A new sudden decline disease of bullet wood in Thailand is associated with *Ceratocystis manginecans*

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Abstract In Thailand, since 2013, sudden decline of bullet wood (*Mimusops elengi*) has been noted. The causal agent of this disease was identified based on both morphological and molecular properties as *Ceratocystis manginecans*. A portion of its ITS, β -tubulin and TEF1- α genes were amplified by PCR with the universal primer pairs PN3/PN16, β t1a/ β t1b and EF1-728F/EF1-986R, respectively. Sequencing of the PCR product confirmed this pathogen was *C. manginecans*. This is the first report on the new sudden decline disease of bullet wood in Thailand associated with *C. manginecans*.

Keywords *Mimusops elengi* · PCR · Phytopathogenic fungi · Universal primers

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, and it is known in Thailand as “Pikul”. These trees range from small to large, and are found in all parts of Thailand where bullet wood is cultivated in gardens as an ornamental tree, and planted along avenues because of its fragrant flowers. Bullet wood is a medicinally important plant; its bark has anti-inflammatory properties, along with analgesic and antipyretic activities (Purnima et al. 2010).

In 2013, wilt and dieback symptoms were observed for the first time on *M. elengi* in the grounds of Prince of Songkla University, Hatyai, Songkhla, Thailand. The aim of this study was to identify the causal agent of this sudden decline disease of bullet wood. Sap stain moulds were isolated from infected

bullet wood, and identified based on morphological characteristics and molecular analysis.

The bullet wood branches were cut transversely and longitudinally and had red to dark red internal discoloration (Fig. 1e and f). Bark beetle emergence holes on the trunks of infected bullet wood were also found (Fig. 1d, arrows). Symptomatic wood pieces were extracted by cutting, sealed in plastic bags, and transported in cooler boxes to the laboratory where isolations were conducted immediately. Three sections of 1–3 cm in length were cut from the margins of lesions, including some healthy wood. These wood sections were surface-sterilised with rapid scorching in a flame. The wood samples were then placed on potato dextrose agar (PDA), and the agar plates were incubated for 10 days at room temperature. The fungal colonies were subcultured on PDA, and incubated for 10 days. Single spore subcultures were maintained for routine use on PDA slants.

The isolated fungi were initially identified based on morphological characteristics of a 10 day old culture. The sexual and asexual state of the fungus were mounted in lactophenol on glass slides, and examined by light microscopy. The white hypha of colonies emerged from infected tissue after a 72 h incubation. Isolates of the fungus grew slowly on PDA with ethylene production. The fungal cultures had light grey mycelia and dark grey to greenish submerged mycelia with few observed ascomata (Fig. 2). The ascomatal bases were black and globose to subglobose (Fig. 2a), and the fungal isolates produced ascomatal necks with divergent ostiolar hyphae at their tips (Fig. 2b), from which hat-shaped ascospores were released (Fig. 2b and f). This fungus also produced young chlamydospores, chains of conidia and barrel-shaped conidia (Fig. 2c, d and e).

Based on morphological characters, the fungus was identified as *Ceratocystis manginecans*, a widely distributed species pathogenic on mango (Ploetz 2003). A culture of the fungus

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Fig. 1 Characteristics of sudden decline disease on bullet wood, observed at Prince of Songkla University (a), wilted leaves of bullet wood (b–c), wounded bark and bark beetle emergence holes (d, arrows), and sap stain mould on bullet wood (e–f)

was deposited in the Culture Collection of Pest Management Department, Faculty of Natural Resources, Prince of Songkla University, Thailand, as PSU-PK01.

To confirm the species identification, isolates were cultured on PDA at room temperature for 2 weeks. Total DNA was

extracted by the cetyltrimethyl ammonium bromide (CTAB) method (Kollar et al. 1990), as follows. Mycelia was collected and cleaned with sterilised distilled water (DW), and then dried and kept at -20°C prior to isolating the DNA. Frozen mycelia were ground to a fine powder with a small mortar and

Fig. 2 Morphological characteristics of *Ceratocystis manginecans* (PSU-PK01), **a** globose ascomata with long neck, **b** divergent ostiolar hyphae release ascospores (arrow), **c** young chlamydospore, **d** chain of conidia, **e** barrel-shaped conidia and **f** hat-shaped ascospores in side view. Scale bars: **a**=100 μm , **b**–**d**=10 μm , **e**=20 μm , **f**=5 μm

Fig. 3 Neighbour-joining tree generated using sequences of the ITS, specifically the β -tubulin and TEF1- α gene regions, of *Ceratocystis manginecans* (PSU-PK01). *Chaetomium globosum* was included as an outgroup. The numbers at branches indicate the percentage support using 1000 bootstrap replications

pestle, mixing with liquid nitrogen. The powders were transferred to 1.5 ml microtubes, CTAB solution (2.0 g CTAB, 10 ml of 1 M Tris pH 8.0, 4 ml of 0.5 M DETA pH 8.0, 28 ml of 5 M NaCl, 40 ml of DW and made up to 100 ml with DW) was added, incubated at 65 °C and mixed with chloroform isoamyl (24:1). The epiphase was removed to a fresh microtube and isopropanol was added. After centrifugation, the pellet was collected, air dried, and finally dissolved in 20 μ l of sterile deionised water (DI).

