Black List of Unreported Pathogenic Bambusicolous Fungi Limiting the Production of Edible Bamboo

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Abstract

Edible bamboo species are now domesticated and commercialized because of their nutraceutical values. The production of edible bamboo species are restrained by diseases caused by pathogenic bambusicolous fungi valued at 40% losses of the total $818.6 million generated annually in bamboo trade in North East India. Based on a systematic survey performed for 2 years in succession, only one Basidiomycota, a Perenniporia sp. was identified and validated by pathogenicity test. Ascomycota was the dominant and diverse group of pathogenic bambusicolous fungi. Some rDNA locus sequences failed to match sequences in the up-to-date databases and indicated novel species or genera. Divergence study based on rDNA locus showed that pathogenic bambusicolous fungi were located in the class of Ascomycetes, Sordariomycetes, Eurotiomycetes, Dothideomycetes and Basidiomycetes. The data demonstrated for the first time that Fusarium, Cochliobolus, Daldinia, Leptosphaeria, Phoma, Neodegittonia, Lasiodiplodia, Aspergillus, Trichoderma, Pseurotinae, Perenniporia, Nigrospora and Hyporales are potent pathogenic bambusicolous fungi genera restraining the production of edible bamboo Dendrocalamus hamiltonii.

Keywords: Fungal diversity; Phylogeny analysis; Pathogenicity test; Trichoderma asperellum; Dendrocalamus hamiltonii; rDNA

Introduction

Woody bamboo species are highly diverse and abundantly represented in Asian countries such as China, Japan and India etc. Raw bamboo products generate annual revenue of $818.60 million in North East India alone [1]. Bamboo is used in paper making, landscaping, soil conservation, handicrafts, construction, as well as food [2,3]. Nonetheless, it is predicted that half of the world woody bamboo species are in risk of extinction [4,5]. Because of the multipurpose usage and the risk of extinction, techniques for in vitro propagation and cultivation of endangered edible bamboo shoots had been developed [6,7].

Remarkably, bush fire, shifting cultivation, flowering boom followed by erratic death [3,8,9], pest and diseases are important factors accelerating the extinction of bamboo species. Although edible bamboo cultivation is plagued by these factors, low level production is exacerbated by harmful bambusicolous fungi. Bambusicolous means organisms life on bamboo [10]. Even though some bambusicolous fungi records are indexed (http://nt.ars-grin.gov/fungal databases), the list is not comprehensive for the following reasons: 1) The bamboo species hosting bambusicolous fungi are often not reported, 2) most bamboo species are in the wild and not domesticated for phytopathological scrutiny, and 3) the complex lifestyle of bamboo species which encompasses fast growth, giant height, often growing in difficult terrain limits surveillance and impedes insights on bamboo pathology.

Fungal diseases weaken the rate of growth and the quality of edible bamboo shoots. This is because bamboo shoots development depends on the health status of mother clump-rhizome and leaf canopy. To achieve the optimal production of edible bamboo, pathogenic fungi limiting cultivation must be identified. Dendrocalamus hamiltonii Nees et Arn. ex Munro is a sympodial commercial species, with erect and curve culms, and highly valued for its nutraceutical values [3,11]. It is richly distributed in North Eastern Himalayan region, India [12]. Young succulent bamboo shoots of D. hamiltonii are consumed fresh or fermented as vegetable; and preferred over other species because its fermented products retained good taste and low water content [13]. At present, there is no report on the diversity of pathogenic bambusicolous fungi of any edible bamboo species. To address this issue, landraces of edible bamboo species of D. hamiltonii were surveyed for a period of 2 years in succession for fungal diseases and pathogenicity test was used to validate the disease causing potential of the fungi. Herein, new pathogenic bambusicolous fungi and their phylogenetic link are established.

