Studies in Fungi 5(1): 358–367 (2020) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/sif/5/1/17

Bionectria pseudochroleuca, a new host record on *Prunus* sp. in northern Thailand

Huanraluek N[1], Jayawardena RS[1,2], Aluthmuhandiram JVS [1, 2,3], Chethana KWT[1,2] and Hyde KD[1,2,4*]

[1]*Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[2]*School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[3]*Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, People's Republic of China*
4Kunming Institute of Botany, Chinese Academy of Science, Kunming 650201, Yunnan, China

Huanraluek N, Jayawardena RS, Aluthmuhandiram JVS, Chethana KWT, Hyde KD 2020 – *Bionectria pseudochroleuca*, a new host record on *Prunus* sp. in northern Thailand. Studies in Fungi 5(1), 358–367, Doi 10.5943/sif/5/1/17

Abstract

This study presents the first report of *Bionectria pseudochroleuca* (Bionectriaceae) on *Prunus* sp. (Rosaceae) from northern Thailand, based on both morphological characteristics and multilocus phylogenetic analyses of internal transcribe spacer (ITS) and Beta-tubulin (TUB2).

Key words – Bionectriaceae – *Clonostachys* – Hypocreales – *Nectria* – *Prunus* spp. – Sakura

Introduction

Bionectriaceae are commonly found in soil, on woody substrates and on other fungi (Rossman et al. 1999, Schroers 2001). *Bionectria* is a member of Bionectriaceae (Rossman et al. 2013, Maharachchikumbura et al. 2015, 2016) and is distinct from other genera in the family as it has characteristic ascospores and ascus morphology, but none of these are consistently found in all *Bionectria* species (Schroers 2001). Some species of this genus such as *B. tonduzii* occur on living plant material (Spegazzini 1919). The species identification remained doubtful and subsequent authors considered *Bionectria* as a synonym of *Nectria*. However, the type species *B. tonduzii* Speg. was never recollected, and the plant-parasitic life-style of the genus was not considered as a significant character for generic delimitation of hypocrealean fungi (Müller & von Arx 1962, Samuels 1988). Further studies were undertaken and found that *Nectria*-like species are distributed in three families of *Hypocreales*: Hypocreaceae, Nectriaceae and Bionectriaceae (Rossman et al. 1999). Recognition of *Bionectria* and its link to *Clonostachys* is based on the similarities between perithecia of *B. tonduzii* and *B. ochroleuca* / *C. rosea* and related species (Dingley 1957). *Bionectria pseudochroleuca* (previously known as *Clonostachys pseudochroleuca*) are common soil fungi and isolated as endophytes, epiphytes, saprotrophs and mycoparasites (Schroers 2001, Moreira et al. 2016). Distinguishing characteristics of the species *B. pseudochroleuca* such as penicillate conidiophores and imbricate conidia held in columns are presented based on the asexual morphs (Schroers 2001, Rossman et al. 2013).

Sakura or cherry blossoms (*Prunus* spp.) are flowering plants that produce stone fruits and are widely distributed in China, Japan, Korea, Myanmar, Taiwan and Thailand. There are many fungal species associated with these plants as endophytes (*Dactylaria* spp. and *Diaporthe* spp.) and

Submitted 7 March 2020, Accepted 12 June 2020, Published 22 June 2020
Corresponding Author: K.D. Hyde – e-mail – kdhyde3@gmail.com

pathogens (Botryosphaeriaceae spp., *Colletotrichum* spp., *Diaporthe* spp., *Fusarium* spp. and *Phomopsis* spp.) (Santos & Phillips 2009, Pérez et al. 2010, Gomes et al. 2013, Marek et al. 2013).

In this paper, we report *Bionectria pseudochroleuca* as the first record on *Prunus* spp. from northern Thailand. A description, photo-plate and phylogenetic analyses are provided for *B. pseudochroleuca*, which is a new host and geographical record.

