First Inventory of Fungi in Symptomless and Symptomatic Chinese Mesona Indicates Phytopathological Threat

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Abstract

Chinese mesona (*Platostoma palustre*) plays an important role as special crop in Southeast Asia and Taiwan for the production of herbal tea, grass jelly, and further processed food. In order to assess the potential threat of fungi to Chinese mesona, we surveyed isolates from symptomless plants in the area of mesona production, as well as from leaf spots of potted plants in a garden shop and a plantation in a botanical garden in Taiwan. From leaves, stems, and roots of 15 symptomless plants sampled at five collection events over two years, 154 isolates from 810 surface-sterilized plant fragments were obtained and identified based on DNA sequence data of the internal transcribed spacer region, and partially of the β -tubulin and histone H3 genes. The most common species belonged to the genera *Cercospora*, *Colletotrichum*, and *Fusarium* and were considered to be potential plant pathogens. Latent pathogenicity was confirmed by an infection experiment

with an endophytic strain of *Corynespora cassiicola*. Observation of leaf spot disease associated with *Cercospora kikuchii* suggested pathogenicity of this fungus, which was also isolated as an endophyte from symptomless leaves. We hypothesize that the most common endophytic fungi are latent pathogens in the host and may cause plant disease when the host becomes weakened by senescence or changed cultivation condition. Leaf spots of plants in the botanical garden were associated with a species of *Pseudocercospora*, which was not found among the endophytic isolates and is newly described based on morphology and analysis of translation elongation factor 1 alpha gene sequences.

Keywords: fungal diversity, latent pathogenic endophytic fungi, *Mesona chinensis*

Chinese mesona [*Platostoma palustre* (Blume) A.J. Paton = *Mesona chinensis* Benth.] of the mint family (Lamiaceae) is mainly distributed in Southeast Asia and Taiwan, where the aerial parts of the herb are dried for oxidation of its compounds and then used for producing herbal tea or jelly dessert (“grass jelly”) as medicine or functional food (Goh et al. 2014; Sasmita and Ling 2017). Because of the health properties, the plant is called *xiancao* (“herb of the immortals”) in Chinese. In Taiwan, promotion of this plant as a priority crop has led to the development of further processed food hardly known outside Asia (Supplementary Fig. S1) and of three cultivars grown altogether on 74–243 hectares per year, but the majority is imported (Chiang et al. 2017). Endophytic bacteria of Chinese mesona were investigated in Fujian Province, China (Liu et al. 2015). Despite its economic importance, unidentified *Phytophthora* species represent the only fungal genus known for this plant (Chuang et al. 2017). A quick access to the fungal diversity associated with a given plant is study of its endophytes (Yeh and Kirschner 2019). Endophytic fungi grow inside living plants, which may be comparable to the human gut bacteria. For using the term “endophyte,” we apply the same usage as in Yeh and Kirschner (2019). Endophytic

fungi are assumed to be latent pathogens, mutualists, or cryptic commensalists that become pioneer saprobes after natural death of plant tissues, although these hypothetical roles are poorly understood (Kirschner 2018). In useful plants, endophytes may have effects on the medical or nutritious quality of the crop (Lledó et al. 2015; Zhang et al. 2013). In order to establish basic data for assessing possible ecological roles of fungi associated with Chinese mesona and their potential threat to crop production, we made a first inventory of fungi from symptomless and symptomatic plants from three types of cultivation. In the commercial production of Chinese mesona with annual turnover, disease symptoms were not observed; therefore, endophytic fungi were isolated. In less or non-commercial plants, such as potted plants sold in a garden shop and plantations in a botanical garden, leaf diseases could occasionally be found and investigated mycologically.

Materials and Methods

Survey of mesona plantations and fungal isolation. The inventory and isolation of fungi associated with Chinese mesona was done at three types of plantations. Endophytic isolates were obtained from the main area of commercial mesona production; isolates from leaf spots of symptomatic plants were taken from potted plants in a gardener shop and from a plantation in a botanical garden.

