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A new host record of *Pseudocercospora atromarginalis* on the medicinal plant *Lycium barbarum*

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ABSTRACT

Lycium barbarum is one of the two species of "gouqi" (枸杞) whose fruits are used in Traditional Chinese Medicine. A parasitic fungus, *Pseudocercospora atromarginalis*, is hitherto only once recorded from the other medicinal species of *Lycium*, *Lycium chinense*, and on further species of Solanaceae. *Lycium barbarum* is recorded here as new host species of *Ps. atromarginalis*. The identification is based on morphology and identities of sequences of the gene coding for the translation elongation factor 1 alpha (*TEF*). Too few data exist for the ca. 25 named *Pseudocercospora* species on Solanaceae to make a sound taxonomic revision. Knowledge of the potential pathogens on medicinal plants in Taiwan is scarce but indispensable for establishing domestic sustainable production and reducing the dependence from imports.

Key words: biodiversity, cercosporoid fungi, Mycosphaerellaceae

Introduction

The berries of *Lycium barbarum* L. and *Lycium chinense* Mill. (Solanaceae) are both used in Traditional Chinese Medicine and known under the Chinese name "gouqi" (枸杞). Gouqi belongs to the twenty most important medicinal plants and for the most part is imported in Taiwan from China (Liu 2017). The cercosporoid fungal pathogen *Pseudocercospora atromarginalis* (Mycosphaerellaceae) is known in Taiwan and other countries mainly on the weeds *Solanum americanum* Mill. and *S. nigrum* L., and occasionally on other hosts among Solanaceae (Braun 2017, Farr and Rossman 2022). Here, *L. barbarum* is presented as new host worldwide.

Materials and Methods

An infected plant of *Lycium barbarum* L. was purchased in 2021 as pot plant in the Jianguo Holiday Flower Market in Taipei City. Infected leaves of *Solanum americanum* Mill. were collected in the wild at roadsides in Pinglin District New of New Taipei City. In the laboratory, conidia were aseptically transferred from leaf spots to corn meal agar complemented with 0.04% chloramphenicol. Mycelium from growing cultures was used for DNA isolation and PCR as described in Yeh and Kirschner (2019). For the internal transcribed spacer of the ribosomal RNA genes (ITS), primers ITS1F and ITS4 were used (Yeh and Kirschner 2019), and for the gene cod-

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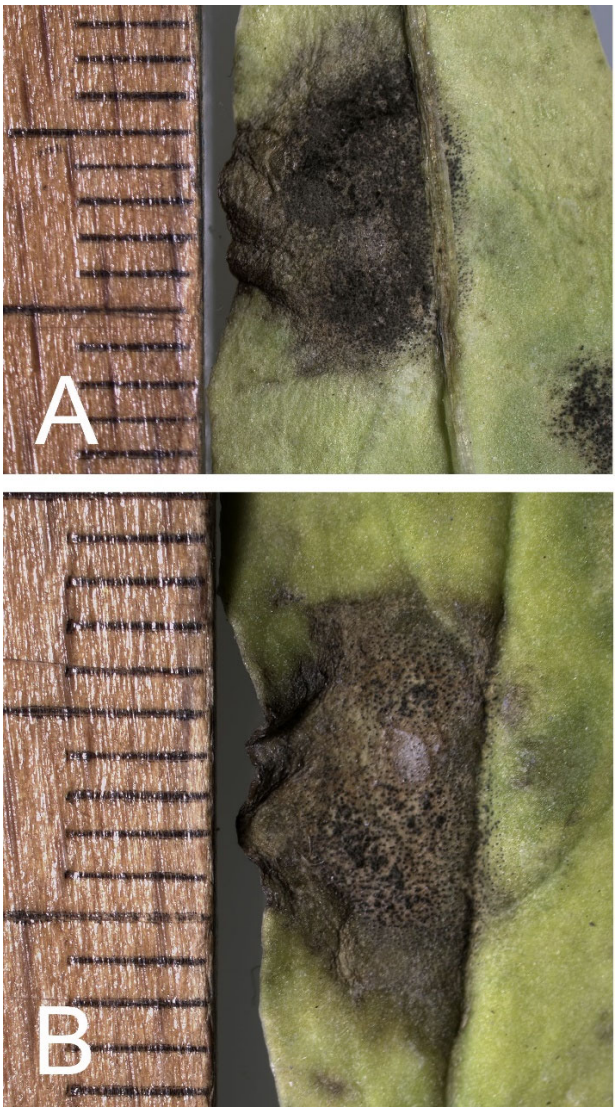