Materials & Methods

Isolates and morphology

Sample collection, morphological examination and isolation

A dead branch of a *Prunus* sp. with fungal fruiting bodies was collected at Mae Fah Luang Botanical Garden, Thailand on August 2018. The specimen was placed in a plastic bag with sterilized cotton dipped in distilled water to maintain high humidity. After one day, the specimen was surface sterilized with 70% ethanol for 1 minute, 5% NaClO for 1 minute, rinsed three times in sterilized water and incubated on potato dextrose agar (PDA) at 25 °C for three days. Pure isolates on PDA plates were incubated for 7 to 10 days at 25 °C and colony morphology was recorded. Morphological observations and capturing of digital images were made following the method in Thambugala et al. (2015). The morphological characteristics were measured by using Tarosoft ® Image Frame Work software (version 0.9.7). The photomicrograph plate was prepared using Adobe Photoshop CS6 version. The culture is deposited in Mae Fah Luang University Culture Collection (MFLUCC), and the fungarium specimen is deposited in the Mae Fah Luang University Herbarium (MFLU), Thailand. Faces of Fungi (FoF) number was obtained, following Jayasiri et al. (2015).

DNA extraction, PCR amplification and sequencing

Genomic DNA was obtained from a pure culture using a Qiagen DNA extraction kit following the protocols in the manufacturer's instructions (Qiagen, USA). The polymerase chain reactions (PCR) were carried out using two partial gene regions ITS (ITS5/ITS4, White et al. 1990) and β-tubulin (BT2A/BT2B, Glass & Donaldson 1995, O'Donnell & Cigelnik 1997, Carbone & Kohn 1999, Rehner 2001). The PCR was performed in a BIORAD 1000 Thermal Cycler in a total volume of 25 µl. PCR mixtures contained TaKaRa Ex-Taq DNA polymerase 0.3 µl, 12.5 µl of 2 × PCR buffer with 2.5 µl of dNTPs, 1µl of each primer, 9.2 µl of double-distilled water and 100–500 ng of DNA template. Giraldo et al. (2017) was followed for the thermal cycling program. The PCR products were visualized under UV light using a GelDoc XR+ Molecular Imager (Bio-Rad, Hercules, CA, USA) on 1% agarose electrophoresis gels stained with ethidium bromide. The PCR products were purified and sequenced at Beijing Biomed Gene Technology Co., Ltd, Beijing, China. All the newly generated sequences in this study were deposited in the GenBank (Table 1).

Phylogenetic analyses

Phylogenetic trees and data files were created from the combined ITS and TUB2 sequence dataset (Table 1). Sequence alignment of each gene partition was automatically aligned with MAFFT (v.7.310) (Katoh & Stanley 2016) and manually aligned wherever necessary in BioEdit version v.7.0.9.1 (Hall 1999). Two separate phylogenetic trees were constructed for topology comparison. In the CIPRES Science Gateway V. 3.3 (Miller et al. 2011), RAxML rapid bootstrapping and subsequent ML search were performed using distinct model/data partitions with joint branch length optimization. Rapid bootstrap inferences were set to 1,000 and thereafter a thorough ML search was done. All free model parameters were estimated by RAxML. Likelihood of the final tree was evaluated and optimized under GAMMA +P-Invar. Model parameters were estimated to an accuracy of 0.001 log-likelihood units. Bayesian inference analysis (BYPP) was determined by using MrBayes 3.2 on XSEDE (Ronquist et al. 2011) in the CIPRES portal (Miller et al. 2011), Simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The first 1,000 trees, representing the burn-in phase of the

analyses, were discarded, while the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree and using Adobe Illustrator CS3 software to present the tree.

Results

Phylogenetic analyses

The final alignment included 68 strains, representing Bionectriaceae. Maximum parsimony, maximum likelihood and bayesian inferences presented similar topologies in their phylogenetic trees. The phylogenetic tree (Fig. 1) was constructed through analyses of the ITS sequence data combined with TUB2 sequence data for Bionectriaceae. Single gene analyses were carried out and the topology of the tree and clade stability were compared. The best scoring tree obtained from maximum likelihood analysis received a final value of -10139.659585. The matrix had 541 distinct alignment patterns, with 35.67% of undetermined characters or gaps. Estimated base frequencies were as follows; A = 0.206628, C = 0.274931, G = 0.247047, T = 0.271394; substitution rates AC = 1.087025, AG = 3.019444, AT = 1.159304, CG = 0.589069, CT = 3.528787, GT = 1.000000; gamma distribution shape parameter alpha = 0.851856 and invar = 0.397405. Our strain *B. pseudochroleuca* (MFLUCC 19–0491) clustered with the other strains of *B. pseudochroleuca* (CBS 192.94, CBS 220.93) with high bootstrap support (99% ML/ 1.00 BYPP) confirming its phylogenetic position (Fig. 1).