Chinese mesona in the Botanical Garden of Taipei City was planted in an outdoor bed for educational purposes. Leaves with spot disease were collected on 4 July, 24 July, and 12 September 2019. The fungus associated with leaf spots was detected under a dissecting microscope and isolated by transferring externally visible conidia to 2% corn meal agar (CMA, Fluka).

Potted plants of Chinese mesona are sold for individual customers in small garden shops. In such a shop in Zhongli District, Taoyuan City, a potted plant with spontaneously developing leaf spots (Fig. 1) was purchased on 12 November 2017. A strain of the fungus associated with these leaf spots was isolated in the same way as above.

Plants for the isolation of endophytic fungi were collected in the center of mesona production with an annual turnover of the plants that constantly appeared symptomless. The main area for cultivation,

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production, and promotion of Chinese mesona in Taiwan is Guanxi Township in northern Taiwan, where marketing of its products accounts for about 40% of the agricultural productivity per year (Lin et al. 2017). Healthy plants of *P. palustre*, cultivar Taoyuan No. 1, were collected at Guanxi Xiancao Museum, Guanxi Township, Hsinchu County 306 (24.801°N, 121.186°E). The aim of this plantation was demonstration and promotion of Chinese mesona so that pesticides were not applied as in other plantations. As common in northern Taiwan, cultivation at the collection site started from stem cuttings in the autumn to harvest of the whole plant prior to fruiting in autumn of the following year. Plants were collected on 20 March 2014, 28 May 2014, 6 October 2014, 10 March 2015, and 24 June 2015. At every collection, three whole plants were dug out from the plantation, cleaned from soil, individually placed into bags, returned to the laboratory, and kept at 4°C until further processing within 24 h after sampling.

The plants were washed with water and cut into leaf, stem, and root. The plant fragments were washed and surface-sterilized under laminar flow at a UV-sterilized clean bench. Different concentrations of sodium hypochlorite (NaClO) and ethanol as well as periods of time for treating plant samples were initially tested in order to determine the final conditions for optimal surface disinfection. After soaking the plant parts in 95% ethanol for 30 s (leaf and root) or 60 s (stem), they were transferred into autoclaved ddH₂O for 30 s, then soaked in 6 to 12% NaClO (5% chloride) for 5 min (leaf and root) or 10 min (stem), and finally washed in sterile water for 30 s and placed into clean dishes for drying. When the water evaporated from the samples, they were temporarily pressed onto 1.3% malt extract agar (MEA, Scharlau) to make imprints as a method to verify the effectiveness of the surface disinfection method, and every stem and root was cut into three ~1 cm long parts with a sterile knife, and circular leaf fragments were cut out by pressing the collar of a sterile

10 µl pipetman tip onto the leaf surface to obtain three 5 mm diameter fragments. Each surface-sterilized fragment was put onto MEA. Beginning at the third day after placing plant fragments onto MEA, the endophytic fungi growing out from the fragments were separated onto 2% CMA (Fluka). Both cultivation media MEA and CMA were complemented with chloramphenicol (0.2 g/liter) to inhibit bacterial growth. Cultures were kept at room temperature and diffuse daylight. The isolates were classified into morphospecies according to their colony appearance on CMA. Morphological identification was done using light microscopy at 1,000× magnification with material from cultures or leaf sections mounted in 5 to 10% KOH. Sizes given for the new species were based on means of *n* measurements ± standard deviation. Representative strains were deposited at the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. Dried specimens of the new species were deposited at the National Museum of Natural Science, Taichung, Taiwan (TNM).

DNA isolation, PCR, and phylogenetic analyses. The methods were applied as in Yeh and Kirschner (2019). The internal transcribed spacer region (ITS) of the ribosomal RNA genes was amplified and sequenced for the representative strains for most species; other DNA regions were amplified and sequenced as recommended for the respective genus or species group in the special mycological literature. For amplifying and sequencing the ITS region, primers ITS1, ITS4, and ITS5 (White et al. 1990), and ITS1-F (Gardes and Bruns 1993) were used, for the partial ribosomal large subunit gene region (LSU), primers NL1 and NL4 (Kurtzman and Robnett 1997), for the β-tubulin gene (TUB), primers Bt2b (Glass and Donaldson 1995) and T1 (O'Donnell and Cigelnik 1997), for the histone 3 gene (HIS), primers H3R1 and H3F1 (Bills et al. 2009), for the gene of the DNA-directed RNA polymerase II second largest subunit (RPB2), primers rRPB2-5F and rRPB2-414R (Quaedvlieg et al. 2013), and for the translation elongation factor 1 alpha gene (TEF), primers EF1-728F and EF1-986R were applied (Carbone and Kohn 1999). The PCR reactions were programmed as in the cited references for the primers.