Materials and Methods

Study area, sampling and morphological identification of fungal pathogens

Landraces of edible bamboo species (Dendrocalamus hamiltonii GenBank accession JX564903) were systematically surveyed in bamboo farms for 2 years in succession for the occurrence of fungal diseases during the month of July–August of 2011 to 2013. The farms are located in Imphal – East District, Manipur, India (Figure 1). The average age of bamboo clumps were 5-7 years old. The area often received an average rainfall of 1320 ± 3 mm and temperature of 29 ± 3°C during the months of July to August. Diseased plant tissue fragments (< 1 cm²) from leaves, nodes and internodes were surface

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Total genomic DNA was isolated from mycelium using UltraClean™ Microbial DNA isolation kits (MO Bio-Laboratories, Carlsbad, CA, USA) as described by the manufacturer. The integrity and quality of DNA was checked by agarose gel electrophoresis and absorbance measurements using a biospectrophotometer (Shimadzu® BioSpec, Japan), respectively. rDNA locus comprising of partial sequence of 5.8S rRNA, complete internal transcribed region two (ITS2) and partial 28S rRNA region was amplified using the primer set (5´-tcctccgcttattgatatgc-3´, 5´-gcatcgatgaagaacgcagc-3´) [14] and the PCR conditions were as follows. PCR was performed in a 25 μl volume containing 2.5 μl of 10 μM of forward and reverse primers each, 0.25 μl of DreamTaq® polymerase (ThermoScientific, UK) 1 μl of 10 ng DNA template and 18.5 μl nuclease free water. DNA template was denatured at 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 1 min for 48s and a final extension at 72°C for 5 min in a thermocycler (Bio-rad, C1000). All products were profiled by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The PCR products were sequenced. Sequences were assigned to molecular species based on standard monograph taxonomic keys with the help of a microscope (Olympus BX61, Japan).

DNA phylogeny

Sporulating fungi and non-sporulating fungi (that could not be identified morphologically) were characterised at the rDNA locus. Total genomic DNA was isolated from mycelium using UltraClean™ Microbial DNA isolation kits (MO Bio-Laboratories, Carlsbad, CA, USA) as described by the manufacturer. The integrity and quality of DNA was checked by agarose gel electrophoresis and absorbance measurements using a biospectrophotometer (Shimadzu® BioSpec, Japan), respectively. rDNA locus comprising of partial sequence of 5.8S rRNA, complete internal transcribed region two (ITS2) and partial 28S rRNA region was amplified using the primer set (5´-tcctccgcttattgatatgc-3´, 5´-gcatcgatgaagaacgcagc-3´) [14] and the PCR conditions were as follows. PCR was performed in a 25 μl volume containing 2.5 μl of 10× DreamTaq buffer green, 1 μl of 2 mM dNTPs, 1 μl of 10 μM of forward and reverse primers each, 0.25 μl of DreamTaq polymerase (ThermoScientific, UK) 1 μl of 10 ng DNA template and 18.5 μl nuclease free water. DNA template was denatured at 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 48s and a final extension at 72°C for 5 min in a thermocycler (Bio-rad, C1000). All products were profiled by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The PCR products were sequenced. Sequences were assigned to molecular species based on 98–100% sequence similarity threshold in the DNA database of Japan (DDBJ®) in accordance with standard monograph taxonomic keys. Multiple sequence alignment was performed in Muscle program [15] at default settings. Best substitution model parameters for phylogenetic inference were determined based on Akaike Information Criterion, corrected (AICc) and Bayesian Information Criterion (BIC). The maximum likelihood (ML) method was used for phylogenetic inference. All analysis was performed in MEGA 6.06 (updated v. 6140226) software [16]. The ML tree was statistically tested by 1000 bootstrap iterations.

Pathogenicity test

To validate Koch’s postulates for the pathogenic bambusicolous fungi, pathogenicity test was performed as follows. Bamboo seeds of D. hamiltonii (GenBank accession JX564903) were propagated in MS culture medium following previously established protocol [17] in a 20 cm long x 15 cm² diameter test tube. Following rooting, plants were progressively transferred to sterile soil (consisting of rice-straw vermin-compose-sand mixture (3:1)) in a 10 cm diameter pots under greenhouse conditions. Following the development of internodal culms with 15-20 true leaves, plants were sprayed with a suspension of 10° conidia/ml of each fungal pathogen under aseptic conditions. Each inoculated plant was enclosed with a plastic bag to create a near 100% humidity. Plants were observed every 12 h for the development of symptoms and pathogenicity test was performed three times. Only fungal pathogens which produced similar symptoms to those observed in the field are reported.

