

Article

Using Culture-Dependent and Molecular Techniques to Identify Endophytic Fungi Associated with Tea Leaves (*Camellia* spp.) in Yunnan Province, China

Saowaluck Tibpromma ^{1,2,3}, Samantha C. Karunarathna ^{1,2,3}, Jayarama D. Bhat ⁴, Nakarin Suwannarach ⁵, Steven L. Stephenson ⁶, Abdallah M. Elgorban ⁷, Salim Al-Rejaie ⁸, Jianchu Xu ^{1,2,*} and Peter E. Mortimer ^{1,*}

- ¹ Centre for Mountain Futures, Kunming Institute of Botany, Kunming 650201, China; saowaluckfai@gmail.com (S.T.); samanthakarunarathna@gmail.com (S.C.K.)
- ² CIFOR-ICRAF China Program, World Agroforestry (ICRAF), Kunming 650201, China
- ³ Center for Yunnan Plateau Biological Resources Protection and Utilization, College of Biological Resource and Food Engineering, Qujing Normal University, Qujing 655011, China
- ⁴ No. 128/1-J, Azad Housing Society, Curca, P.O. Box, Goa Velha 403108, India; bhatdj@gmail.com
- ⁵ Research Center of Microbial Diversity and Sustainable Utilization, Chiang Mai University, Chiang Mai 50200, Thailand; suwan.462@gmail.com
- ⁶ Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA; slsteph@uark.edu
- ⁷ Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 1451, Saudi Arabia; aelgorban@ksu.edu.sa
- ⁸ Department of Pharmacology & Toxicology, College of Pharmacy, King Saud University, Riyadh 1451, Saudi Arabia; rajaie@ksu.edu.sa
- * Correspondence: jxu@mail.kib.ac.cn (J.X.); peter@mail.kib.ac.cn (P.E.M.)



Citation: Tibpromma, S.; Karunarathna, S.C.; Bhat, J.D.; Suwannarach, N.; Stephenson, S.L.; Elgorban, A.M.; Al-Rejaie, S.; Xu, J.; Mortimer, P.E. Using Culture-Dependent and Molecular Techniques to Identify Endophytic Fungi Associated with Tea Leaves (*Camellia* spp.) in Yunnan Province, China. *Diversity* **2022**, *14*, 287. <https://doi.org/10.3390/d14040287>

Academic Editor: Ipek Kurtboke

Received: 21 February 2022

Accepted: 6 April 2022

Published: 11 April 2022

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Abstract: The association of endophytic fungi with the host plant is called a symbiotic relationship. Studies of the endophytic fungi from tea have been reported in numerous documents, but researchers still largely focus on tea endophytic fungi as they have ability to produce bioactive compounds which have numerous applications. The present work characterizes the fungal endophytic communities associated with healthy tea leaves in Yunnan Province, China. A total of 287 fungal strains were isolated from healthy leaf tissues of tea plants using a culture-dependent approach. Based on nuclear ribosomal DNA internal transcribed spacer (ITS) sequence analyses taken from the fungal cultures, strains were classified into 28 fungal genera with high similarity matches to known sequences in GenBank. The majority of genera (98.25%) belong to the phylum Ascomycota and most of the dominating fungal endophytes are from the genera *Colletotrichum* and *Clonostachys*.

Keywords: Ascomycota; *Clonostachys*; *Colletotrichum*; healthy tissues; ITS

1. Introduction

Tea surged in global popularity in the 21st century, and nowadays nearly half of the world's population drinks tea [1]. Currently, tea is cultivated in over 52 countries, with China and India being the two largest producers [2–4]. In southwestern China, Yunnan Province is one of China's most important tea-growing regions, and several species of *Camellia* are used for tea production in this region [5]. Moreover, Yunnan Province has long been assumed to be one of the original tea domestication centers of the world [6,7].

Endophytic fungi, also known as mycosymbionts, live asymptotically within various tissues of host plants [8,9]. Endophytic fungi are a diverse polyphyletic group of microorganisms that can thrive in various plant tissues above and/or underground, including stems, leaves, and/or roots [10]. In general, leaves feature a more diverse fungal endophytic community compared to other parts of the plant [11,12]. According to Faeth and Fagan [13], it is estimated that there are more than one million species of endophytic

fungi. These endophytic organisms confer a number of benefits to the host plant, such as improving resilience to abiotic stress, tolerance to fungal pathogens, and growth performance of the host plant [14–22].