Fig. 1. Leaf spots on *Lycium barbarum* caused by *Pseudocercospora atromarginalis* (R. Kirschner 5333) with mm ruler on the left side. A. Abaxial side. B. Adaxial side.

ing for the translation elongation factor 1 alpha (*TEF*), the primer pair EF1-728F/EF1-986R was applied following Carbone and Kohn (1999). Successful PCR was verified under UV light after gel electrophoresis. PCR products were sent to Mission Biotech (Nangang) for Sanger sequencing by using the same primers as for the PCR. The forward and reverse sequences were aligned and edited in CodonCode Aligner and deposited in GenBank (ITS) and the DNA Databank of Japan (*TEF*). The sequences were also subjected to MegaBLAST searches at GenBank. A living strain of the fungus on *L. barbarum* was deposited in the Bioresource Collection and Research Center, Hsinchu (BCRC). The original specimens were dried and deposited in the National Museum of Natural Science, Taichung (TNM). For microscopy and morphological characterization, material was scraped from the leaf spots, and leaf sections were cut by hand with a razor blade. The material was mounted in water and investigated at 1000× magnification. Drawings were made by hand by using scaled paper; photographs were made with an Olympus digital microscope EP50 camera. For measuring sizes, the length and broadest width of each structure was measured in n replicates and calculated as mean ± standard deviation of measurements. Extreme values were noted in brackets. Illustrations were compiled and adjusted in Adobe Photoshop CS2.

Results and Taxonomy

Pseudocercospora atromarginalis (G. F. Atk.) Deighton, Mycol. Pap. 140: 139. 1976.

Figs. 1, 2

Associated with premature leaf dehiscence of *Lycium barbarum*. Leaf spots amphigenous, dark brown by fungal structures, while leaf tissues remaining green in small spots and becoming pale green or ochre in large leaf spots, 3–11 mm diam. External hyphae absent. Internal hyphae intercellular, smooth, hyaline with greenish droplets, or pale brown when close to stroma, 1–5 µm wide. Stromata amphigenous, subglobose to irregular, sometimes constricted between a major substomatal part and a smaller part distal to guard cells, adaxial stromata usually larger than abaxial