Bionectriaceae Samuels & Rossman

Bionectriaceae is found as soil inhabitants, plant decomposers and endophytes in tropical and subtropical areas (Schroers 2001, Domsch et al. 2007, Lucas et al. 2014). Both sexual and asexual morphs have been recorded for the species in this family (Rossman et al. 1999, Maharachchikumbura et al. 2015, 2016).

Bionectria Speg

Bionectria (syn. *Clonostachys*) (Rossman et al. 2013, Maharachchikumbura et al. 2015, 2016, Hongsanan et al. 2017), has 42 species epithets in Index Fungorum (2020). Species of this genus have found on barks of recently dead trees, decaying leaves, rarely on lichens, frequently close to or on fungal hosts, particularly ascomycetes, or with stroma incorporating a host. The asexual morphs are often associated with sexual morphs on various decaying plant materials or obtained separately when soil-borne (Schroers 2001, Rossman et al. 2013). Some species of this genus are known as destructive mycoparasites, growing on or in the host mycelium, sometimes on animal substrata (Schroers 2001). In this study, we would like to use the name *Bionectria*, as this name is commonly used in the plant pathology associated with trunk diseases of numerous hosts. Therefore, even though *Bionectria* has been synonymized to *Clonostachys* we would retain the use of *Bionectria* to avoid confusion within the plant pathology community.

Bionectria pseudochroleuca Schroers & Samuels, Stud. Mycol. 46: 122 (2001) Fig. 2

Index Fungorum number: IF485135; Facesoffungi number: FoF06563

Saprobic on dead branch of *Prunus* sp. Sexual morph: not observed. Asexual morph: secondary conidiophores 81–141 × 2–6 μm ($\overline{x}$ = 101 × 3 μm, n = 6), solitary or aggregated, arising from strands of aerial mycelium or directly from medium, bi- to quarter verticillate terminating in moderately divergent metulate and adpressed phialides. Phialides 9–24 × 2–3 μm ($\overline{x}$ = 15 × 2 μm, n = 3), in whorls of 2–6, almost cylindrical tapering in upper part, straight to slightly curved. Conidia 4–6 × 2–3 μm ($\overline{x}$ = 5 × 3 μm, n = 10), formed by phialides on secondary conidiophores hyaline, ellipsoidal, slightly curved with one almost straight side, hilum typically laterally displaced.

Culture characteristics – Colonies reaching 25 mm diam in 16 days at 25 °C on PDA colony reverse yellowish white or pale white.

Material examined – THAILAND, Chiang Rai Province, Mae Fah Luang Botanical Garden, on dead branch of *Prunus* sp. (Rosaceae), 21 August 2018, Ruvishika S. Jayawardena, Fungarium no: MFLU 19–2644, Culture collection no: MFLUCC 19–0491.