Studying endophytes is largely based on molecular techniques. However, the reliability of molecular findings can be verified using Bill’s concept, which is a culture-dependent approach [23,24]. Guo et al. [25,26] mentioned that the use of morphological characteristics alone is not sufficient to identify fungal endophytes, especially when they do not sporulate, and thus, DNA data are needed for identification. The internal transcribed spacer (ITS) region is the most widely sequenced genetic marker for fungi and has also been formally proposed as the standard fungal barcode marker [27,28]. Thus, depending on the objectives of the endophytic fungal study, some authors only use the ITS region to identify endophytic fungi [29,30]. However, multi-gene analyses are more accurate for identifying endophytic fungal species [31,32].

Tea plants are rich in endophytic fungi. The systematic study of tea endophytes began in the early 21st century [1,33]. Endophytic fungi reported from tea have been isolated from several parts of the plant, viz. flowers, leaves, roots, shoots, and stems [34]. Past studies relating to tea endophytes have focused mainly on the composition, diversity, and distribution of endophytes in tea plants, as well as studies relating to the impact of climate and the age of tea plants on the make-up of tea endophyte communities [33,35–38]. Despite an increase in attention, tea endophytic fungal studies remain limited in scope compared to other economically important crops [1]. One reason for this could be since the number and types of endophytic fungi existing in tea plants vary with time as well as across different altitudinal locations, tissue types, and cultivars [38,39]. Furthermore, different types of tea plant tissues have their own dominant microflora [35]. In China, endophytic fungal studies in tea have been carried out in several areas and most of the endophytic fungi were isolated from leaves and branches of tea plants [35,38,40,41].

The current study was designed to determine the endophytic fungal communities in tea leaf (*Camellia* spp.) tissues collected in Yunnan Province, China. Using culture and DNA-barcode (ITS) methods, we isolated and identified endophytic fungal communities from 11 tea plantations in different areas of Yunnan Province.

2. Materials and Methods

2.1. Study Site and Sampling

The study was conducted in Baoshan, Honghe, Lijiang, and Xishuangbanna of Yunnan Province, China, from 2019 to 2020 (Figure 1 and Table 1). Healthy tea leaves were randomly collected from different kind of tea plantations, viz. shade tea, wild tea, terraced tea, and mix planting, and immediately placed in plastic bags, ice boxes, labeled and subjected to fungal isolation within 48 h.

Table 1. Samples collection details.

Original Code	Location	Collection Date	GPS	Tea Plantation Type and Location Details (Yunnan, China)	Basic Climate Information
CN	Baoshan	26 August 2019	24°29′09.2″ N 99°20′44.2″ E	Terraced tea, Wandianxiang, Changning County, Baoshan	Baoshan is located with mountainous and semi-mountainous areas with characterized by a climate that changes with altitude, experiencing a monsoon climate in the low-latitude plateau and six climate types between 535 m and 3780 m above mean sea-level [42–44].
LYA		25 August 2019	25°10′25.2″ N 99°06′51.5″ E	Terraced tea, Banqiaozen, Longyang District, Baoshan	
LYB		25 August 2019	25°10′25.2″ N 99°06′51.5″ E	Terraced tea, Banqiaozen, Longyang District, Baoshan	
BS		24 August 2019	25°08′00.2″ N 99°08′07.3″ E	Terraced tea, Hanzhuangzhen, Longyang District, Baoshan	
HT		23 August 2019	25°09′59.3″ N 99°11′00.5″ E	Shade tea with pine trees, Banqiaozen, Longyang District, Baoshan	

Table 1. Cont.