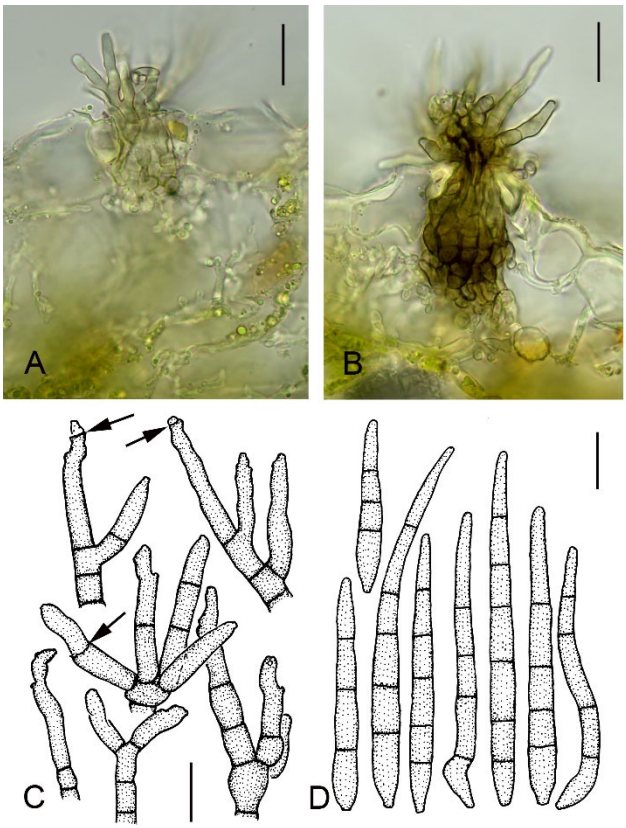

Fig. 2. Light microscopy of *Pseudocercospora atromarginalis* (R. Kirschner 5333). A, B. Hypophyllous fascicles penetrating between the guard cells from substomatal stromata (A: young, B: mature) and hyaline smooth intercellular hyphae in transversal sections of *Lycium barbarum* leaves. C. Conidiophores. Percurrent extensions of conidiogenous cells indicated with arrows. D. Conidia. Scale bars A, B = 20 µm; C, D = 10 µm.

ones, 25–50 µm diam. or 25–100 µm high and 20–90 µm wide, composed of medium brown, 3–7 µm wide cells. Fascicles amphigenous, arising from stromata through stomata. Conidiophores erect or prostrate, simple or with 1–2 basal or apical branches, straight but often apically geniculate, medium to pale brown, smooth, 0–3-septate, septa 5–9 µm apart, (16–)22–35(–42) × (3–)4–5(–6) µm (n = 30). Conidiogenous cells cylindrical to slightly subulate, straight or becoming slightly curved in the apical half by 1–2 geniculations, occasionally with a percurrent proliferation, apical cells (8–)11–21(–30) × 3–4(–5) µm (n = 30), with 1–4 conidiogenous loci at the apex or ca. upper third of the cell, 1–1.5 µm wide, cell wall not thickened, not darkened. Conidia solitary, pale brown (paler than conidiophores), smooth, mostly straight or in some cases slightly curved, with 3–6(–8) transversal septa, (28–)41–63(–69) × (3–)3.5–4(–4.5) µm (n = 30), apex broadly rounded, basal hilum 1.5–2 µm, cell wall not thickened, not darkened.

Specimen examined: On leaves of potted plant of *Lycium barbarum* L. (labeled with name tag as 寧夏枸杞), Taiwan, Taipei City, Jianguo Holiday Flower Market, 4. Oct. 2021, R. Kirschner 5333 (TNM), living strain BCRC FU31691, GenBank ITS OQ061476, *TEF* LC743855.

Additional specimen examined: On leaves of wild *Solanum americanum* Mill., New Taipei City, Pinglin District, wayside near Pinglin Tea Museum, ca. 24.933419, 121.712535, ca. 200 m, 14. Nov. 2022, R. Kirschner 5370 (TNM), GenBank ITS OQ061477, *TEF* LC743856.

Comparison of *TEF* and ITS sequences

Our *TEF* sequences of *Ps. atromarginalis* from *L. barbarum* and *S. americanum* (GenBank LC 743855 and LC743856, respectively) differed from each other by three bps, with two of them within the first ten positions at the 5’ end of the sequences. BLAST searches with the *TEF* sequence of *Ps. atromarginalis* from *L. barbarum* among *TEF* sequences exceeding 200 bp in GenBank showed 99–100% identities and 0–2 different bps between the fungus from *Lycium barbarum* and five sequences of *Ps. atromarginalis*

from Solanaceae in GenBank as well as one sequence of *Ps. chengtuensis* [GU 384390, CBS 131924, in Crous et al. (2013) corrected to *Ps. atromarginalis*], one sequence of *Ps. cruenta* from Fabaceae (GU384404, CBS 132021), and one sequence of *Pseudocercospora fuligena* from *Solanum lycopersicum* L. (GU 384428, MUCC 533). The TEF sequences with comparable lengths of all other *Pseudocercospora* species differed for nine or more bps.

