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First report of '*Candidatus* Liberibacter asiaticus' in *Diaphorina communis*

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Abstract Huanglongbing (HLB) or citrus greening is one of the most destructive diseases of citrus in the world and one of the major factors limiting citrus production in south east Asia including Bhutan. The presence of '*Candidatus* Liberibacter asiaticus', associated with the Asiatic form of HLB, was confirmed by conventional and real-time PCR in adults of the black psyllid, *Diaphorina communis* Mathur. This is the first formal detection of '*Ca*. L. asiaticus' in *D. communis*, and the first detection of the pathogen in a psyllid other than *D. citri* Kuwayama in Asia, excluding Arabia. This study is also the first to report the presence of *D. communis* in Bhutan.

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Huanglongbing (HLB) is a bacterial disease affecting citrus and other Rutaceae. The disease is caused by an endogenous, phloem-limited, α-Proteobacterium: '*Candidatus* Liberibacter asiaticus' found in Asia, the Arabian Peninsula and North and South America (Garnier *et al.* 2000; Bové 2006); '*Ca.* L. americanus' found in South America (Teixeira *et al.* 2005a, b); and '*Ca.* L. africanus' found in Africa and the Arabian Peninsula (McClean 1974; Garnier and Bové 1996; Pietersen *et al*. 2010). '*Ca*. L. asiaticus' has been previously reported in mandarin (*Citrus reticulata* Blanco) orchards in Bhutan (Doe Doe *et al*. 2003; Ahlawat *et al*. 2003). A survey of citrus orchards conducted in the districts of Punakha, Wangdue Phodrang and Tsirang of Bhutan by the authors of this study in May 2009 found symptoms consistent with HLB on mandarin trees (Fig. 1) of the one local variety grown. Severe symptoms were observed including leaf defoliation, mottling, twig and tree dieback, poor fruit production and lopsided fruit.

Two insect vectors of HLB and associated '*Ca.* Liberibacter sp.' have been identified in the literature. The first is the African citrus psyllid, *Trioza erytreae* del Guercio [Hemiptera: Psyllidae], which is associated with transmission of the African form of the disease and considered a vector of '*Ca*. L. africanus' (McClean and Oberholzer 1965a; b). The second is the Asiatic citrus psyllid, *Diaphorina citri* Kuwayama [Hemiptera: Psyllidae], which is associated with transmission of both the Asian and American forms of the disease (Capoor *et al.* 1967; Martinez and Wallace 1967; Yamamoto et al. 2006). Experimentally, *T. erytreae* has been shown to transmit the Asiatic form of HLB (Massonié *et al.* 1976) and *D. citri* the African form

Fig. 1 Symptoms typical of huanglongbing in Bhutan: **a** mandarin orchard, Baychu November 2010; **b** lopsided fruit and mottled leaves, Baychu November 2010; **c** mandarin orchard, Kamichu May 2009; and **d** mandarin orchard, Lower Suntaley May 2009

(Lallemand *et al.* 1986). Several other psylloid species are known to feed on Rutaceae including the black psyllid, *Diaphorina communis* Mathur (Fig. 2), which has been recorded on *Murraya paniculata* (L.) Jack, curry leaf (*Bergera koenigii* L.) and occasionally on *Citrus* spp. (Mathur 1975).

During the survey in Bhutan, specimens of the black psyllid were collected from symptomatic mandarin trees and nearby asymptomatic curry leaf plants in orchards in two out of the three districts surveyed: Wangdue Phodrang (Baychu N27.29864, E089.96828, 763 m asl and Kamichu N27.27016, E090.03854, 637 masl) and Tsirang (Lower Suntaley N27.03280, E090.11140, 1009 masl). Additional specimens of *D. communis* were collected from both mandarin and curry leaf plants in the orchard in Baychu, Wangdue Phodrang in October 2010. All specimens were fixed and preserved in 70% ethanol for transport to Australian laboratories. Psyllid specimens were identified as *D. communis* by Daniel Burchardt, Naturhistorisches Museum, Basel, Switzerland and lodged with him under number NMB-ENT 2010–003. Specimens of *D. communis* collected under this name or the synonym, *D. mathuri* Loginova, have only been described from India and Nepal (D. Burchardt, pers. comm.). Therefore, to our knowledge, this is the first report of *D. communis* in Bhutan.