Results and Discussion

In Manipur, India, landraces of D. hamiltonii are densely populated in Impal East District (Figure 1). This region often witness sporadic rainfall, foggy weather, and strong wind movement during July–August each year. D. hamiltonii is rich in nutraceutical values and highly demanded by consumers [3,11]. Because of the nutritional attributes and important population size of D. hamiltonii in Manipur, the study was focused on the fungal pathogens of this edible bamboo species.

A total of 32 bambusicolous pathogenic fungi identified and validated by Koch’s postulates was deposited in DDBJ accessions (Table 1) and were used for phylogenetic reconstruction. Of the 32 fungal pathogens, 31 were Ascomycota distributed within the class of Dothideomycetes, Eurotiomycetes, Sordariomycetes and one was unclassified. Nonetheless, it has been shown that most fungi in these subclasses are pathogens [18,19]. Only one of the fungal pathogen (i.e. Perenniporia sp.) was Basidiomycetes (Table 1). Additionally, the pathogenic bambusicolous fungi belonged to the genera of Fusarium, Cochliobolus, Daldinia, Leptosphaeria, Phoma, Neodeightonia, Lasiodiplodia, Aspergillus, Trichoderma, Perenniporia, Nigrospora and Hyporales. It is estimated that there are over 630 Ascomycetes, 150 Basidiomycetes and 330 mitosporic taxa (100 coelomycetes and 230 hyphomycetes) infecting bamboo [10,20]. The finding in this study is in accordance with other data [10,20], that predominant bambusicolous fungi of bamboo are Ascomycetes (Table 1). Although Hypocreaceae is understood to be the common bambusicolous fungi [10], only one - Hypocreales sp. strain B101 was identified as a pathogenic bambusicolous via Koch’s postulates (Table 1).

The combined sequences had an estimated transition/transversion bias ratio of 1.26. The Kimura 2-parameter [21] substitution model (+G, 5 categories, parameter = 3.50) produced the following nucleotide frequencies: A = 25.00%, T/U = 25.00%, C = 25.00%, G = 25.00% and transition/transversion ratio of 1.26. The Kimura 2-parameter [21] substitution model (+G, 5 categories, parameter = 3.50) produced the following nucleotide frequencies: A = 25.00%, T/U = 25.00%, C = 25.00%, G = 25.00% and 4.71% (Table 1). Although Perenniporia was understood to be the common bambusicolous fungi [10], the best substitution model used was T92 + G + I. Initial ribotype tree for the heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach.

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The ML tree indicated that all *Fusarium* taxa formed a main node (at bootstrap value \(= 67\%\)) and strongly supported by internal branches with bootstrap values > 98\% (Figure 3). *Fusarium chlamydosporum* (2 isolates), *Fusarium proliferatum* (2 isolates), *Fusarium incarnatum* (2 isolates) were the most common *Fusarium* species (Figures 3 and 4). As shown (Figure 3), bumbusicolous fungus population on edible bamboo *D. hamiltonii* is highly diversified. Generally, predominant species of fungi life in bamboo *D. hamiltonii* is the Ascomycota, estimated to fit in about 228 genera and 70 families [10]. In decreasing frequency of occurrences, *Hypocreaceae*, *Xylariaceae*, *Lasiosphaeriaceae*, *Clavicipitaceae*, *Phyllachoraceae*, *Lophiostomataceae*, *Diatrypaceae*, *Hyaloscyhaceae*, *Paradiopsidaceae*, *Valsaceae* and *Pseudoperisporaceae* are reported families that successfully thrived on bamboo species [10].

Some fungi species were encountered only once or twice (Table 1), suggesting that the fungal community could change over time or natural fluctuation in the populations. Regardless of the 99\% bootstrap values at the node associating *Ascomycetes* strain b119 and *Peronosclerella glomerata* strain b116 (Figure 3), we did not find similarity at the morphological level using standard monographs. Furthermore, *Ascomycetes* strain b119 did not match sequences in the databases at 100\% threshold value. This may be an indication of the weakness in public DNA repositories to delineate all fungi. Within the surveillance period, dominant fungal genera were *Peronosclerella glomerata*, *Aspergillus* spp., *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Chociobolus lunatus* (Figure 3b).