Our two ITS sequences differed for 1 bp. BLAST results with the ITS sequence of *Ps. atromarginalis* from *L. barbarum* presented 1–2 different bps for the 5 most similar sequences of *Pseudocercospora* species, which were all isolated from Solanaceae, while there were at least 3 different bps compared to the sequences of all other *Pseudocercospora* species from other host families.

Discussion

Species identification

Three species of *Pseudocercospora* have been recorded from *Lycium* hosts, but hitherto only from *L. chinense* and not *L. barbarum*, namely *Ps. atromarginalis*, *Ps. chengtuensis* (F.L. Tai) Deighton, and *Ps. lyciicola* (J.M. Yen) J.M. Yen (Crous et al. 2013, Hsieh and Goh 1990, Yen and Lim 1980). The hypophyllous sporulation of *Ps. chengtuensis* with longer conidiophores (up to ca. 100 µm) was emphasized as diagnostic characteristic for this species, while amphigenous sporulation with shorter conidiophores (max. 60 µm) was recorded for *Ps. atromarginalis* (Hsieh and Goh 1990, Yen and Lim 1980). Except for the occasionally much larger stromata, the morphology of the fungus on *L. barbarum* conforms to the descriptions of *Ps. atromarginalis* (Braun 2017, Hsieh and Goh 1990). By its longer and wider conidia (up to 148 × 6 µm), *Ps. lyciicola* may differ from *Ps. atromarginalis* (Yen and Lim 1980), but too few data exist for either synonymizing both species or keeping them clearly separate.

Our *TEF* sequences of *Ps. atromarginalis* from *L. barbarum* and *S. americanum* differed for three bps, but two of these deviating positions were located within the first ten positions at the 5’ end of the sequences, which are particularly prone to technical sequencing errors. This deviation indicates that one or two different bps should not be overemphasized for assessing differences between species, but in the contrary, however, could also indicate the presence of different species. Hitherto only one *TEF* sequence of a specimen of *Ps. chengtuensis* is available, and data of this single strain may suggest synonymy with *Ps. atromarginalis* (Crous et al. 2013). Apparently some of the ca. 25 *Pseudocercospora* species described on Solanaceae (Farr and Rossman 2022) are not strictly specific on a single host species or genus (Bakhshi et al. 2014, Wang et al. 1995), while the specificity of most other species has not been tested. No DNA data are available for at least 50% of the *Pseudocercospora* species described on Solanaceae, whereas their morphology in most cases is highly similar (Braun 2017). More detailed information about variations in the hosts, morphology, and DNA sequences are necessary for revising the taxonomy of these species. Among these species, *Ps. atromarginalis* is the oldest one (Atkinson 1892).

The high similarity of the ITS and *TEF* markers among *Ps. atromarginalis* and *Ps. chengtuensis* and a close relationship to *Ps. fuligena* on tomato were noted by previous researchers (Crous et al.

2013, Silva et al. 2016). The species on solanaceous hosts in phylogenetic analyses form a distinct clade with an apical subclade comprised of two strains of *Ps. fuligena* (Bakhshi et al. 2014, Crous et al. 2013, Silva et al. 2016), while the basal polytomy for the other species in this clade indicate insufficient resolution and misapplied species names. The above mentioned sequence of *Ps. cruenta* GU384404 differs from all other published *TEF* sequences in GenBank also labeled as *Ps. cruenta* (GU384405, Crous et al. 2013; MW848613, MW848602, MW848606, Meswaet et al. 2021) and *Ps.* cf. *cruenta* (MW 685835–MW685839, Bakhshi et al. 2021). These sequences have identities among each other of ca. 99%, but only ca. 88% identity with the above mentioned *Ps. cruenta* GU384404. This discrepancy indicates an erroneous labeling of *Ps. cruenta* GU384404. The sequence from *Ps. fuligena* GU384428 also differs for at least 12 bps from the other available ones also labeled as *Ps. fuligena* in GenBank (GU384427, JQ 837455), so that we believe that strain MUCC 533 from tomato may be rather *Ps. atromarginalis* than *Ps. fuligena*, particularly since the host range of *Ps. atromarginalis* is not limited to a single species or genus, but includes species of different genera of Solanaceae (Bakhshi et al. 2014). The differences among sequences of *Ps. fuligena* do not support the synonymy with *Ps. atromarginalis* and other, more poorly known species as suggested by Braun (2017). On the other hand, *Ps. solanacea* U. Braun, kept as separate species in Braun (2017) has the same main host, *Solanum nigrum*, and very similar morphology as *Ps. atromarginalis*. If the larger stromata of the new specimen on *L. barbarum* are considered in using the key in Braun (2017), the specimen would be identified as *Ps. solanacea*.