Original Code	Location	Collection Date	GPS	Tea Plantation Type and Location Details (Yunnan, China)	Basic Climate Information
ML	Xishuangbanna	4 August 2020	2192120101.27	Shade tea with rubber trees, Xishuangbanna botanical garden, Xishuangbanna	Xishuangbanna is located with mountainous and historically highly forested area with elevations ranging between 477 and 2429 m, annual mean temperatures ranging between 15.1 and 21.7 °C, and a monsoonal climate [45,46].
LJ	Lijiang	1 July 2020	26°51'00.5" N 99°50'16.7" E	Wild tea, Shitouxiang, Yulong Naxi Autonomous County, Lijiang	Yulong Naxi Autonomous County of Lijiang City, 2370 m above sea level [47].
SG		30 June 2020	26°50'58.1" N 99°50'34.2" E	Wild tea, Shitouxiang, Yulong Naxi Autonomous County, Lijiang	
AMA	Honghe	1 September 2020	23°14'44.6" N 102°11'46.6" E	Mix planting, Jiachexiang, Honghe County, Hani and Yi Autonomous Prefecture	Hani-Yi Autonomous Prefecture of Honghe belonging to the plateau subtropical monsoon climate region. The average annual rainfall is 1491 mm. The average annual sunshine is 1065–2300 h. [48].
AMB		1 September 2020	23°14'39.1" N 102°11'20.4" E	Shade tea with rubber trees, Jiachexiang, Honghe County, Honghe Hani and Yi Autonomous Prefecture	
AMC		1 September 2020	23°14'40.8" N 102°11'26.3" E	Shade tea with rubber trees, Jiachexiang, Honghe County, Honghe Hani and Yi Autonomous Prefecture	

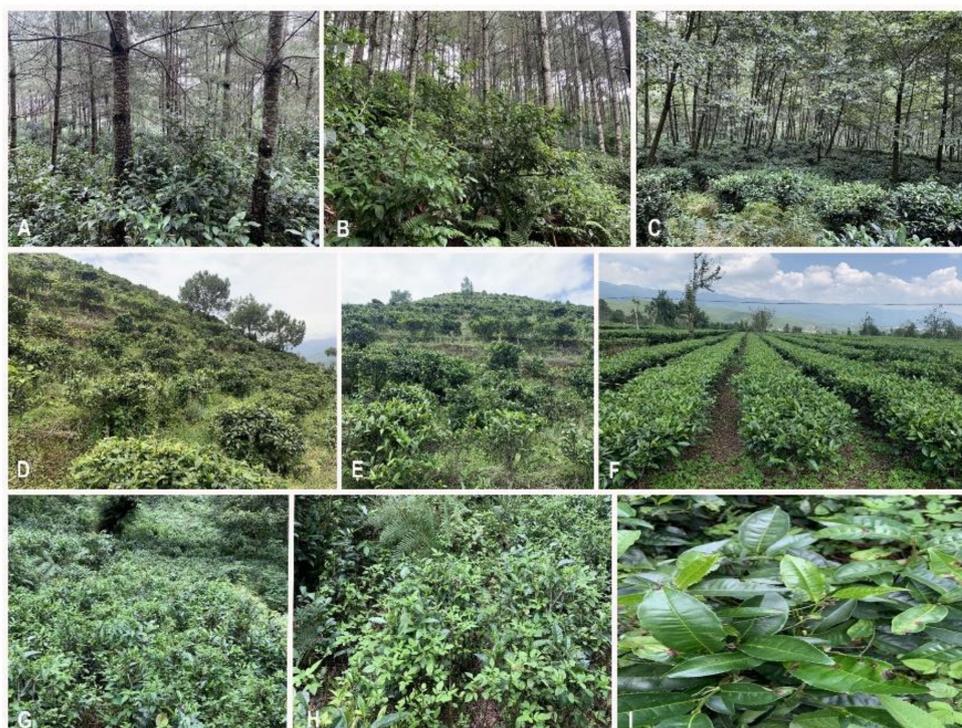


Figure 1. Tea plantations in Yunnan Province. (A–C) = Shade tea. (D–F) = Terraced tea. (G–I) = Mix planting.

2.2. Isolation and Identification of Endophytic Fungi

At each site, 20 leaves randomly selected from healthy tea plants were first washed in running tap water to remove soil and dust. Tibpromma et al. [32] was followed for the surface sterilization procedures. Leaf pieces (0.5 cm size pieces) were placed on potato dextrose agar (PDA) dishes with amoxicillin added to prevent bacterial growth (50 mg of amoxicillin per 1 L of PDA). All dishes were incubated at room temperature (20–25 °C) for five days and periodically checked. Mycelia emerging from the leaf bits were aseptically transferred to new PDA dishes and incubated at 28 °C. A total of 287 strains were isolated from the Yunnan tea leaf samples.