Total DNA was extracted from all samples (1 or 5 psyllids per sample) using the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA extracts were tested using conventional

Fig. 2 *Diaphorina communis* Mathur in Bhutan on: **a** mandarin (*Citrus reticulata* Blanco); and **b** curry leaf (*Bergera koenigii* L.)

Table 1 Detection of '*Candidatus* Liberibacter asiaticus' by conventional and real-time PCR in specimens of *Diaphorina communis* collected in Bhutan

District	Area	No. samples '*Ca*. L. asiaticus detected / No. samples tested	
		Conventional PCR	Real-time PCR
2009			
Wangdue Phodrang	Baychu	0/2	2/2
	Kamichu	3/21	4/4
Tsirang	Lower Suntaley	0/1	1/1
2010			
Wangdue Phodrang	Baychu	2/10	8/8
		2/5[A]	4/5 [A]

[A] 5 psyllids / sample, all other samples referred to in the table contained 1 psyllid / sample

PCR reactions using primers A2 and J5, which were designed to amplify part of the β operon containing the ribosomal protein genes *rpl*A and *rpl*J of '*Ca.* Liberibacter asiaticus' and 'africanus' (Hocquellet *et al*. 1999) and were multiplexed with primers, fD1 and rP2, designed to amplify the 16S rDNA gene of various bacterial species to serve as an amplification (internal) control (Weisberg et al. 1991). Negative 'water' controls and known positive samples from leaves and psyllids (collected in Papua New Guinea) were also included. The final reaction volume was 20 μL and contained 1 mM $MgCl_2$, 750 nM of each of the target primers (A2, J5), 100 nM of each of the internal control primers (fD1, rP2) and REDExtract-N-Amp reagents. The reaction conditions were 92°C for 2 min followed by 40 cycles of 92°C for 45 s, 65°C for 45 s and 72°C for 1 min, including touchdown annealing steps from 69°C to 65°C during the first 5 cycles; and a final extension step of 72°C for 10 min. Eight μL of PCR products were analysed on a 2% agarose gel and the remaining amplification product was purified using UltraClean® PCR Clean-Up Kit (MOBIO laboratories). The purified DNA amplification product from one adult specimen collected in Kamichu was directly sequenced in both directions at the Australian Genome Research Facility Ltd with automated sequencing using an Applied Biosystems 3730*xl* capillary sequencer (www.agrf.org.au) to confirm the identity of the amplified fragment. Real-time PCR was performed as per Li *et al*. (2006) with an internal control for monitoring the quality of psyllid DNA extraction coding for the wingless gene using the primers and probe (DCF, DCR and DCP) described by Manjunath *et al*. (2008).

'*Ca*. L. asiaticus' was detected by conventional and real-time PCR in specimens of adult *D. communis* collected from mandarin and curry leaf trees in 2009 and 2010 (Table 1). Real-time PCR was found to be more sensitive than conventional PCR and detected '*Ca*. L. asiaticus' in a larger proportion of samples. The sequence from one adult psyllid specimen collected from a mandarin tree in Kamichu was identical with published sequences for '*Ca*. L. asiaticus' using BLAST analysis. The sequence was lodged with GenBank under accession number JF346109. This is the first formal detection of '*Ca*. L. asiaticus' in *D. communis* and, given the ability of *T. erytreae* and *D. citri* to transmit the different *Ca.* Liberibacter species associated with HLB, it will be important to determine the ability of *D. communis* to transmit '*Ca*. L. asiaticus' to host plants.

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