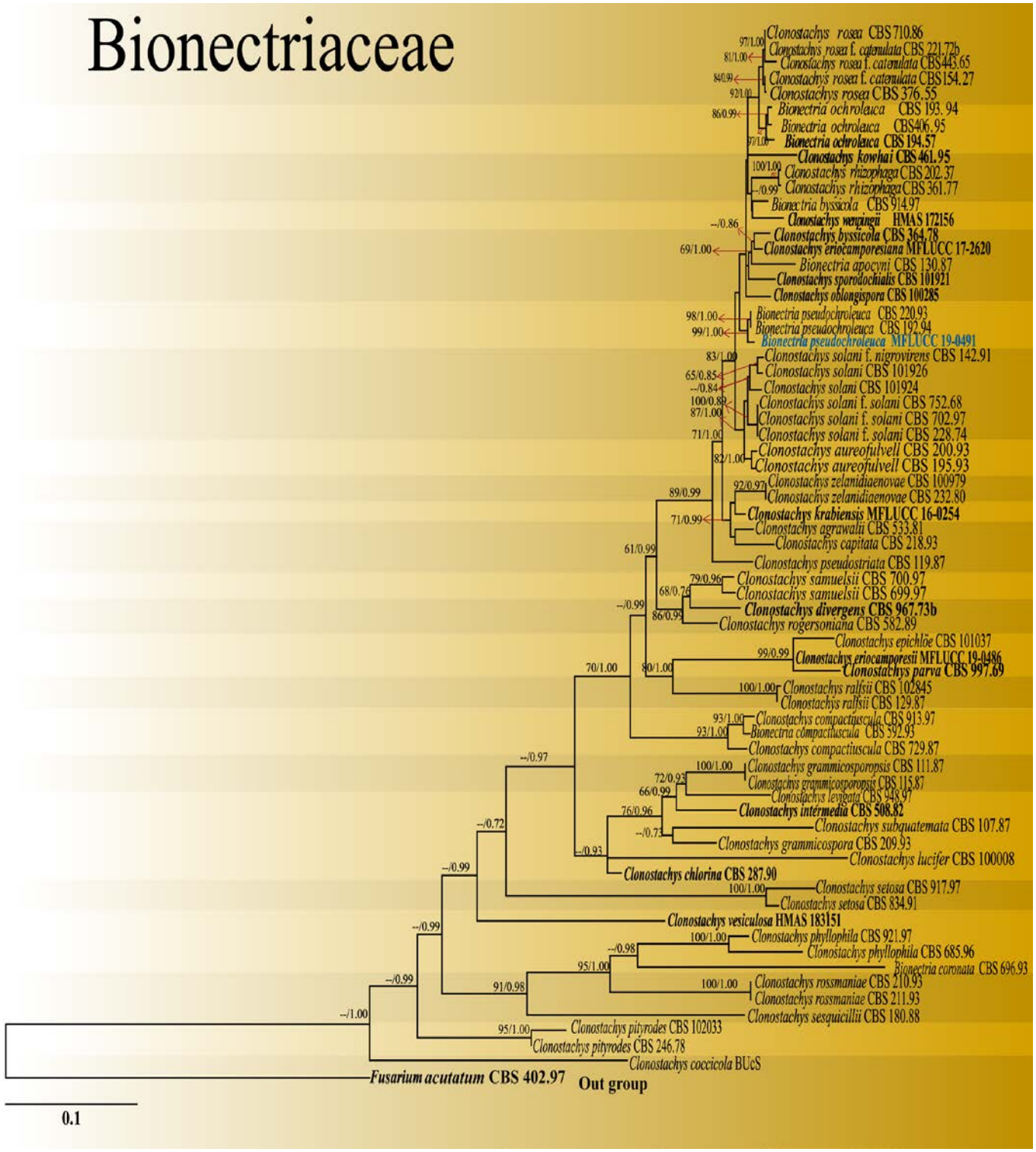

Fig. 1 – Phylogram generated from maximum likelihood analysis of combined ITS and TUB sequence data of the family Bionectriaceae. Bootstrap (ML/BYPP) support values greater than or equal 60% are given above the nodes. Culture accession number is given along with the species name, and the tree is rooted to *Fusarium acutatum* (CBS 402.97). Our stain is in blue bold and ex-types are in black bold.

Table 1 GenBank accession numbers, culture accession numbers, host information and countries reported of the taxa used in the phylogenetic analyses. Sequences generated in this study are in blue bold and ex-type strains are in black bold.