Edited sequences were deposited at GenBank (Supplementary Table S1) and used for BLAST searches at GenBank. For comparison of sequence identities, only published sequences with an identity of at least 97% were considered (O'Brien et al. 2005). For species identification in *Cercospora* and *Colletotrichum*, sequences of TUB were used; in *Fusarium* those of HIS were used. In addition to using BLAST, some strains were also identified by phylogenetic analyses. Our edited sequences and related ones selected from BLAST searches and the literature were aligned with the default options of MEGA 7 (MUSCLE) (Kumar et al. 2016) or MAFFT with E-INS-I strategy (Katoh and Toh 2008). Uneven ends of sequences in the alignment blocks were trimmed, but no other manipulation was done within the alignments. Phylogenetic analyses were done with the Maximum Likelihood method and Kimura-2 parameter model with uniform rates (in *Colletotrichum*) or with Gamma distribution (in *Pseudocercospora* and *Xylariales*) as implemented in MEGA 7. Strains without sufficiently matching reliable records in GenBank were identified above the species level. Updates of names and classification as well as authors of scientific names were retrieved from Index Fungorum (<http://www.indexfungorum.org/Names/Names.asp>). For details see Supplementary Table S1 and the below Discussion section.

Preliminary pathogenicity test. Two potted plants of *P. palustre* without disease symptoms were acquired at a garden shop in Zhongli District, Taoyuan City. The potentially infectious mesona endophyte, *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei strain CHD 002 (= BCRC FU30291, Fig. 1) could be identified earliest in this study and was therefore chosen for an infection experiment, while the identification of the other strains was still in progress. It was grown on fresh CMA plates for 1 week. The 7 mm diameter bottom of a sterilized pipetman tip was pressed onto the edge of the growing fungal colony in order to punch out a small cylindrical agar plug with the fungus. In order to infect the plants, 10 leaves per plant were provided with one agar plug per leaf. The same agar plate was used for treating the same plant. The other plant served as control without

Fig. 1. Examples of two pathogenic fungi also isolated as endophytes from Chinese mesona in Taiwan. **A, B,** Conidiophore (**A**) of *Corynespora cassiicola* in culture (BCRC FU30291) and conidium (**B**) showing thickened lateral cell walls and thin transversal distosepta (scale bar = 20 µm). **C,** Leaves became discolored after artificial inoculation with mycelium. **D,** Leaf spots (arrows) of potted plant associated with spontaneous infection by *Cercospora kikuchii* (R. Kirschner 4507, BCRC FU30931, Zhongli, 13 Nov 2017).

fungi, which was inoculated with sterile CMA plugs. The pots were watered in order to increase humidity; the plants in the pots were covered with a plastic bag and incubated in the laboratory at room temperature and diffuse daylight for ~1 week, during which the plants were controlled daily. After 1 week, the treated leaves were checked macroscopically for symptoms of leaf discoloration and counted, and microscopically for the presence and morphology of fungi.

Results

Pseudocercospora xiancao R. Kirschner, sp. nov. Index Fungorum IF557370 (Fig. 2). Etymology: referring to the Chinese name of the plant.

