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Phylogeny and pathogenicity of fungal species in the family botryosphaeriaceae associated with mango (*Mangifera indica*) in Thailand

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Abstract Fungal species belonging to the family Botryosphaeriaceae are cosmopolitan and important pathogens of a wide range of plant hosts which include mango. Based on only morphological study, these fungi have been traditionally identified as genus *Lasiodiplodia* on mango in Thailand and mainly named as *Lasiodiplodia theobromae*. In this study a combination of morphology and phylogenetic inference were used to correctly identify isolates resembling botryosphaeriacous fungi that obtained from mango in Thailand. Phylogenetic inference applied here clearly revealed six clades together with the isolates obtained from Thailand grouping with *Lasiodiplodia* spp., which included *L. pseudotheobromae*, *Lasiodiplodia* sp., and *L. viticola*. To our knowledge, this study represented the first report of *L. pseudotheobromae* associated with mango in Thailand. Pathogenicity test was performed on mango fruits, stem-ends, and trees of the Mahachanok and Keawmorakot cultivars indicated that endophytic fungi might change to be opportunistic pathogens and pathogenic as latent pathogens when plant under stress or favorable environments for diseases development. In this study *L. pseudotheobromae* and *L. viticola* were the most pathogenic on the fruits and stem-ends, whereas *L. pseudotheobromae* and *Lasiodiplodia* sp. were more pathogenic to the inoculated trees.

Keywords: Phylogeny, Pathogenicity, Botryosphaeriaceae, Mango, Thailand

Introduction

Mango (*Mangifera indica*) is an economically important tropical fruit and agricultural commodity in Thailand that is exported to several countries, especially the Asian and European markets (Jitareerat *et al*., 2005). The Food and Agriculture Organization of the United Nations (FAO) has estimated that in

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2010-2011, Thailand represented the third biggest mango producer globally (http://faostat.fao.org). A limiting factor for mango production and marketability is diseases caused by fungi, and these fungi included several species in the family Botryosphaeriaceae which is a species-rich family in the Ascomycota (Crous *et al*., 2006). These fungi are known to have a cosmopolitan distribution and have a wide range of plant hosts, included mango (Abdollahzadeh *et al*., 2010; Ismail *et al*., 2012). Diseases associated with these fungi during mango production included pre and post-harvest diseases such as canker, dieback, panicle brown rot, fruit rot, and stem-end rot (Abdollahzadeh *et al*., 2010; de Oliveira Costa *et al*., 2010; Sakalidis *et al*., 2011). These fungi are generally regarded as opportunistic pathogens with a latent endophytic stage causing numerous diseases when the host plants are exposed to stress or favorable conditions for disease development (Slippers and Wingfield, 2007). Since the taxonomy of these fungi has traditionally been based on morphological characteristics, the overlap of some characters causes some confusion and has led to incorrect identification of some species, especially in Thailand. The present study represents the first attempt to correctly characterize these fungi from mango collected in Thailand using a combination of morphological characteristics and a multi-gene phylogenetic inference. The pathogenicity of these fungi is also tested on two important Thai mango cultivars.

Materials and methods

Isolation and morphology

Twigs of mango cultivars Aokrong, Fahbandan, Keawmorakot, Mahachanok, and R2E2 were collected from Chiang Mai province, Thailand. They were incubated in moist chamber at room temperature for 1-2 weeks to allow the development of fungal structures. Species belonging to Botryosphaeriaceae were isolated and single spored on Potato Dextrose Agar (PDA). Morphological characteristics were determined as described by Alves *et al*. (2008) and investigated on stereo (Olympus SZ50) and compound microscope (Olympus BX51).

Molecular and phylogenetic analysis

Total genomic DNA was extracted from 7 day-old cultures using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) following to the manufacturer's instructions. The ITS region of the nuclear rDNA was amplified using primers ITS5 and ITS4 (White *et al*.,

1990). A partial region of the translation elongation factor 1-α (EF1-α) was amplified using primes EF1-728 (Carbone and Kohn, 1999) and EF2 (O'Donnell *et al*., 1997). Sequences were generated in both directions, using the same primer pairs as were used for the amplifications, using the Big Dye terminator sequencing kit v.3.1 and run on an ABI PRISM™ 3730 DNA automated sequencer (Perkin-Elmer Applied BioSystems, Foster City, CA, USA).

The generated sequences were edited and aligned together with other sequences obtained from GenBank using MEGA v 5.1 and MAFFT v 7, respectively. Bayesian Inference analyses were performed on the combined datasets of ITS and EF1-α sequences using MrBayes v 3.2.1, rooted with *Guignardia citricarpa* (CBS 111.20). The analyses included two independent runs of the Markov Chain Monte Carlo (MCMC) algorithm with four MCMC chains run simultaneously applying the sixth substitution model that was general time-reversible (GTR) with rate variation of gamma-distribution (G) and proportion of invariable site (I). The analyses were run until the average standard deviation of split frequency came below 0.01 within 100,000,000 generations and the posterior probabilities and consensus tree were determined after the first 25% of trees were discarded as the "burnin phase".