Aspergillus species have not been reported among the bumbusicolous fungi in previous studies [10,22,23]. In this present study, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* (Figure 2a), *Neodeightonia subglobosa* (Figure 3b), *Trichoderma* species (Figure 3c and 3d), *F. incarnatum* (Figure 3e) were identified. All the *Aspergillus* species sporulated on *D. hamiltonii* during the infestation period of 72 h (Figure 5a-5c). *Trichoderma* species and *Aspergillus* species were recently shown to be pathogens of *Guadua* species, which are abundantly distributed in Ecuador, Chile and Peru ([ftp://ftp.fao.org/docrep/fao/010/ah782e/AH782e00.pdf](ftp://ftp.fao.org/docrep/fao/010/ah782e/AH782e00.pdf)) only. This present study provide the first report of *Trichoderma* species (Figure 3c and 3d) and *Aspergillus* species causing diseases on edible bamboo *D. hamiltonii* (Figure 5a-5c). Although some *Aspergillus* spp. and *Trichoderma* spp. are used as biocontrol agent [24-26], they are important cellulase producers [27,28], which is an important factor for pathogenicity. On this basis, some *Aspergillus* spp. and *Trichoderma* spp. are opportunistic colonizers of economic importance [29-31].

| Pathogens                  | DDBJ Accession | Strain | Tissue | Phylum          | Class/Subclass | Collection date | Period of occurrence |
|----------------------------|----------------|--------|--------|-----------------|----------------|-----------------|----------------------|
| *Fusarium incarnatum*      | AB918015       | B120   | Leaf   | Ascomycota      | Sordariomycetes| 10-06-2012      | May-June             |
| *Fusarium chlamydosporum*  | AB918016       | B121   | Internode | Ascomycota     | 18-07-2012      | June-July        |
| *Fusarium camptoceras*     | AB918017       | B122   | Node   | Ascomycota      | 07-07-2013      | June-July        |
| *Fusarium proliferatum*    | AB918018       | B124   | Internode | Ascomycota     | 12-07-2013      | June-July        |
| *Nigrospora oryzae*        | AB918019       | B125   | Leaf   | Ascomycota      | 19-08-2013      | September-July    |
| *Fusarium chlamydosporum*  | AB918020       | B126   | Leaf   | Ascomycota      | 21-08-2012      | July-August      |
| *Nigrospora sphaerica*     | AB918021       | B127   | Leaf   | Ascomycota      | 15-03-2014      | March-April       |
| *Fusarium oxysporum*       | AB918022       | B129   | Leaf   | Ascomycota      | 03-05-2012      | May-June          |
| Chaetomium bostrychodes     | AB918027       | L3     | Internode | Ascomycota     | 06-06-2012      | June-July        |
| *Trichoderma reesi*        | AB918031       | L9     | Leaf   | Ascomycota      | 15-07-2013      | July-August      |
| *Fusarium proliferatum*    | AB918023       | B130   | Leaf   | Ascomycota      | 04-07-2014      | July-August      |
| *Trichoderma asperellum*   | AB918007       | L7     | Leaf   | Ascomycota      | 10-11-2012      | October-November  |
| *Fusarium incarnatum*      | AB918010       | B110   | Leaf   | Ascomycota      | 10-07-2012      | July-August      |
| *Hypocreales*              | AB918034       | B101   | Leaf   | Ascomycota      | 09-07-2012      | June-July        |
| *Daldinia eschscholzii*     | AB918033       | S1     | Internode | Ascomycota     | 10-09-2013      | August-September  |
| *Phoma plurivora*          | AB918009       | B104   | Leaf   | Ascomycota      | 10-07-2013      | June-July        |
| *Phoma herbarum*           | AB918006       | L29    | Leaf   | Ascomycota      | 04-10-2013      | October-November  |
| *Cochliobolus lunatus*     | AB918004       | L26    | Leaf   | Ascomycota      | 09-06-2014      | June-July        |
| *Lasiodiplodia theobromae* | AB918000       | L1     | Node   | Ascomycota      | 15-07-2012      | July-August      |
| *Lasiodiplodia miyabeanaus*| AB918003       | L17    | Leaf   | Ascomycota      | 13-08-2013      | July-September   |
| *Peyronellaea glomerata*   | AB918011       | B116   | Leaf   | Ascomycota      | 05-08-2012      | August-November   |
| *alternaria sp.*           | AB918012       | B117   | Leaf   | Ascomycota      | 07-10-2013      | September-December|
| *Lasiodiplodia theobromae* | AB918025       | L14    | Leaf   | Ascomycota      | 07-07-2014      | June-August      |
| *Phoma herbarum*           | AB918026       | L16    | Internode | Ascomycota     | 06-08-2013      | July-August      |
| *Lasiodiplodia theobromae* | AB918028       | L18    | Leaf   | Ascomycota      | 07-07-2014      | June-August      |
| *Cochliobolus miyabeanaus* | AB918032       | LUN1   | Leaf   | Ascomycota      | 13-06-2012      | July-October      |
| *Neodeightonia subglobosa* | AB918035       | S3     | Leaf   | Ascomycota      | 19-11-2013      | October-November  |
| *Aspergillus fumigatus*    | AB918018       | B118   | Leaf   | Ascomycota      | 03-08-2013      | June-August      |
| *Aspergillus flavus*       | AB918002       | L12    | Leaf   | Ascomycota      | 08-05-2012      | June-August      |
| *Aspergillus niger*        | AB918001       | L11    | leaf   | Ascomycota      | 07-05-2013      | June-August      |
| *Ascomycetes sp.*          | AB918014       | B119   | Leaf   | Ascomycota      | 14-06-2013      | June-August      |
| *Perenniporia sp.*         | AB918008       | B100   | Leaf   | Basidiomycota   | 02-06-2013      | June-July        |