Leaf spots on mature leaves, amphigenous, ochre to reddish brown, irregular-angular, vein-limited, 1 to 11 × 1 to 3 mm, becoming confluent. Fascicles amphigenous but epiphyllous ones more conspicuous. External hyphae absent. Intercellular hyphae pale brown, smooth or occasionally appearing rough with some external adcrustations, 1 to 3.5 µm wide. Stromata absent or composed of few cells, or small, at maximum 30 µm wide and 25 µm deep, giving rise to few conidiophores rarely exceeding 10 per stroma. Conidiophores arising through stomata, erect, rarely somewhat prostrate when located at the margin of the fascicle, straight to slightly undulate or geniculate, occasionally with 1 to 3 percurrent extensions along the whole length, medium to pale brown, composed of 1 to 3 cells, (9–) 15 to 27 (–30) × 3 to 4 µm ($n = 30$). Conidiogenous cells terminal, lageniform, straight, slightly curved, or geniculate, pale brown, smooth, (8–) 11 to 18 (–22) × (2.5–) 3 (–4) µm ($n = 30$). Conidiogenous loci not thickened, not darkened, 1.5 to 2 µm. Conidia solitary, obclavate-cylindrical, straight to only slightly curved, pale brown, smooth, with 2 to 9 indistinct transversal septa, (26–) 35 to 61 (–75) µm long ($n = 30$, R. Kirschner 4813 only) or (26–) 35 to 80 (–105) µm long ($n = 40$, R. Kirschner 4813 including 10 measurements from R. Kirschner 4762), (2.5–) 3 µm wide. Conidial hila not thickened, not darkened, 1.5 to 2 µm.

Material examined. On living mature leaves of *Platostoma palustre* (Blume) A.J. Paton (= *M. chinensis* Benth., *M. procumbens* Hemsl.), Taiwan, Taipei, Botanical Garden, 4 July 2019, R. Kirschner 4762 (paratype), same place, 24 July 2019, R. Kirschner 4762-B (paratype), same place, 12 September 2019, R. Kirschner 4813 (holotype, TNM). Ex-type strain: BCRC FU31285, DNA sequences ITS MN582999, RPB2 LC506511, TEF LC506512.

Notes. During the study, three subsequent collections at the same place were necessary because attempts to cultivate the fungus so that DNA could be extracted were successful only on the third collection. Morphological study was done with the fresh specimen of the first collection (R. Kirschner 4762) and the last one (R. Kirschner 4813) in the dried state. There was no obvious difference in the characteristics of conidiophores and conidiogenous cells as well as the width of conidia. Conidium length, however, was considerably longer in the first specimen (exceeding 80 µm) compared with the last one (less than 80 µm), which indicates that conidium length should not be used as sole distinguishing characteristic for morphological species delimitation, especially when only a single specimen is available.

Several species of *Pseudocercospora* on Lamiaceae, however, are characterized by reduced stromata and lack of external hyphae so that they differ morphologically mainly by minor differences of sizes of conidiophores and conidia. Among these species, *Ps. physostegiae* (W.A. Jenkins ex Chupp) U. Braun & Crous on *Physostegia virginiana* (L.) Benth. appears most similar and differs only by its slightly wider conidiophores (up to 6 µm) and conidia (up to 4 µm) from the new taxon (Braun and Crous 2007). The host plant, however, belongs to the subfamily Lamioideae, whereas *Platostoma* is placed in Nepetoideae and thus is only distantly related to *Physostegia* (Li et al. 2016). Due to several molecular phylogenetic studies of Lamiaceae, the taxonomy of subfamilies and tribes can be considered quite well resolved (Li et al. 2016; Suddee et al. 2005). *Platostoma* belongs to the tribe Ocimeae of the subfamily Nepetoideae (Li et al. 2016). Because of the high host specificity of most *Pseudocercospora* species confirmed by molecular analyses (Bakhshi et al. 2014),