2.3. DNA Extraction, PCR Amplification and DNA Sequencing

The pure mycelia of endophytic fungal cultures grown on PDA at room temperature for four weeks were used for DNA extraction. The pure fungal mycelia were scraped off with a sterile scalpel and transferred to 1.5 mL micro-centrifuge tubes under aseptic conditions. The Biospin Fungal Genomic DNA Extraction Kit (BioFlux, China) was used to perform DNA extraction on the fungal cultures following the manufacturer's protocols. Polymerase chain reaction (PCR) was used to amplify partial gene regions of Internal Transcribed Spacers (ITS) using ITS5 and ITS4 primer [49]. The total volume of PCR mixtures for amplifications was set as described in Tibpromma et al. [50]. Purification and sequencing of PCR products were carried out by Sangon Biotech Co., Shanghai, China. ITS sequence data produced in this study were checked for the quality of chromatograms, and raw forward and reverse sequences were assembled using Geneious Pro.v4.8.5. Assembled sequences were trimmed out from the LSU and SSU sequence regions with an online program (<https://plutof.ut.ee>, accessed 6 January 2022), leaving only the ITS1-5.8S-ITS2 sequence region, and the size of the ITS gene was approximately 400–500 bp. ITS sequences were used in a BLAST search of the GenBank (<http://blast.ncbi.nlm.nih.gov>, accessed 15 January 2022) database to determine their most-probable closely related genus. Identifications of ITS sequences were made using the highest hit score of listed species (Supplementary Table S1). By this method, each isolate was identified and assigned to a specific genus.

3. Results

The isolates obtained during the course of this study belong to 28 known genera. Twenty six genera belonging to ascomycetes (*Alternaria*, *Annulohyphoxylon*, *Aquapteridospora*, *Cercospora*, *Cladosporium*, *Clonostachys*, *Colletotrichum*, *Coniochaeta*, *Daldinia*, *Diaporthe*, *Epicoccum*, *Fusarium*, *Gliomastix*, *Kretzschmaria*, *Melanconiella*, *Nemania*, *Neosetophoma*, *Nigrospora*, *Penicillium*, *Pestalotia*, *Pestalotiopsis*, *Phomatospora*, *Phyllosticta*, *Pseudopestalotiopsis*, *Trichoderma*, and *Xylaria*) and two genera belonging to Basidiomycetes (*Fomitopsis* and *Psathyrella*) and an additional unidentified fungal endophyte.

The dominant fungal genera derived from each collecting site and among all isolates, belonged to the phylum *Ascomycota* (98.25%), 0.70 belonged to the phylum *Basidiomycota*, and 1.05% could not be identified and were thus marked as unknown fungal endophytes (Figure 2). In order level, *Glomerellales* was reported as the predominant order, while *Xylariales* and *Hypocreales* were the most diverse orders with other isolates from the orders *Agaricales*, *Amphisphaeriales*, *Botryosphaeriales*, *Capnodiales*, *Coniochaetales*, *Diaporthales*, *Eurotiales*, *Mycosphaerellales*, *Phomatosporales*, *Pleosporales*, and *Polyporales*. At the genus level, *Colletotrichum* and *Clonostachys* were the dominant genera (42.16 and 21.25%, respectively) and other isolates within 1–10% included *Diaporthe*, *Fusarium*, *Nemania*, *Nigrospora*, *Pestalotia*, *Phomatospora*, *Phyllosticta* and unidentified fungal endophytes (Figure 3). Nineteen endophytic genera with <1% were regarded as rare genera, viz. *Alternaria*, *Annulohyphoxylon*, *Aquapteridospora*, *Cercospora*, *Cladosporium*, *Coniochaeta*, *Daldinia*, *Epicoccum*, *Fomitopsis*, *Gliomastix*, *Kretzschmaria*, *Melanconiella*, *Neosetophoma*, *Penicillium*, *Pestalotiopsis*, *Psathyrella*, *Pseudopestalotiopsis*, *Trichoderma*, and *Xylaria* (Figure 3).

