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

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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...
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 been 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)  
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 ($\bar{x} = 101 \times 3$ 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 ($\bar{x} = 15 \times 2$ µm, $n = 3$), in whorls of 2–6, almost cylindrical tapering in upper part, straight to slightly curved. Conidia 4–6 × 2–3 µm ($\bar{x} = 5 \times 3$ 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.

**Bionectriaceae**

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**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 |
|------------------------------|--------------------|--------------------------------------|--------------------|---------------------------|
| Bionectria apocyni           | CBS 130.87         | dead stem of *Apocynum cannabinum*   | U.S.A, New York    | AF210688 AF358168         |
| Bionectria byssicolica       | CBS 914.97         | *Alchornea 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 agravalii       | 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         | palrn                               | 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 cocciola        | 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 eriocamporesia** | **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 grammicosporopsis | 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         |
| Species                        | Culture collection | Host                                      | Country                | GenBank accession numbers |
|-------------------------------|--------------------|-------------------------------------------|------------------------|--------------------------|
| *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 AF35812          |
| *Clonostachys pseudostriatia* | 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 rogersonian*    | CBS 582.89         | soil                                      | Brazil                 | AF210691 AF358189        |
| *Clonostachys rosea*          | CBS 376.55         | on *Acer palatum*                        | U.S.A., Massachusetts  | MH857520 AF358162        |
| *Clonostachys rosea*          | CBS 710.86         | soil, on sclerotia of *Sclerotinia minor* | Netherlands            | MH862010 –               |
| *Clonostachys rosea f.*       | CBS 154.27         | soil                                      | U.S.A., Utah           | MH854911 AF358160        |
| *catenulata*                  |                    |                                           |                        |                          |
| *Clonostachys rosea f.*       | CBS 221.72b        | soil                                      | Germany                | AF358234 AF358203        |
| *catenulata*                  |                    |                                           |                        |                          |
| *Clonostachys rosea f.*       | CBS 443.65         | soil                                      | U.S.A., Wyoming        | MH858662 AF358166        |
| *catenulata*                  |                    |                                           |                        |                          |
| *Clonostachys rosea f.*       | CBS 142.91         | egg of *Arion ater*                     | Germany                | AF358244 AF358178        |
| *nigrovirens*                 |                    |                                           |                        |                          |
| *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 | GenBank accession numbers |
|--------------------------------|--------------------|----------------------------------------------|------------------------|---------------------------|---------------------------|
| *Clonostachys solani*          | CBS 101924         | *Hypoxylon* sp. on bark                      | Jamaica                | AF358232                  | AF358180                  |
| *Clonostachys solani* f. solani| CBS 101926         | decaying palm inflorescence                  | Venezuela              | AF358230                  | AF358179                  |
| *Clonostachys solani* f. solani| CBS 228.74         | *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 orchroleuca* 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 *Tranzschelia 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.

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