Taxa names	Culture collection	Host	Country	GenBank accession numbers	
				ITS	TUB
Bionectria apocyni	CBS 130.87	dead stem of *Apocynum cannabinum*	U.S.A, New York	AF210688	AF358168
Bionectria byssicola	CBS 914.97	*Alchorea branches*	Uganda	AF358252	AF358151
Bionectria compactiuscula	CBS 592.93	bark of *Fagus* sp.	France	AF358247	AF358192
Bionectria coronata	CBS 696.93	leaves of *Buxus sempervirens*	France	AF210667	AF358215
Bionectria ochroleuca	CBS 193.94	live rachis of *Pteridium aquilinum*	Guyana	AF210686	AF358159
Bionectria ochroleuca	CBS 406.95	bark of *Salix* sp.	France	AF358249	AF358167
Bionectria ochroleuca	**CBS 194.57**	**decaying bulb of *Lilium auratum***	**U.S.A**	**AF358237**	**AF358165**
Bionectria pseudochroleuca	CBS 192.94	decaying palm	French Guiana	AF358238	AF358171
Bionectria pseudochroleuca	CBS 220.93	palm	French Guiana	–	AF358172
Bionectria pseudochroleuca	**MFLUCC 19–0491**	***Prunus* sp.**	**Chiang Rai, Thailand**	**MN647544**	**MN688570**
Clonostachys agrawalii	CBS 533.81	decomposing buffalo horn from animal house floor sweepings	India	AF358241	AF358187
Clonostachys aureofulvella	CBS 195.93	root of tree unknown host	New Zealand	AF358226	AF358181
Clonostachys aureofulvella	CBS 200.93	palm	French Guiana	–	AF358182
Clonostachys byssicola	**CBS 364.78**	**wood**	**Venezuela**	**MH861151**	**AF358153**
Clonostachys capitata	CBS 218.93	bark unknown host	Japan	AF358240	AF358188
Clonostachys chlorina	**CBS 287.90**	**soil**	**Brazil**	**MH862212**	**–**
Clonostachys coccicola	BUcS	*Unaspis citri*	Australia	KU720552	–
Clonostachys compactiuscula	CBS 913.97	bark of dead *Fagus* sp.	U.S.A., North Carolina	AF358245	AF358194
Clonostachys compactiuscula	CBS 729.87	soil	Germany	AF358242	AF358193
Clonostachys divergens	**CBS 967.73b**	**soil**	**Germany**	**AF210677**	**AF358191**
Clonostachys epichloë	CBS 101037	*Sasa* sp.	Japan	AF210675	AF358209
Clonostachys eriocamporesiana	**MFLUCC 17-2620**	**Botryosphaeriaceae**	**Thailand**	**MN699132**	**MN699965**
Clonostachys eriocamporesii	**MFLUCC 19-0486**	***Cenchrus polystachios***	**Thailand**	**MN699133**	**–**
Clonostachys grammicospora	CBS 209.93	standing dead tree	French Guiana	AF210678	AF358206
Clonostachys gramnicosporopsis	CBS 111.87	bark of *Coprosma* sp.	New Zealand	AF358255	–
Clonostachys grammicosporopsis	CBS 115.87	bark of *Metrosideros* sp.	New Zealand	AF210679	AF358204
Clonostachys intermedia	**CBS 508.82**	**soil**	**Netherlands**	**AF210682**	**AF358205**

Table 1 Continued.

Species	Culture collection	Host	Country	GenBank accession numbers	
				ITS	TUB
Clonostachys kowhaii	**CBS 461.95**	**bark of *Sophora microphylla***	**New Zealand**	**AF358250**	**AF358170**
Clonostachys krabiensis	**MFLUCC 16-0254**	**Pandanaceae**	**Thailand**	**MH388335**	–
Clonostachys levigata	CBS 948.97	branch of dead *Buxus sempervirens*	France	AF210680	AF358196
Clonostachys lucifer	CBS 100008	Bark of recently dead *Casearia arborea*	U.S.A., Puerto Rico	AF210683	AF358208
Clonostachys oblongispora	**CBS 100285**	**bark of dying tree of *Orixa japonica***	**Japan**	**AF358248**	**AF358169**
Clonostachys parva	**CBS 997.69**	**soil**	**Netherlands**	**AF210677**	**AF358210**
Clonostachys phyllophila	CBS 685.96	–	Cuba	AF210663	–
Clonostachys phyllophila	CBS 921.97	leaves of *Viscum album*	France	AF210664	–
Clonostachys pityrodes	CBS 246.78	bark	Brazil	AF210673	–
Clonostachys pityrodes	CBS 102033	bark	Mauritius	AF210672	AF358212
Clonostachys pseudostriata	CBS 119.87	bark	Indonesia	AF358251	AF358183
Clonostachys ralfsii	CBS 129.87	bark	New Zealand	AF210676	AF358195
Clonostachys ralfsii	CBS 102845	bark	Australia, Victoria	AF358253	AF358219
Clonostachys rhizophaga	CBS 202.37	root of *Ulmus americana*	U.S.A., Ohio	AF358225	AF358156
Clonostachys rhizophaga	CBS 361.77	culture contaminant	Switzerland	AF358228	AF358158
Clonostachys rogersoniana	CBS 582.89	soil	Brazil	AF210691	AF358189
Clonostachys rosea	CBS 376.55	on *Acer palmatum*	U.S.A., Massachusett	MH857520	AF358162
Clonostachys rosea	CBS 710.86	soil, on sclerotia of *Sclerotinia minor*	Netherlands	MH862010	–
Clonostachys rosea f. *catenulata*	CBS 154.27	soil	U.S.A., Utah	MH854911	AF358160
Clonostachys rosea f. *catenulata*	CBS 221.72b	soil	Germany	AF358234	AF358203
Clonostachys rosea f. *catenulata*	CBS 443.65	soil	U.S.A., Wyoming	MH858662	AF358166
Clonostachys rosea f. *nigrovirens*	CBS 142.91	egg of *Arion ater*	Germany	AF358244	AF358178
Clonostachys rossmaniae	CBS 210.93	bark of twigs	French Guiana	AF358227	AF358213
Clonostachys rossmaniae	CBS 211.93	bark of living liana	French Guiana	MH862393	–
Clonostachys samuelsii	CBS 699.97	bark	Venezuela	AF358236	AF358190
Clonostachys samuelsii	CBS 700.97	bark	U.S.A., Puerto Rico	AF210689	–
Clonostachys sesquicillii	CBS 180.88	twigs and lichen	Guyana	AF210666	AF358214
Clonostachys setosa	CBS 834.91	*Trophis racemose*	Cuba	AF210670	AF358211
Clonostachys setosa	CBS 917.97	decaying twig	U.S.A., Puerto Rico	MH862683	–