Different fungal groups among tea plantation types are shown as: (1) *Clonostachys* was the most abundant genus in terraced tea plantations; (2) in the three shade tea sites (AMB, AMC, ML), the most abundant genus was found to be *Colletotrichum* and the other shade tea site (HT), it is *Clonostachys*; (3) in the two wild tea sites, the dominant fungal groups were *Colletotrichum* and *Nigrospora*; (4) and the most abundant genus in the mixed planting site was *Colletotrichum* (Figure 2).

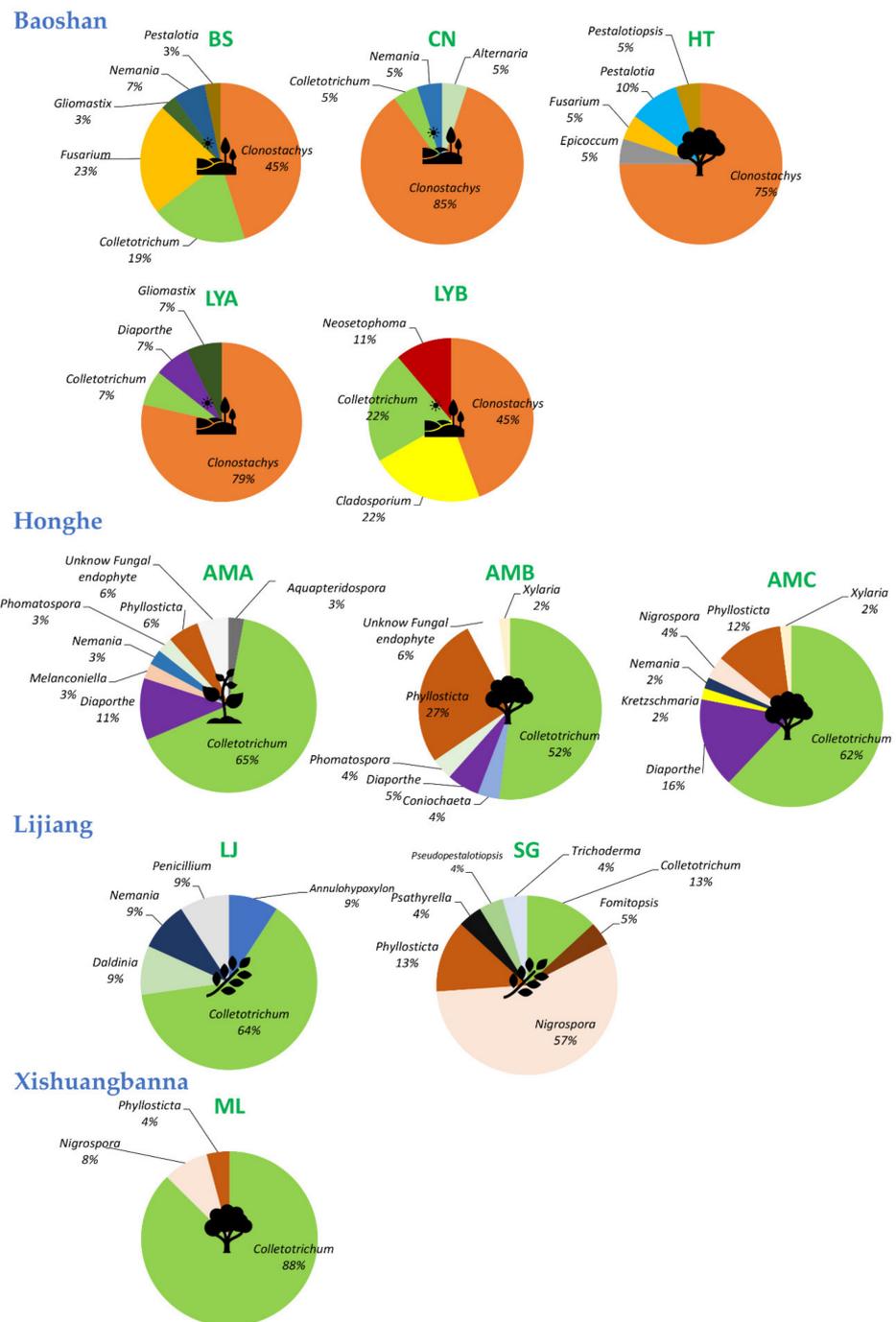


Figure 2. Distribution of tea endophytic fungi (genus level) according to plantation type, with percentages in each collection site and tea plantation types. Symbols represent as = Terraced tea, = Shade tea, = Mix planting, = Wild tea.