The internal transcribed spacer (ITS), β -tubulin (β t) and transcribed elongation factor 1- α (TEF1- α) gene regions were amplified using a BIO-RAD T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplification of the gene regions used the primers PN3 and PN16 (Neuveglise et al. 1994), β t1a and β t1b (Glass and Donaldson 1995) and EF1-728 F and EF1-986R (Jacobs et al. 2004). The PCR was carried out sequentially in a final volume of 50 μ l containing 10 pmol of each primer, 2x DreamTaq Green PCR Master Mix (Thermo Scientific) and 50 ng of template DNA. An initial denaturation step for 3 min at 95 °C was followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 50 °C,

and extension for 1 min at 72 °C, with a final extension step of 10 min at 72 °C. The PCR products were visualised by agarose gel electrophoresis. The portion of ITS gene region was sequenced at Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand, by automated DNA sequencing with ABI Prism 377 (Applied Biosystems, USA) using the same primers used in the PCR reaction.

For the ITS, β -tubulin, and TEF1- α gene regions, PCR amplification gave fragments of about 500 base pairs in size. The sequences of the amplified products were then deposited in the GenBank database and assigned accession numbers AB905498, AB919056, AB919057 for the ITS, β -tubulin and TEF1- α gene, respectively, and they were compared with the sequences of *C. manginecans* available at GenBank. BLAST searches in GenBank indicated that our isolates grouped within *C. manginecans* species with 99 % identity of the sequences. The phylogenetic relationships of this select isolate with closely related taxa was analysed using neighbour-joining methods. Phylogeny was assessed using a bootstrap analysis with 1000 replicates. According to the phylogenetic relationships derived

Table 1 *Ceratocystis* spp. used in this study

Species	Host	GenBank accession number	Geographical origin
<i>C. albifundus</i>	<i>Terminalia sericea</i>	KC691452 KC691476 KC691500	South Africa
<i>C. albifundus</i>	<i>Acacia</i> sp.	AF388947 DQ371649 EF070401	South Africa
<i>C. atrox</i>	<i>Eucalyptus</i> sp.	EF070417 EF070433 EF070405	Australia
<i>C. atrox</i>	<i>Eucalyptus</i> sp.	EF070414 EF070430 EF070402	Australia
<i>C. cacaofunesta</i>	<i>Theobroma cacao</i>	DQ520637 EF070428 EF070399	Ecuador
<i>C. cacaofunesta</i>	<i>Theobroma cacao</i>	DQ520636 EF070427 EF070398	Costa Rica
<i>C. coerulescens</i>	<i>Larix kaempferi</i>	KC305119 KC335997 KC405274	Japan
<i>C. coerulescens</i>	<i>Picea abies</i>	KC305117 KC335995 KC405272	Germany
<i>C. colombiana</i>	<i>Citrus</i> sp.	AY233862 AY233872 EU241489	Colombia
<i>C. colombiana</i>	<i>Schizolobium parahyba</i>	AY233861 AY233873 EU241490	Colombia
<i>C. colombiana</i>	<i>Citrus</i> sp.	AY233863 AY233871 EU241488	Colombia
<i>C. fimbriata</i>	<i>Ipomoea batatas</i>	DQ520629 EF070442 EF070394	USA
<i>C. fimbriata</i>	<i>Ipomoea batatas</i>	AF264904 EF070443 EF070395	Papua New Guinea
<i>C. fujiensis</i>	<i>Larix</i> sp.	KC305115 AY233951 KC405270	Japan
<i>C. laricicola</i>	<i>Larix</i> sp.	KC305112 KC335990 KC405267	United Kingdom
<i>C. manginecans</i>	<i>Acacia crassicarpa</i>	EU588665 EU588644 EU588654	Indonesia
<i>C. manginecans</i>	<i>Mangifera indica</i>	EF433304 EF433313 EF433322	Pakistan

Table 1 (continued)

