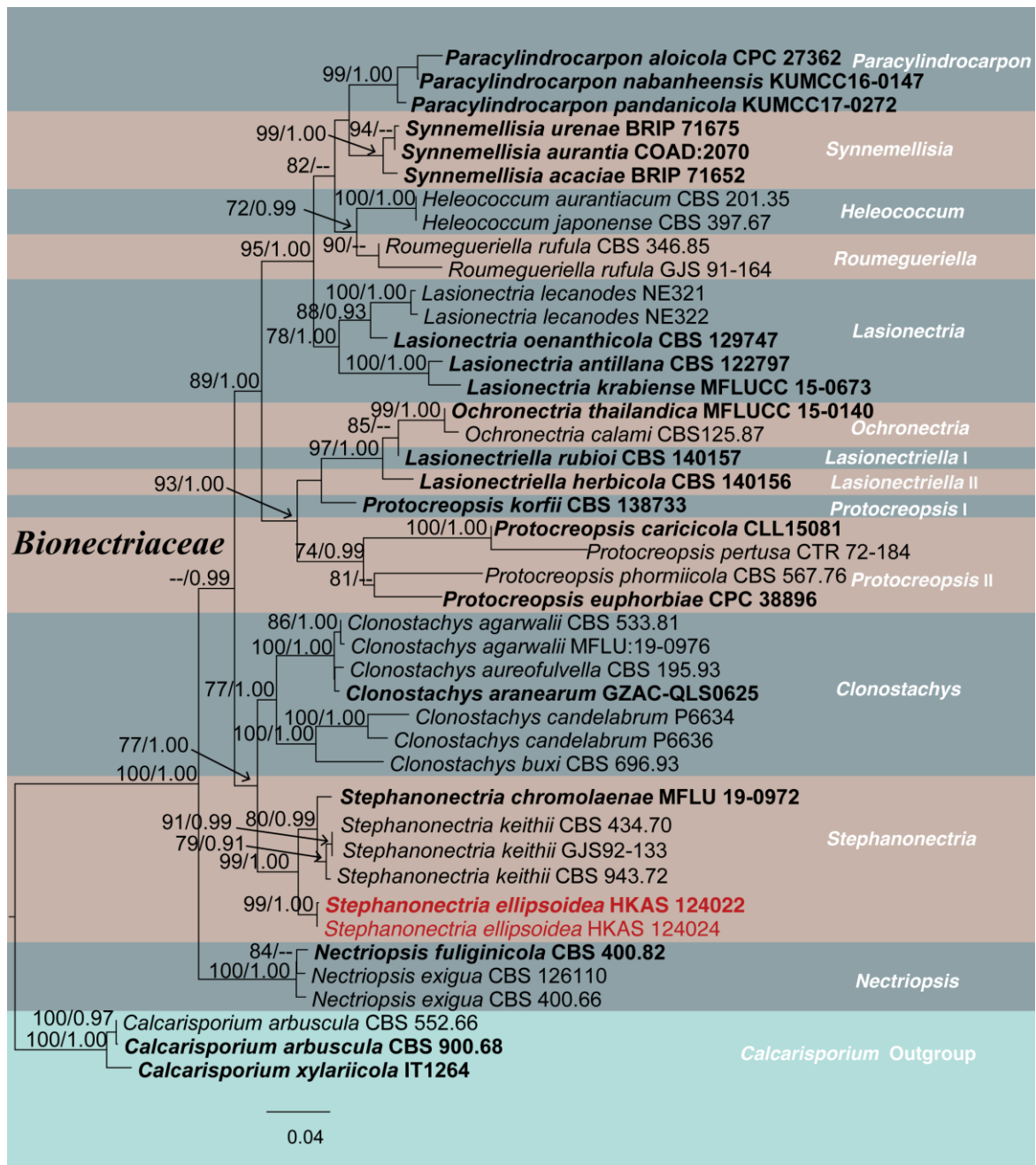


Fig. 1 – Phylogram generated from maximum likelihood analysis based on concatenated LSU and ITS sequence data. Maximum likelihood bootstrap support values (MLBS) equal to or greater than 70% and Bayesian posterior probabilities (BYPP) equal to or higher than 0.90 are placed on the nodes. The type strains are in bold, and the newly generated sequences are indicated in red bold.