morphological comparison is limited to members on hosts belonging to the Nepetoideae, *Ps. kirishimensis* (Naito) C. Nakash. & Tak. Kobay., *Ps. lamiacearum* H.S.G. Rao, A.S. Moses & S. Narayan, *Ps. ocimi-basilici* Crous, M.E. Palm & U. Braun, *Ps. ocimicola* (Petr. & Cif.) Deighton, *Ps. plectranthi* G.C. Hunter, Crous, U. Braun & H.D. Shin, *Ps. salviae* Goh & W.H. Hsieh, and *Ps. velutinomaculans* U. Braun & F.O. Freire. Among these species, *Ps. ocimicola* differs mainly by its very pale and slightly wider conidiophores (up to 5 µm) and conidia (3 to 4 µm) (Chupp 1954; Hsieh and Goh 1990), but otherwise seems to be most similar to the new species. The single available DNA sequence of *Ps. ocimicola*, however, is an ITS sequence (GenBank GU214678) with a too-low identity (92%) with that of *Ps. xiancao* to indicate conspecificity. ITS sequence identities of different *Pseudocercospora* species is often 100% and thus resolution too poor (Chen and Kirschner 2018). If ITS sequence identity is considerably lower than 100%, we can conclude that different species are involved. RPB2 and TEF sequences provide a better resolution (Bakhshi et al. 2014; Chen and Kirschner 2018), but data are even fewer than for the ITS region. TEF sequences but no RPB2 sequences were available for *Ps. ocimi-basilici* and *Ps. plectranthi* on hosts belonging to the subfamily Nepetoideae and some other subfamilies of Lamiaceae. A phylogenetic estimate of TEF sequences further supports that *Ps. xiancao* is not closely related to any other known *Pseudocercospora* species on Lamiaceae (Fig. 3).

Diversity and identification of endophytic isolates. From 15 plants (810 plant segments) altogether during the 2-year period (five collection events) from the same plantation, 154 strains were isolated, comprising 20 genera (or taxa above genus rank) and 34 species (including species complexes) (Supplementary Table S1). Among the 34 species, two species were Basidiomycota and the others Ascomycota. Among the 34 species, 20 were identified to species (or species complexes). *Colletotrichum* was the most frequently found genus (12 strains; 22%), which was particularly dominant in the leaf (44%), *Fusarium* the second most (nine strains; 17%), and *Cercospora* the third most frequent genus (six strains; 11%). These latter two genera were especially dominant during the spring season. *Hypoxyylon investiens* (Schwein.) M.A. Curtis was represented by five strains (7%, Supplementary Table S1, Supplementary Fig. S2). Comparing the fungal communities in plant organs, the colonization

Fig. 2. *Pseudocercospora xiancao*, morphological characteristics. **A**, Leaf spots of *Platostoma palustre* associated with *Ps. xiancao* (R. Kirschner 4762). **B**, **C**, Fascicles of conidiophores penetrating through stoma (**B**: R. Kirschner 4813) or epidermis (**C**: R. Kirschner 4762). **D**, **E**, Conidiophores (**D**: R. Kirschner 4813, **E**: R. Kirschner 4762). **F**, Conidia (R. Kirschner 4762). Scale bars = 10 µm, except **F** = 20 µm.

frequency of leaf was the highest (46%), followed by that of stem (32%) and root (22%). Comparing the fungal communities in different seasons, the colonization frequency of spring (March 2014 and 2015, altogether 52% of strains) was highest, followed by summer (May 2014 and June 2015, 28%) and autumn (based on a single collection event in October 2014).

Since except for *Phytophthora* species (Chuang et al. 2017), no fungi associated with *P. palustre* have been recorded previously, all fungi from *P. palustre* identified in our study are new records from this host, namely *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner, *Chaetomium globosum* Kunze, *Colletotrichum karsti* You L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, *Co. siamense* Prihast., L. Cai & K.D. Hyde, *Corynespora cassicola*, *Fusarium oxysporum* Schltdl., *Hypoxyton monticulosum* Mont., *Microbotryozyma collariae* S.O. Suh, D.A. Maslov, Molestina & J.J. Zhou, *Neocosmospora rubicola* L. Lombard & Crous, *Phyllosticta capitalensis* Henn., *Plectosphaerella cucumerina* (Lindf.) W. Gams, *Pl. oligotrophica* T.T. Liu, D.M. Hu & L. Cai, and *Pseudozyma aphidis* (Henninger & Windisch) Boekhout. The ITS region served as primary barcode for these identifications. *Corynespora cassicola* strain CHD 002 (= BCRC FU30291) was identified based on morphology as well as ITS sequence identity with entries in GenBank. Species of *Cercospora* and *Colletotrichum* were additionally identified through distinctive matches in BLAST searches with TUB sequences. *Cercospora kikuchii* was identified mainly due to the highest match of our TUB sequences (endophytic strains CHD 105 and 106; parasitic strain BCRC FU30931; Fig. 1) with that of the ex-type strain [CBS128.27 = CPC 5068, with 0 to 1 different bp (382/382; 393/394 bp)] in BLAST searches, whereas other named species differed by at least 9 bp. This was shown in a phylogenetic analysis of the *Co. boninense* Moriwaki, Toy. Sato & Tsukib. species complex (Fig. 4)