Pathogenicity test

Pathogenicity test on mango fruits and stem-ends

Unripe mature mango fruits of cultivars Mahachanok and Keawmorakot were collected from Chiang Mai province. Healthy fruits were surface sterilized using 70% ethanol and immersed into 1.5 % NaOCl (Clorox ®), followed by rinsing with sterile water for 5 minutes and finally air dried for 10-15 min. Wounds inoculations were performed following the protocol described by de Oliveira Costa *et al*. (2010) with six replications for each treatment. A mycelium plug of 6 mm diam. of a 7day-old culture was placed, mycelial side down into the wound and a sterile PDA plug was used as control. Inoculated fruits were incubated in plastic boxes with moist paper towel for 7 days at room temperature. The inoculated fruits were monitored daily for lesions formation and measured accordingly.

Pathogenicity test on mango trees in greenhouse

One year-old of mango trees of cultivars Mahachanok and Keawmorakot with average heights of 800-900 mm and 40-60 mm diam. stem, were maintained in the greenhouse to acclimatize for 2 weeks. The inoculation area,

at the height of 200-250 mm height or the second node above the soil level, was surface sterilized using 70% ethanol. A wound of 6 mm^2 was made with a sterile blade by removing the bark of the stem and exposing the cambium. A mycelial plug of 6 mm diam. was inserted into the wound with the mycelium facing the cambium. A sterile PDA plug was used as control treatment. The inoculated area was wrapped with laboratory film to prevent desiccation and contamination.

Results

Isolation and morphology

Six isolates were obtained from the twigs representing fungal species in the family Botryosphaeriaceae. The morphological characteristics broadly indicated that the isolates represented *Lasiodiplodia* spp. with ovoid to ellipsoid, hyaline conidia, becoming dark brown and developing longitudinal striations with aged, and having cylindrical hyaline paraphyses between the conidiogenous cells (Crous *et al*., 2006; Alves *et al*., 2008).

Conidia of *L. pseudotheobromae* isolates B031/1, B035/1, B041/1, and B041/2 were larger (23.5-32 x 14-18 µm) and more ellipsoid than isolates B042/1 and B047/1 (Alves *et al*., 2008). The conidia of *Lasiodiplodia* sp. isolate B042/1 were smaller (18.7-22.7 x 12.1-13.9 µm) but the paraphyses were longer (up to 127 µm) than *L. psuedotheobromae* (Abdollahzadeh *et al*., 2010). By contrast conidia of *L. viticola* isolate B047/1 were smaller (19.5 x 9.5 µm) than *L. pseudotheobromae* (Urbez-Torres *et al*., 2012) and *Lasiodiplodia* sp.

Molecular and phylogenetic analysis

Amplicons of approximately 570 bp for ITS and 500 bp for EF1-α were determined for each isolate. Bayesian Inference analyses of the combined dataset, revealed six clades with high posterior probability support. These clades were distinguished as follows: Clade I representing *Pseudofusicoccum* sp., Clade II representing *Neofusicoccum* sp., Clade III representing *Botryosphaeria* sp., Clade IV representing *Neoscytalidium* sp., Clade V representing *Lasiodiplodia* sp., and Clade VI representing *Barriopsis* sp. Isolates from Thailand were cluster within Clade V, with isolates B031/1, B035/1, B041/1, and B041/2 placed with *L. pseudotheobromae*, isolate B042/1 *Lasiodiplodia* sp. was closely related with *L. iraniensis*, and isolate B047/1 placed with *L. viticola* (Fig 1).