Table 1: Hit list of unreported pathogenic bumbusicolous fungi limiting edible bamboo production.

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Figure 2: Multiple sequence alignment depicting the variations in bambusicolous fungi the alignment was performed in CLC workbench (Qiagen, Valencia, CA) and variable nucleotides are colored.

Figure 3: Taxonomical placement of unreported pathogenic bambusicolous fungi of edible bamboo. a: A maximum likelihood tree of highest log likelihood (-1116.47), associated taxa clustered together and supported with 1000 bootstrap reiterations. The ribotype tree is scaled, with branch lengths measured as the number of substitutions per site. b: Brown macroconidia of Neodeightonia subglobosa. c: Conidiophore of Trichoderma asperellum and close-up shows detail of hyphae branching. d: Conidiophore of Trichoderma reesei. e: Conidia of Fusarium incarnatum. All micrographs were acquired with Olympus DP70 camera (Olympus BX61, USA) at 1000× magnification and scale bars represent 15 μm.
F. incarnatum causing rot disease of bamboo in India. Under field conditions, Fusarium infected culms were bend and fallen. Also, F. moniliforme var. intermedium has been reported to be associated with rot of emerging culms in B. bambos [22]. Severe rot and blight diseases of bamboo have been observed in Bangladesh [32,33] and in India [22,34] caused by Fusarium species.

Recently in India, it was shown that Fusarium semitectum caused both blight and rot disease of Bambusa tulda [35]. Also, F. oxysporum and F. chlamydosporum have been reported in India on Solanum tuberosum L and Capsicum annum L, respectively [36,37]. Cochliobolus species caused foliar and sheath blight diseases, manifested by brownish oval-shaped and water-soaked lesions which became black as the bamboo leaf turned yellowish (Figure 5f). Cochliobolus species causes diseases on Bambusa bambos and Dendrocalamus longispathus [22], with similar characteristic symptoms to those described herein. Symptoms caused by C. lunatus in bamboo are similar to leaf spot disease of rice (Oryza sativa), wheat (Triticum aestivum), cassava (Manihot esculenta), sorghum (Sorghum bicolor) and potato (Solanum tuberosum) [38-42]. It was suggested that C. lunatus produced brown-to-black symptoms in many plant hosts because of its melaninated colonizing hyphae [42-44]. Nonetheless, other recurrent leaf spot diseases of bamboo are caused by many species of Phyllachora [44]. Interestingly, other studies [35,45,46] have reported new bambusicolous fungi causing a major threat to bamboo production (Table 2). The danger of all the reported bambusicolous pathogenic fungi is that, once bamboo shoots are infected in the field, fungal proliferation continues up to the market level and account to severe economic losses.