where two strains from *P. palustre* nested within a well-supported clade comprising strains of *Co. karsti* supported the feasibility of TUB sequences as secondary barcode in this systematic group (Damm et al. 2012).

Further species could not be identified to the species level, namely *Cercospora* sp. 1, *Cercospora* sp. 2, *Cladosporium* sp., *Co. gigasporum* Rakotonir. & Munaut species complex, *Co. gloeosporioides* (Penz.) Penz. & Sacc. species complex, *Co. orchidacearum* Allesch. species complex, *Didymellaceae* sp., *Fusarium fujikuroi* Nirenberg species complex, *Magnaportheales* sp., *Metarhizium* sp., *Pezizomycotina* sp., *Pleosporales* sp. 1, *Pleosporales* sp. 2, *Sordariomycetes* sp., *Talaromyces* sp., *Xylariales* sp. 1, and *Xylariales* sp. 2. In some cases, the strains became lost due to contamination; in others there were no sufficient species matches in GenBank.

Preliminary pathogenicity test. In the infection experiment on 10 leaves of a potted Chinese mesona plant with agar plugs from an endophytic isolate of *Cor. cassicola*, whole agar plugs were coated by dark mycelium after 3 days, and the lower side of all leaves became black with tissue rot (10/10, 100%), whereas the leaves of the control plant did not change (0/10, 0%). Microscopic observation of the darkened leaves confirmed the presence of conidiophores and conidia of *Cor. cassicola*.

Discussion

Successful surface disinfection and species identification are the most critical and often neglected points in studies of endophytic fungi. We applied the imprint technique in order to adjust and control the efficacy of the surface sterilization. Species of *Phytophthora* (Oomycota/Stramenipila) were not found, which might be explained by the specific locality or because oomycete-specific isolation methods were not applied. As in most other studies of endophytes from

Fig. 3. Phylogenetic hypothesis derived from a maximum likelihood analysis of TEF sequences of *Pseudocercospora xiancao* and other *Pseudocercospora* species on Lamiaceae and most similar sequences according to BLAST searches (scientific name followed by strain or specimen number and GenBank accession number). Analysis with 1,000 bootstrap replicates, values lower than 70% not shown. *Cercospora kikuchii* used as outgroup in the unrooted tree.

herbaceous plant tissues, most taxa were Ascomycota; only two isolates belonged to basidiomycetous yeasts. For species identification, the ITS sequences are widely applied as primary barcode with a threshold of 97% identity for species as rule of thumb (O'Brien et al. 2005). This range of identity indicates identification of five strains from *P. palustre* as *Hypoxylon investiens*. This fungus was represented by five strains from all plant organs and collection seasons, whereas other *Xylariales* species were collected only once each. As suggested through a phylogenetic analysis of ITS sequences (Supplementary Fig. S2), however, endophytic strains from different plant species in Taiwan (Ho et al. 2012) labeled as *Hypoxylon* sp. and our strains from *P. palustre* occurred together with a morphologically identified field-collected specimen from Taiwan (Hsieh et al. 2005) in a strongly supported subclade. All the unidentified endophytic strains could be shown to belong to *H. investiens* for the first time. This subclade also contained sequences of endophytes from mainland China (Chen et al. 2011), Papua New Guinea (Vincent et al. 2016), and air spores from Germany (Fröhlich-Nowoisky et al. 2009); thus, no clear correlation between biogeography and subclades could be found. This compilation of data indicates the widespread occurrence of this fungus as an endophyte. The sequences from this subclade differed by 0 to 4 bp (99 to 100% identity), whereas sequences from other strongly or weakly supported subclades differed

by at least 6 bp (98% identity). The ITS, however, provides weaker resolution in this genus than TUB (Kuhnert et al. 2014), which may explain the weak support for the overall clade including all sequences of *H. investiens*. For the highly supported subclade containing the endophytes from Taiwan, however, only ITS sequences were available.