When assessing the diversity of species according location, in Baoshan, *Clonostachys* was most abundant; in Honghe, *Colletotrichum* was most abundant; whereas in Lijiang, abundance was split according to the two sites, *Colletotrichum* was most abundant in one site (LJ) and *Nigrospora* is the other site (SG); and finally, in Xishuangbanna, the most abundant genus was also found to be *Colletotrichum*. Furthermore, members of *Colletotrichum* were found in all types of tea plantations) (Figure 2).

Colletotrichum comprises fungi that are classed as endophytes, saprobes, entomopathogens, as well as many species of phytopathogens [51–54]. Several *Colletotrichum* species have been

reported as endophytes in living plant tissues (with the majority from *C. boninense*, *gloeosporioides*, and *graminicola* species complexes) [51,52,54–57]. Further complicating the issue regarding *Colletotrichum* (species in the *C. gloeosporioides* species complex) is that these species are able to switch their lifestyle from endophytic to pathogenic modes [58,59]. According to Tibpromma et al. [60], *Colletotrichum acutatum* and *Colletotrichum camelliae* are candidate pathogens of emergent diseases on the tea plant, with the added potential of shifting to novel areas or hosts under future climate change scenarios. Members of the genus *Clonostachys*, which has a global distribution, are known as mycoparasites, lichenicolous fungi, endophytes, and saprobes [61,62]. Many species of *Clonostachys* have been studied for their secondary metabolites [61]. For example, *Clonostachys rosea* is an excellent biocontrol agent that can control a wide range of plant pathogens [63]. *Clonostachys rosea* is also commonly found as an endophyte of healthy palm trees and is a good candidate for further study as a potential biological control agent of date palms diseases [64].

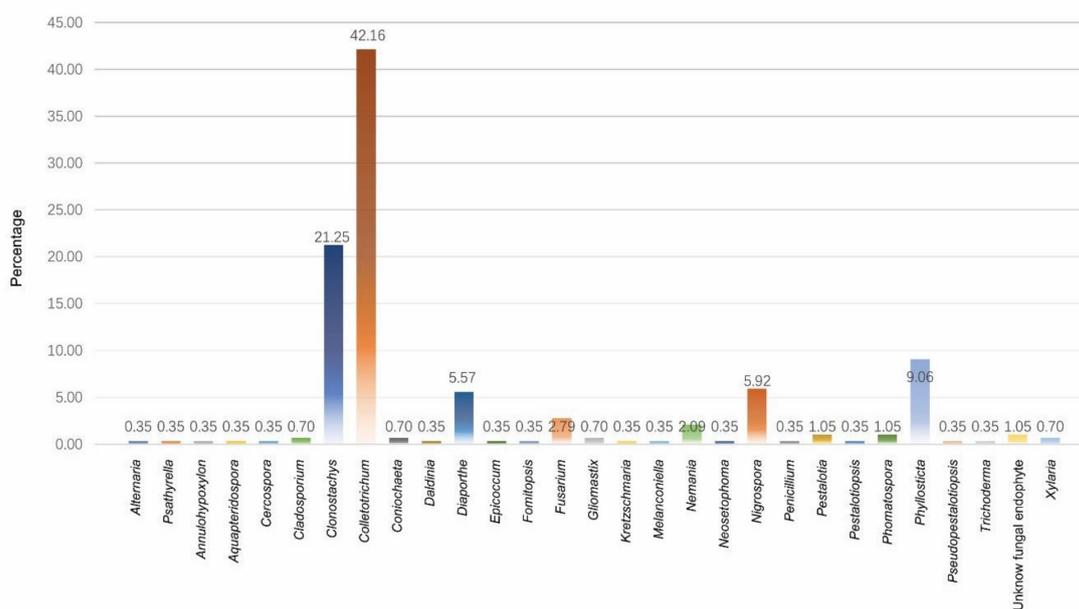


Figure 3. Overall distribution of tea endophytic fungi (genus level) with percentages.