New host

A single record of *Ps. lyciicola* on *Lycium chinense* exists from Singapore (Yen and Lim 1980), while most records on this host in East Asia refer to *Ps. chengtuensis* (Farr and Rossman 2022, Hsieh and Goh 1990). Crous et al. (2013) suggested identifying a strain of *Ps. chengtuensis* on *Lycium chinense* from South Korea as *Ps. atromarginalis*, which may represent the first record of *Ps. atromarginalis* on a *Lycium* host. *Lycium barbarum* is recorded here as new host for a species of *Pseudocercospora*. While *Lycium chinense* was introduced in Taiwan some centuries ago (Wu et al. 2010), we did not find a record about the introduction of *L. barbarum* in Taiwan. Gouqi is not yet grown in Taiwan at quantities sufficient to the need of the Taiwanese market for Chinese herbal medicines so that the fruits are mainly imported from China (Liu 2017). The Taiwanese government recently started to boost the hitherto negligible domestic production of medicinal plants (https://www.taipeitimes.com/News/taiwan/archives/2022/06/06/2003779426). The pathogens on *Lycium* species hitherto did not receive attention in Taiwan. We recently recorded a powdery mildew on *L. chinense* in Taiwan (Yeh et al. 2021). This further new record of a fungal pathogen on gouqi indicates that more measures for the control of pathogens will inevitable in the enhanced domestic gouqi production than have hitherto been envisaged. Since medicinal plants such as gouqi exported from China often exceed the maximal legal levels of pesticide residues (https://www.ua-bw.de/pub/beitrag.asp?subid=1&Thema_ID=5&ID=1276&Pdf=No&lang=DE), e.g. the German public is regularly

warned against all imports of medicinal plant products from East Asia. For sustainable production of gouqi in Taiwan with high quality standards such as low pesticide residues, establishing knowledge about diagnosing, preventing and controlling plant pathogens of medicinal plants is urgently needed.

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藥用植物寧夏枸杞為黑緣假尾孢菌之新寄主紀錄

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摘　　要

寧夏枸杞(*Lycium barbarum*)為日用枸杞的兩種物種的其中一種，兩者之果實經常作為傳統中醫藥材使用。寄生真菌—*Pseudocercospora atromarginalis* 迄今為止只有在枸杞(*Lycium chinense*)和其他茄科植物(Solanaceae)上發現，此次於寧夏枸杞上發現之紀錄，則為一個新宿主之紀錄。基於形態學和比對 the translation elongation factor 1 alpha (*TEF*) 基因序列進行真菌物種的鑑定。由於茄科植物上假尾孢菌屬(*Pseudocercospora*)真菌的資料過於稀少，目前已知茄科植物上僅約有 25 種假尾孢菌屬真菌之紀錄，因此不能進行合理的分類學修訂。台灣藥用植物之潛在病原菌的相關資訊很稀少，但是這方面的病原菌資料對於建立藥用植物在國內的永續生產和降低進口依賴是不可或缺的。

關鍵詞：生物多樣性，尾孢屬類似真菌，球腔菌科