Pleosporales, which in other studies often comprise the majority of endophytes (Yeh and Kirschner 2019), were comparatively rare. The other most common genera were *Cercospora* (Mycosphaerellales), *Colletotrichum* (Glomerellales), and *Fusarium* (including *Neocosmospora*, Hypocreales). For these genera, ITS sequences can exceptionally be used for distinguishing between different species, since the sequences show 100% identity among several species. Based on ITS sequences, species complexes can sometimes be recognized, although the cryptic species within the complex cannot be resolved. Optimally, several genes are applied for species identification in critical genera, which may not be a practical solution for ecological/environmental studies comprising hundreds of strains of diverse systematic groups. Applying a specifically selected second barcode region already provides a better approximation toward species identification in certain genera than the ITS alone. For species of *Colletotrichum*, TUB has been recommended as additional barcode for identification with BLAST (Damm et al. 2012; Weir et al. 2012). In studies focusing on *Colletotrichum* as the single genus of pathogenic

Fig. 4. Maximum likelihood phylogenetic estimate of TUB sequences of the *Colletotrichum boninense* species complex including two endophytic isolates from Chinese mesona (arrows). Bootstrap support values (1,000 replicates) above 50% shown. Species names followed by GenBank accession number. Strains derived from typified material marked with "T". *Colletotrichum gloeosporioides* was included as outgroup.

fungi, phylogenetic analysis of TUB alone led to the same results as the multigene analysis (Mayorquin et al. 2019). Two isolates of *Colletotrichum* in our study did not only have sufficiently distinctive matches in BLAST searches, but also nested within a well-supported clade comprising strains of *Co. karsti* (Fig. 4). Only for this species, however, we obtained sufficient resolution in our phylogenetic analyses with a single secondary barcode. For species of *Fusarium*, HIS has been shown to provide sufficient resolution (O'Donnell and Cigelnik 1997; O'Donnell et al. 2004). For *Cercospora*, hitherto no barcode region with sufficient resolution of species has been discovered. Even with multigene analyses, many strains remained unidentified or only tentatively identified (Groenewald et al. 2013; Soares et al. 2015). Although Soares et al. (2015) demonstrated that the strains from soy plants belonged to different species and the name *Ce. kikuchii* was applicable only to their lineage 1 including the ex-type strain of *Ce. kikuchii*, the same name also remained applied to the species of the other clades in the publication and in GenBank. The data from Soares et al. (2015) were not compatible with those of Groenewald et al. (2013), who focused on other strains and DNA regions. The TUB sequences of three strains from Chinese mesona fitted to lineage 1 of Soares et al. (2015) and had highest identity with the ex-type strain of *Ce. kikuchii* so that this species name was applied here, whereas no species name was assigned to other *Cercospora* strains. In symptomatic plants from the garden shop, leaf spots bore conidiophores and conidia of *Ce. kikuchii*, which indicates that the endophytic isolates become associated with leaf spots in a later stage of development. On leaf spots from the botanical garden, however, a species of *Pseudocercospora* was detected that did not occur as endophyte. These findings confirm that with the cultivation approach as well as direct collection of symptomatic plants in the field, a saturation (rarefaction) cannot be achieved (Gams 2002; Piepenbring et al. 2012). In these approaches, however, morphological traits and the specific barcodes depending on the group of fungi allow a high resolution on the species level, and identification is reproducible and correctible by deposit of voucher strains or specimens. Culture-independent molecular approaches toward fungal diversity can reveal higher diversity, but since in most cases they rely on ITS sequences, resolution on the species level remains comparatively more insufficient in certain genera, such as in the above mentioned phytopathogenic fungi, and by the lack of physical vouchers are not reproducible.