Stephanonectria ellipsoidea S.C. He, D.P. Wei & R.S. Jayawardena sp. nov. Fig. 2

Index Fungorum number: IF900157; Facesoffungi number: FoF 13351

Etymology – The specific epithet refers to the ellipsoidal conidia of this fungus

Saprobic on decaying fruit of an unidentified plant. Sexual morph: Undetermined. Asexual morph: Hyphomycetous. *Colonies* on the substrate surface pale orange, irregularly shaped, effuse, dense, smooth. *Conidiophores* micronematous, penicillate, sporodochial, straight or flexuous, irregularly branched, aseptate, hyaline, smooth-walled, terminal branches developing into phialides, 67–101 × 2.4–3.3 μm (\bar{x} = 84 × 3 μm, n = 10). *Phialides* monopialidic, flask-shaped, discrete, hyaline, smooth-walled, 11.3–14.3 × 2.1–2.9 μm (\bar{x} = 12.8 × 2.5 μm, n = 20). *Conidia* solitary, acrogenous, oblong with obtuse ends, amerspores, minutely guttulate, hyaline, smooth-walled,

6.2–6.8 × 3.3–3.7 μm (\bar{x} = 6.5 × 3.5 μm, n = 30), germinating on the substrate. On PDA, *Conidiophores* monomorphic, penicillate, sporodochial, straight or flexuous, irregularly branched, smooth-walled, hyaline. *Phialides* monopialidic, flask-shaped, discrete, hyaline, 11.2–14.2 × 2.2–2.8 μm (\bar{x} = 12.7 × 2.5 μm, n = 20). *Conidia* amerospores, acrogenous, ellipsoidal, smooth-walled, guttulate, hyaline, 6.2–6.6 × 3.3–3.5 μm (\bar{x} = 6.4 × 3.4 μm, n = 30).