| Figure 4: Micrographs of predominant fungi pathogens of edible bamboo Dendrocalamus hamiltonii cultured on V8 agar medium. a: Peyronellaea glomerata strain b116 showing details of hyphae, conidia and bar=30 µm. b: Alternaria sp. strain b117 showing details of hyphae, conidium and bar=10 µm. c: Cochliobolus lunatus strain L26 showing details of conidia and bar=20 µm. d: Fusarium oxysporum strain b129 and bar=25 µm. e: Aspergillus flavus strain L12 and scale bar=10 µm. Images were acquired with Olympus DP70 camera (Olympus BX61, USA) at 1000× magnification. |
| Figure 5: Pathogenicity test performed with plantlets of D. hamiltonii in test tube to verify Koch’s postulates. a: Sporulating Aspergillus niger and colonization leaf tissue (400× magnification). b: Sporulating Aspergillus fumigatus and colonization of leaf tissue (400× magnification). c: Sporulating Aspergillus flavus and colonization of leaf tissue (400× magnification). d: Leaf rot disease caused by Fusarium proliferatum. e: Colonization marked by leaf rot caused by Fusarium incarnatum with evidence of fruiting bodies. e: Brown-to-black leaf lesion disease caused by Cochliobolus lunatus. |

It was observed that all the Fusarium species caused rot disease of bamboo shoots, rot of growing culms, and rot of leaf tissues and damping-off of seed plantlets during pathogenicity test (Figure 5d and 5e). Noteworthy, this is the first report of F. chlamydosporum, F. oxysporum, F. camptoceras, F. oxysporum, F. proliferatum and Blight and rot diseases of B. tulda caused by Fusarium semitectum [35].

Bamboo rust disease of B. vulgaris caused by Uredium sp [45].

Kwellingia rust of B. vulgaris caused by Kwellingia divina (syn. Dasturella divina) [45].

Bamboo witches broom disease of Phyllostachys bambusoides caused by Aciculusporium take [46].

*Permission for images was granted by Scot N, Matthew G, Tanaka E and Teron R.

Table 2: Some significant rare bamboo diseases recently communicated.
Conclusion

The study shows that poor pathological management of bambusicolous fungi is valued at 40% losses of the total $818.6 million generated annually in North East India. Until 2010, it was thought fungi belonging to the genera of *Kewelingia*, *Pucinia*, *Uredo*, *Phakospora*, *Stereostratum*, and *Tunicospora* which caused bamboo rust diseases was the most predominant pathogenic bambusicolous fungi and distributed worldwide. In our study, two principal damages are often caused by these pathogenic bambusicolous fungi, viz., 1) staining of bamboo shoots and 2) structural decay of bamboo shoots which leads to economic losses to all stakeholders in the commercial chain. Our data indicated that *Fusarium*, *Cochliobolus*, *Daldinia*, *Leptosphaeria*, *Phoma*, *Neodeightonia*, *Lasiodiplodia*, *Aspergillus*, *Trichoderma*, *Peyronellaea*, *Perenoporia*, *Nigropora* and *Hyporales* are new pathogenic bambusicolous fungi genera limiting the production of *D. hamiltonii*. Given most bamboo species are endangered and threatened with extinction [4,5], further studies are required to understand the mechanism of bamboo invasion. The emergence of bambusicolous fungi reported on edible bamboo *D. hamiltonii* in this study illustrated the urgent need for developing a piecemeal control strategy [47].

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