In Taiwan, root-knot nematodes (*Meloidogyne* spp.) have been identified as major pest (Lai 2017), but except for *Phytophthora* fungi, which had not been identified to species (Chuang et al. 2017), no potentially pathogenic fungi are known for this plant (<https://nt.ars-grin.gov/fungalatabases/>). The preliminary pathogenicity test with *Cor. cassiicola* and the leaf symptoms associated with *Ce. kikuchii* and *Ps. xiancao* indicate these three fungi as possible candidates of leaf pathogens of Chinese mesona. The symptoms caused by *Cor. cassiicola* in the host leaves were the same as in our infection experiment with another strain of this fungus on leaves of *Vitex rotundifolia* (Yeh and Kirschner 2017), whereas symptoms were absent in the control. In *V. rotundifolia*, however, *Cor. cassiicola* was only found as a plant pathogen and not as an endophyte (Yeh and Kirschner 2017, 2019). In contrast, *Ce. kikuchii* was isolated as an endophyte from symptomless Chinese mesona as well as a potential parasite from leaf spots of this host plant after surveying additional plants in a garden shop. Species of *Cercospora* are usually only found to be associated with plants (Groenewald et al. 2013) and not in soil samples. Further potential plant pathogens belonged to the genera *Colletotrichum* and *Fusarium* (including *Neocosmospora*, Coleman 2016), which had the most common isolates in this study. Both genera are known to contain widespread virulent plurivorous plant pathogens, but a few *Colletotrichum* species are occasionally and several *Fusarium* species frequently isolated from soil (Domsch et al. 2007). For example, *Co. karsti* was recently shown not only to cause leaf anthracnosis on a wide range of plants, but also to cause twig dieback in citrus (Mayorquin et al. 2019). The most common *Fusarium* strains from Chinese mesona were identified as *F. oxysporum* s. str. or members of the *F. oxysporum* species complex, which

belong to most important fungal pathogens, but also contain non-pathogenic strains that may even suppress the pathogenic ones (Domsch et al. 2007; Edel-Hermann and Lecomte 2019). *Neocosmospora rubicola*, which belongs to *Fusarium*, was recorded as an endophyte in Korea and as a plant pathogen in China (Kim et al. 2017; Zheng et al. 2018). *Hypoxylon investiens* was the single comparatively frequent species of Xylariaceae found in this study. This fungus species as well as the family are known for their abundance among endophytes and production of antifungal and other bioactive compounds (Chen et al. 2011, 2014; Ho et al. 2012; Liu et al. 2008; Vincent et al. 2016) and may have a beneficial effect on the host plant. It was the single comparatively common species with a presumably mutualistic rather than parasitic relationship to the host plant.

The function of endophytic fungi in plants is frequently debated (Herre et al. 2007; Kirschner 2018). The fungi may be saprobic commensals that cryptically survive in the living plant but become dominant pioneer saprobic decomposers after the death of the tissues and by this strategy outcompete secondary saprobic invaders. Certain species of endophytes have been proven to be mutualists that share benefits with the host plant (Pérez et al. 2016). In some cases, latent pathogens have also been found among endophytic isolates (Paul et al. 2014). The finding that potentially plant pathogenic fungi were most abundant in Chinese mesona, however, contrasts with most other studies on endophytes. In our case, an herbaceous commercially grown plant with quick turnover was selected for study of endophytes in contrast to those other studies, where perennially grown or wild plants were investigated. A similar pattern of fungal diversity, i.e., a majority of *Colletotrichum* and *Fusarium* strains and a single genus of Xylariaceae (*Nemania*) was found in a commercial plantation of *Lycium chinense* (Paul et al. 2014). The *Colletotrichum* and some of the *Fusarium* strains may be rather pathogenic, whereas some strains of *Fusarium* and Xylariaceae may represent potential antagonists against the pathogens.

Our pioneer mycological inventory in Chinese mesona indicates that endophyte study from symptomless herbaceous plants grown as annuals may quickly reveal the hidden diversity of potentially plant pathogenic fungi and their antagonists. Since endophytic isolates often only present sterile mycelia, however, field-collected material from symptomatic plants provides more characteristics for comparison with similar known species with insufficient DNA data. Data and strains of diverse genera become available for more refined species identification, pathogenicity tests, and experiments with potential biological control agents.

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