Species	Host	GenBank accession number	Geographical origin
<i>C. manginecans</i>	<i>Acacia crassicarpa</i>	EU588664 EU588643 EU588653	Indonesia
<i>C. manginecans</i>	<i>Mangifera indica</i>	AY953385 EF433310 EF433319	Oman
<i>C. manginecans</i>	<i>Mimusops elengi</i>	AB905498 AB919056 AB919057	Thailand
<i>C. moniliformis</i>	<i>Schizolobium parahybum</i>	FJ151426 FJ151460 FJ151482	Ecuador
<i>C. moniliformis</i>	<i>Eucalyptus grandis</i>	HQ203212 HQ203229 HQ236431	South Africa
<i>C. papillata</i>	<i>Annona muricata</i>	AY233868 AY233878 EU241483	Colombia
<i>C. papillata</i>	<i>Theobroma cacao</i>	GQ478239 GQ478241 GQ478237	Colombia
<i>C. papillata</i>	<i>Theobroma cacao</i>	GQ478240 GQ478242 GQ478238	Colombia
<i>C. paradoxa</i>	<i>Cocos nucifera</i>	JQ963886 JQ963884 JQ963883	Brazil
<i>C. platani</i>	<i>Platanus</i> sp.	DQ520630 EF070425 EF070396	USA
<i>C. platani</i>	<i>Platanus</i> sp.	EU426554 EF070426 EF070397	Greece
<i>C. polonica</i>	<i>Picea abies</i>	AY233907 AY233932 KC583316	Austria
<i>C. polychroma</i>	<i>Syzygium aromaticum</i>	AY528971 AY528967 AY528979	Indonesia
<i>C. populicola</i>	<i>Populus</i> sp.	EF070419 EF070435 EF070407	USA
<i>C. savannae</i>	<i>Pterocarpus angolensis</i>	GU078453 GU078457 GU078461	South Africa
<i>C. savannae</i>	<i>Colophospermum mopane</i>	KC691469 KC691493 KC691517	South Africa
<i>C. smalleyi</i>	<i>Carya</i> sp.	EF070420 EF070436 EF070408	USA

Table 1 (continued)

Species	Host	GenBank accession number	Geographical origin
<i>C. variospora</i>	<i>Prunus</i> sp.	EF070421 EF070437 EF070409	USA
<i>C. rufipenni</i>	<i>Picea glauca</i>	KC305129 KC336007 HM569655	Canada
<i>C. variospora</i>	<i>Prunus</i> sp.	EF070422 EF070438 EF070410	USA
<i>C. virescens</i>	<i>Fagus americana</i>	KC305133 KC336011 EF070413	USA

The sequences in bold were generated in this study

from the neighbour-joining analysis, our *C. manginecans* isolate (PSU-PK01) in *Mimusops elengi* was closely related to *C. manginecans* in *Mangifera indica* and *Acacia crassicarpa* (Fig. 3 and Table 1). This sequence similarity to prior cases of *C. manginecans* corroborates the identification by phenotypic characteristics, suggesting that the causal agent of sudden decline disease on bullet wood in Thailand, represented by the PSU-PK01 isolate, should be regarded as *C. manginecans*.

To determine the pathogenicity of fungi isolated, an inoculation study was conducted on 3 month old bullet wood seedlings using an injection method. A conidial suspension was prepared by flushing 10 day old cultures with sterile DW, while the control treatment used sterile DW alone. The inoculation amount of 0.2 ml was injected into the stem by using a disposable needle and syringe. The inoculated plants were then incubated under greenhouse conditions (30 °C, 50 % relative humidity). Fungal isolates were re-isolated and re-identified using morphological characteristics for Koch's postulates confirmation. The fungi were shown to be pathogenic in young bullet wood, with plants exhibiting wilt symptoms 14 days after inoculation (data not shown). When re-isolated, the fungus was phenotypically identical to the prior isolate of *C. manginecans* (PSU-PK01).

To our knowledge this is the first confirmed record of *C. manginecans* associated with decline of *M. elengi*. *Ceratocystis* spp. is a species complex that has been reported worldwide, causing vascular discoloration, decline, wilt, canker and root diseases (Kile 1993). Thousands of mango trees have been killed by *C. manginecans* in Oman and Pakistan (Kazmi et al. 2005; Al Adawi et al. 2006; Van Wyk et al. 2007). The pathogen has also been reported from Indonesia (Tarigan et al. 2011). The disease may be

associated with high temperatures and humidity in the local fields. Stem wounds, such as from pruning the tree to reduce branching, may be visited by insects such as *Hypocryphalus mangiferae*, a known vector of *C. manginecans* in mango (Al Adawi et al. 2013) and other beetles known to carry *Ceratocystis* species (Appel et al. 1990; Hayslett et al. 2005; Heath et al. 2009).

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