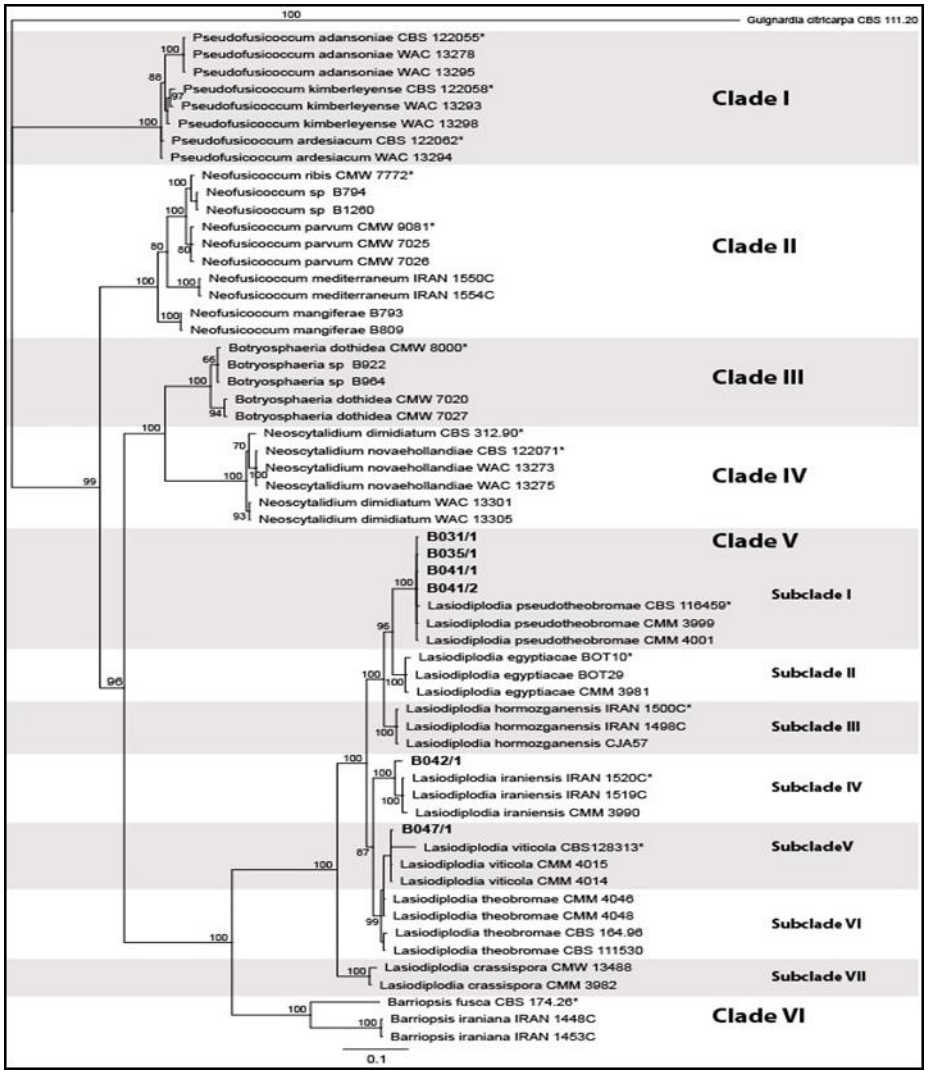

Fig. 1. The consensus tree obtained from Bayesian inference of the combined sequence datasets of ITS and EF1-α with posterior probability values indicated at the branches. Isolates from Thailand were indicated in bold and * represented ex-type isolates.

Pathogenicity test

Pathogenicity test on mango fruits and stem-ends

Brown-black lesions with circular to irregular shapes were observed around the inoculation points three days after inoculation. No significant difference was observed between the two mango cultivars. Isolates B035/1 and B047/1 represented the most virulent isolates with the average lesion diam. of

80 mm on both cultivars. Additionally, it was observed that lesions development at the stem-end was slower than the rest of the fruit surface for both cultivars (Fig 2).