4. Discussion

The 287 fungal endophytes isolated from 11 tea plantations in Yunnan Province, China, were successfully identified at the genus level. The results indicate a high diversity of endophytic fungi with consisting of 28 genera, the majority belonging to *Sordariomycetes* in the phylum *Ascomycota* with *Colletotrichum* (related to *C. gloeosporioides* species complex) and *Clonostachys* (related to *Clonostachys rosea*) being the most dominant (Figure 3).

Our results confirm the work of Lu and Wu [40] and Wu et al. [41] who reported a high diversity of fungal endophytes in tea plants from China. These authors noted that tea leaves maintained a high level of endophytic diversity, and no endophytes were found in tea seeds. Similar to our findings, Rodriguez et al. [15] analyzed endophytic fungi from woody plants and reported a high diversity of non-clavicipitaceous endophytes, most of which belonged to the phylum *Ascomycota*, while only a few were *Basidiomycota*. Xie et al. [1] categorized endophytic fungi previously reported from tea into three phyla, five classes, 14 orders, 24 families, and 34 genera. Members of *Pleosporales* (*Dothideomycetes*), *Diaporthales*, *Glomerellales*, *Hypocreales*, and *Xylariales* (*Sordariomycetes*) were reported as the dominant strains. Our results also showed similar fungal groups in *Ascomycota* to the results of Xie et al. [1] but different groups in *Basidiomycota*. Lu and Wu [40] isolated endophytic fungi from tea trees in southern Henan Province and found that *Colletotrichum* sp., *Pestalotiopsis* sp., *Phomopsis* sp., and *Macrophoma* sp. were the predominant fungi which

is consistent with our results as *Colletotrichum* sp. is the dominant fungal group, however we found a strong presence of *Clonostachys* in our study, which was not reported by Lu and Wu [40].

In this study, we cultured endophytic fungi, and the results will facilitate further research into valuable bioactive compounds and the biocontrol potential of tea fungal endophytes for sustainable agricultural development. However, our work lacked enough rigorous sampling between sites to allow for in depth statistical analyses on the distribution of fungal endophytes and how this may be influenced by environmental factors. Future work should make added efforts to include such data.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14040287/s1>, Table S1: A list of species based on the highest hit score of ITS sequences in NCBI.

Author Contributions: Conceptualization, S.T. and S.C.K.; formal analysis, S.T.; funding acquisition, S.T., A.M.E., S.A.-R., J.X. and P.E.M.; methodology, S.T. and S.C.K.; software, S.T.; supervision, P.E.M.; writing—original draft, S.T., S.L.S., A.M.E., S.A.-R. and P.E.M.; writing—review and editing, S.T., S.C.K., J.D.B., N.S., S.L.S., A.M.E., S.A.-R., J.X. and P.E.M. All authors have read and agreed to the published version of the manuscript.

Funding: Saowaluck Tibpromma thanks the International Postdoctoral Exchange Fellowship Program (number Y9180822S1), CAS President’s International Fellowship Initiative (PIFI) (number 2020PC0009), China Postdoctoral Science Foundation and the Yunnan Human Resources, and Social Security Department Foundation for funding her postdoctoral research. Samantha C. Karunarathna thanks the CAS President’s International Fellowship Initiative (PIFI) young staff under the grant number: 2020FYC0002, and the National Science Foundation of China (NSFC) under the project code 31851110759. Peter E. Mortimer thanks the National Science Foundation of China (NSFC), project codes 41761144055 and 41771063 for financial support. The authors extend their appreciation to the researchers supporting project number (RSP-2021/120) King Saud University, Riyadh, Saudi Arabia, Department of Sciences and Technology of Yunnan Provincial Government, China (grant number 202101AS070045) and Yunnan Provincial Science and Technology Department (grant number 202003AD150004). This research work was partially supported by Chiang Mai University.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Saowaluck Tibpromma thanks Chengjiao Dao and Li Huili for their help and valuable suggestion for this work. Austin G. Smith at World Agroforestry (ICRAF), Kunming Institute of Botany, China, is thanked for English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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