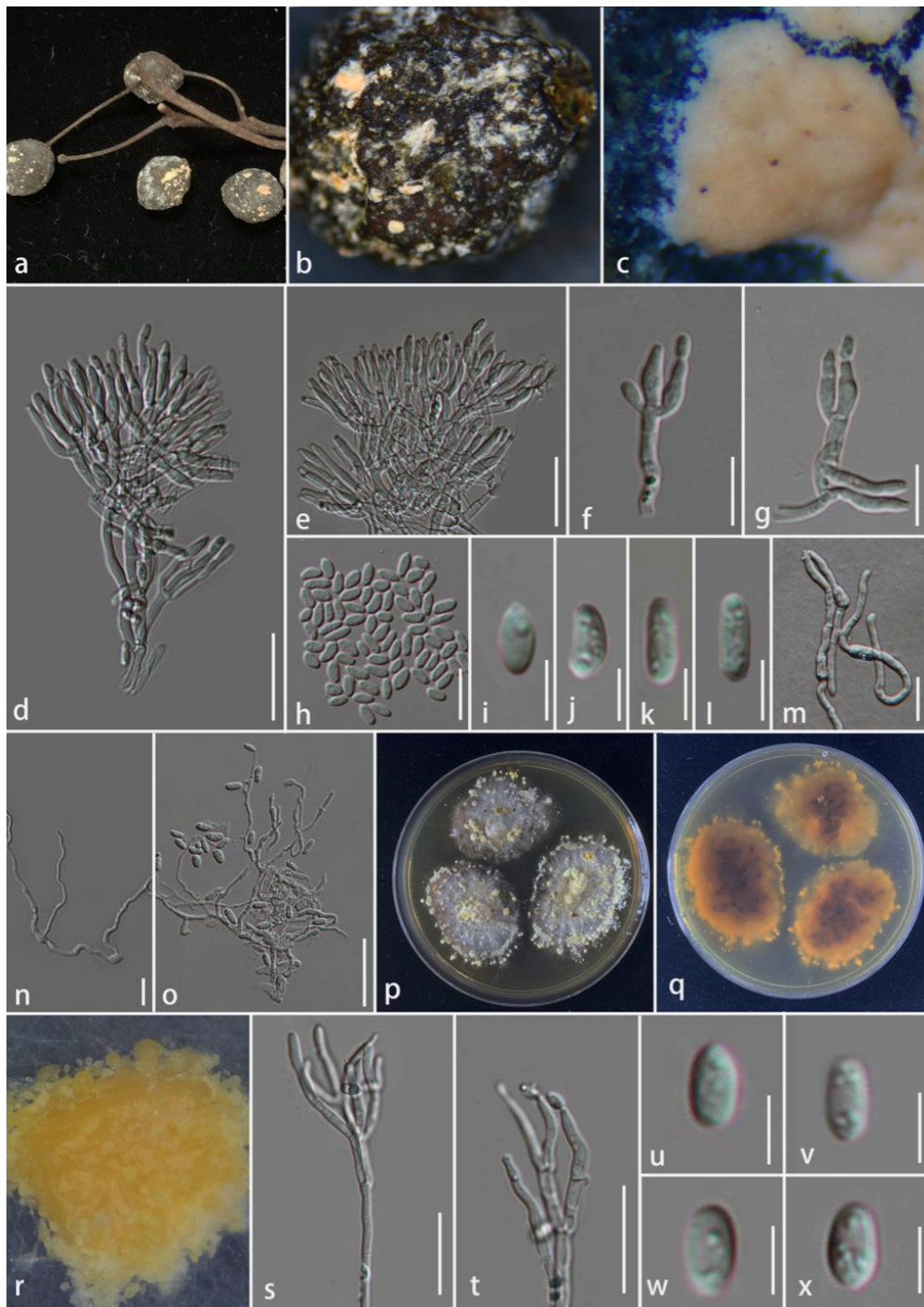


Fig. 2 – *Stephanonectria ellipsoidea* (HKAS124022, holotype). (a–o from the substrate, p–x from culture). a–c Colonies on natural substrate. d, e, s Conidiophores. f, g, t Phialides. h–l, u–x Conidia. m–o Germinating conidia. p, q Upper and lower view of culture on PDA. r Sporodochia formed on the PDA surface. Scale bars: m, o, s, t = 25 μm, d, e, h = 20 μm, f, g, n = 10 μm, i–l, u–x = 5 μm.

Culture characteristics – Conidia germinating within 12 h on the PDA media at 25 °C, reaching 2.3–2.5 cm after 20 days of incubation, colony white, nearly circular, flat, surface rough, fimbriate, mycelia medium sparse, sporodochia formed on PDA in concentric rings.

Material examined – China, Yunnan province, Kunming City, Panglong District, forest near to Songhuaba reservoir, on dried fruit of a woody plant, 1 December 2021, Shu-Cheng He, HSC203 (HKAS 124022, holotype), ex-type living culture, KUNCC 22-12394; *ibid.* HSC323 (HKAS 124024, paratype); living culture, KUNCC22-12395.

Notes – Phylogenetically, our isolates (HKAS124022) formed a monophyletic clade with *Stephanonectria keithii* and *S. chromolaenae* with 99% ML bootstrap support and 1.00 posterior probability support (Fig. 1). *Stephanonectria ellipsoidea* morphologically resembles *S. keithii* and *S. chromolaenae* in having penicillate conidiophore, flask-shaped phialides and ellipsoidal conidia (Schroers et al. 1999). It is hard to distinguish *S. ellipsoidea* from *S. keithii* and *S. chromolaenae* solely based on morphological observation. However, the comparison of nucleotide sequences between our new species and *S. keithii* (CBS 434.70) shows 35 bp differences and 6 bp differences across 521 bp ITS and 795 bp LSU sequences, respectively. Our new species and *S. chromolaenae* (MFLUCC 18-0589) are different in 28 bp and 14 bp across 516 bp ITS and 795 bp LSU sequences, respectively.

Discussion

In this study, the phylogenetic analysis was conducted based on concatenated LSU and ITS sequence data of 41 ingroup taxa representing 11 genera of *Bionectriaceae*. The other 36 genera of this family have not been included in this phylogenetic analysis due to the lack of molecular sequence data. Even though the generic relationship in this tree was well supported, *Lasionectriella* and *Protocreopsis* were polyphyletic groups. The asexual morph has been observed from 11 genera of *Bionectriaceae*. They are *Bullanockia* (Crous et al. 2016), *Clonostachys* (Hyde et al. 2020a, Torcato et al. 2020), *Heleococcum* (Bilanenko et al. 2005), *Lasionectriella* (Lechat & Fournier, 2016), *Nectriella* (Crous et al. 2022), *Paracylindrocarpon* (Tibpromma et al. 2018), *Protocreopsis* (Crous et al. 2021), *Stephanonectria* (Schroers et al. 1999), *Stromatonectria* (Jaklitsch & Voglmayr 2011), and *Xanthonectria* (Lechat et al. 2016). Morphologically, *Stephanonectria* is similar to *Gliocladium* and *Clonostachys* (Schroers et al. 1999). This indicates that molecular data and morphological features of fresh collections are needed to determine the natural classification of taxa in *Bionectriaceae*.

Stephanonectria has been reported in hosts, including *Brassica* sp, soil and dead stem of *Chromolaena odorata* (*Asteraceae*), mainly distributed in Europe (England, France), Japan, New Zealand, Netherlands and Thailand (Perera et al. 2023, Schroers et al. 1999). In this study, we collected a saprobic *S. ellipsoidea* that occurs on dried fruit from a deciduous forest plant in Yunnan Province, China. Morphologically, our isolate is characterized by forming penicillate conidiophores that aggregate in clusters forming superficial sporodochia with pale orange mucoid spore masses. The phialides are monophialidic with minute collarete, cylindrical, smooth-walled, and tapering toward the apex. These features have been reported in *Clonostachys*, *Protocreopsis* and *Stephanonectria*. However, the phylogenetic analysis supports our isolates having a close relationship with *Stephanonectria*. Thus, we determine our isolate as a new species of *Stephanonectria*.

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