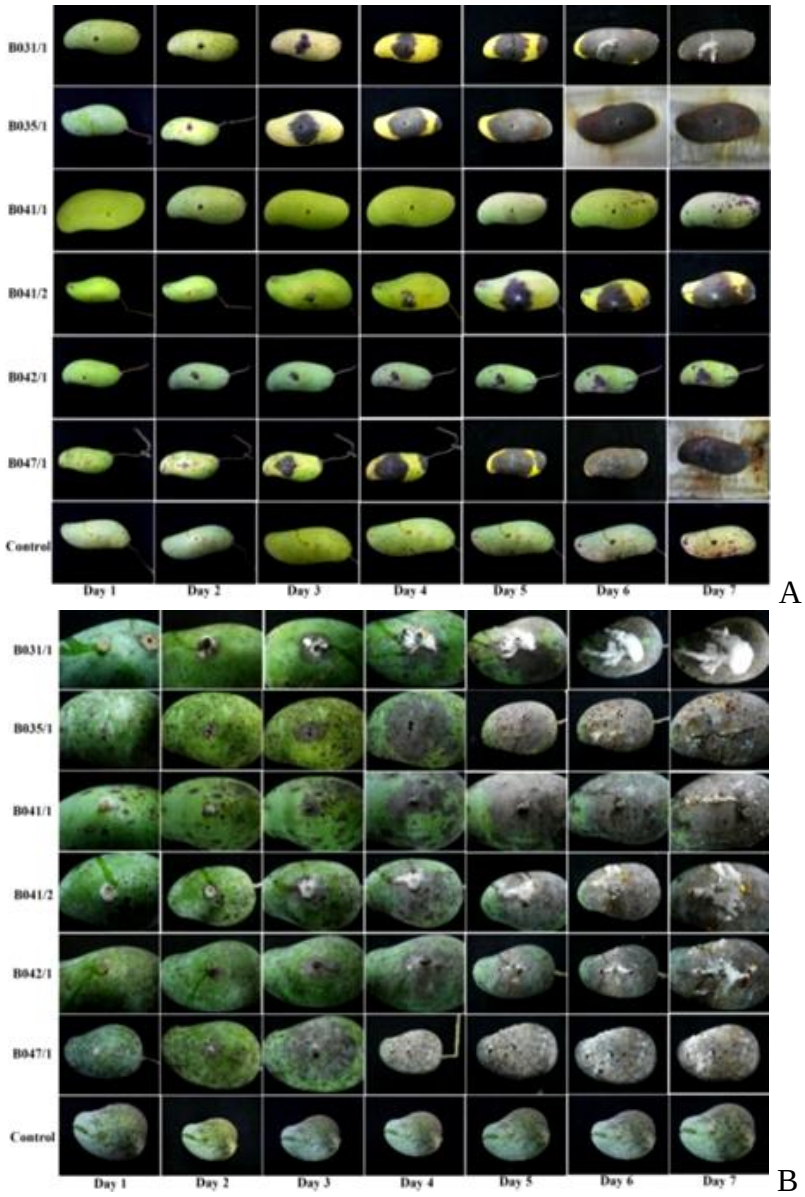

Fig. 2. Pathogenicity test on fruits and stem-ends of Mahachanok (A) and Keawmorakot cultivars (B) with *L. pseudotheobromae* isolates B031/1, B035/1, B041/1, and B041/2, *Lasiodiplodia* sp. isolate B042/1, and *L. viticola* isolate B047/1 over a period of 7 days.

Pathogenicity test on mango trees in greenhouse

Stem inoculation with the tested isolates on the two mango tree cultivars resulted in the formation of leave malformation and blight resembling leave scorch, with brown-reddish sap exuding from the inoculation points. After six weeks, brown necrotic lesions, longitudinally along the stems, were observed radiating from the inoculation points. *L. pseudotheobromae* isolates B041/1, B041/2 and *Lasiodiplodia* sp. isolate B042/1 produced brown necrotic lesion with an average penetration of 4 mm into vascular tissue of the stems (Fig 3).

Fig. 3. Pathogenicity test on mango trees of Mahachanok (A) and Keawmorakot cultivars (B) with *L. pseudotheobromae* isolates B031/1, B035/1, B041/1, and B041/2, *Lasiodiplodia* sp. isolate B042/1, and *L. viticola* isolate B047/1 over a period of 7 days.

Discussion

In this study, a combination of morphology and phylogenetic inference was employed to identify a number of fungal isolates belonging to the family Botryosphaeriaceae, obtained from mango twigs. Bayesian Inference revealed six clades using the combined sequence datasets of ITS and EF1-α. Isolates from this study were placed into subclades representing *L. pseudotheobromae*, *Lasiodiplodia* sp., and *L. viticola*. Since *L. pseudotheobromae* can be easily

misidentified as *L. theobromae*, to our knowledge, this study represented the first report of *L. pseudotheobromae* associated with mango in Thailand.

Pathogenicity test showed that these fungi were pathogenic to mango. These results were also supported by Sakalidis *et al*. (2011), which demonstrated that endophytic fungi from the native plants caused diseases on mango fruits and stems. Lesion development was observed faster in fruits than at the stem-ends and it could be influenced by the thicker peel at the stem-end of the fruit. The pathogenicity of the tested isolates was not significantly different for both cultivars. This was supported by the observation of Slippers and Wingfield (2007) and Abdollahzadeh *et al*. (2013) that the plant hosts of these fungi are generally unspecific whereas environmental factors play a more important role in diseases development. *Lasiodiplodia pseudotheobromae* (B035/1) and *L. viticola* (B047/1) were the most virulent isolates on both cultivars. These results were supported by Sakalidis *et al*. (2011) which found that *L. pseudotheobromae* was the most virulence on mango in Western Australia.

Symptoms observed during the pathogenicity test on mango trees included leave malformation and scorch, with brown-reddish sap exuding from the inoculation points and brown necrotic lesions penetrating deep into vascular tissue of inoculated stem. These symptoms were similarly to canker diseases caused by Botryosphaeriaceae fungi previously observed in another study (Faber *et al*., 2010). *Lasiodiplodia pseudotheobromae* (B041/1 and B041/2) and *Lasiodiplodia* sp. (B042/1) represented the most virulence pathogenic on inoculated stem. This study showed that *Lasiodiplodia* spp. was associated with mango diseases. The correct identification is of utmost important to understand the ecology and distribution of these fungi on mango in Thailand. This, in turn, will lead to more effective disease management strategies allowing the mango industry of Thailand to manage these fungi in the plantations and during post-harvest treatment of the fruits.

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