Table 1 Continued.

Species	Culture collection	Host	Country	GenBank accession numbers	
				ITS	TUB
Clonostachys solani	CBS 101924	*Hypoxylon* sp. on bark	Jamaica	AF358232	AF358180
Clonostachys solani	CBS 101926	decaying palm inflorescence	Venezuela	AF358230	AF358179
Clonostachys solani f. solani	CBS 228.74	tuber of *Solanum tuberosum*	Netherlands	AF358243	–
Clonostachys solani f. *solani*	CBS 702.97	rotten fruit of *Aesculus hippocastanum*	France	AF210687	AF358177
Clonostachys solani f. *solani*	CBS 752.68	bark	Germany	AF358246	AF358221
Clonostachys sporodochialis	**CBS 101921**	**bark**	**U.S.A., Puerto Rico**	**AF210685**	**AF358149**
Clonostachys subquatemata	CBS 107.87	wood	Venezuela	–	AF358207
Clonostachys vesiculosa	**HMAS 183151**	**–**	**China**	**HM050304**	**–**
Clonostachys wenpingii	**HMAS 172156**	**–**	**–**	**NR_119651**	**HM054127**
Clonostachys zelanidiaenovae	CBS 232.80	bark of *Coprosma* sp.	New Zealand	AF210684	AF358185
Clonostachys zelanidiaenovae	CBS 100979	bark of *Agathis australis*	New Zealand	AF358229	–
***Fusarium acutatum* (outgroup)**	**CBS 402.97**	**–**	**India**	**MH862652**	**KU603870**

Discussion

A blast search in NCBI of our strain (MFLUCC 19–0491) showed 92.71% similarity to *Clonostachys* sp. (MH421858) in ITS and 98.31% similarity to *Bionectria pseudochroleuca* (FJ904909) in TUB2 gene regions. In the phylogenetic tree, our strain, *B. pseudochroleuca* (MFLUCC 19–0491) clustered with *B. pseudochroleuca* (CBS 192.94), (CBS 220.93) with high support (99% ML/1.00 BYPP). Our strain formed colonies with secondary conidiophores after 16 days, which is similar to the type strain of *B. pseudochroleuca*. However, our strain did not produce bright yellow to greenish yellow pigment on media, which was observed in the ex-type culture of this species (Moreira et al. 2016). There are only two records for the occurrence of *Bionectria* on *Prunus* spp. in the U.S. National Fungus Collections Fungus-Host Database (USDA, Farr & Rossman 2019). *Bionectria orcholeuca* has been recorded from *Prunus persica* in New Zealand (Gadgil 2005) and *B. sporodochialis* from *P. jamasakura* in Japan (Hirooka & Kobayashi 2007). In Thailand, six fungal species have been identified associated with *Prunus* spp. so far (Farr & Rossman 2019): *Apiosordaria striatispora* (*P. arborea*, Hyde et al. 1997), *Neofusicoccum parvum* (*P. cerasoides*, Trakunyingcharoen et al. 2015), *Passalora rubrotincta* (causing leaf spot of *P. persica*, Giatgong 1980), *Phyllosticta capotalensis* (*P. cerasoides*, Okane et al. 2003), *Podosphaera* sp. (*P. mume, P. persica*, Meeboon et al. 2016) and *Tranzchelia pruni-spinosae* (*P. persica*, Lorsuwan 1984). Therefore, to our knowledge this study provides the first host and geographical record of *B. pseudochroleuca* associated with *Prunus* spp. in Thailand.

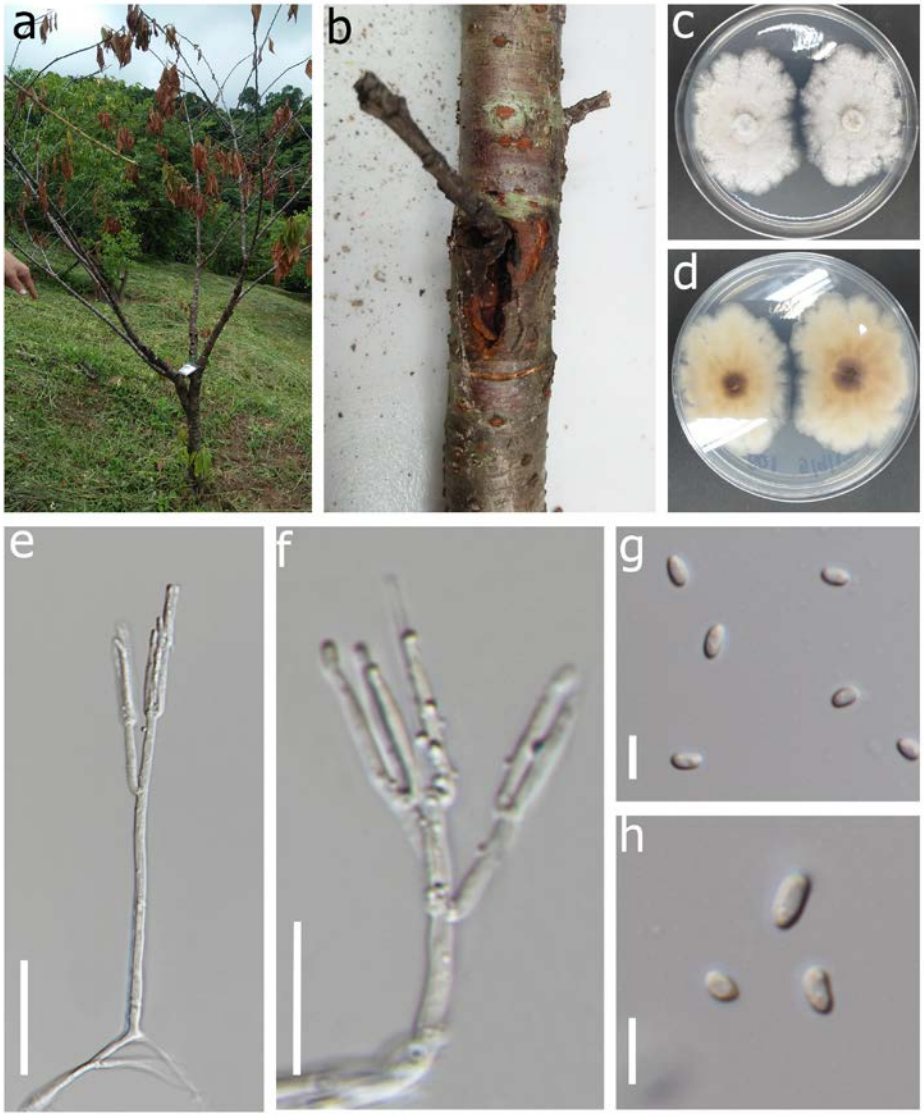

Fig. 2 – *Bionectria pseudochroleuca.* a–b Appearance on host surface. c, d Colonies on PDA 16 days old incubated at 25 °C. e, f Secondary conidiophores with appressed branches and Phialides. g, h Conidia. Scale bars: e–f = 20 µm, g–h = 5 µm.

Acknowledgements

The authors would like to thank the Mae Fah Luang University Botanical garden director J. Tovaranonte and the botanist A. Songssangchun for help with collecting the samples. Authors would like to thank the Mae Fah Luang University for providing financial support for the research work and Kevin D. Hyde would like to thank the grants entitled: 1. The future of specialist fungi in a changing climate: baseline data for generalist and specialist fungi associated with ants, *Rhododendron* species and *Dracaena* species (Grant number: DBG6080013); 2. Impact of climate change on fungal diversity and biogeography in the Greater Mekong Subregion (Grant number: RDG6130001).

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