

Confirmation of *Xanthomonas citri* subsp. *citri* causing citrus canker in Timor-Leste

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Abstract The bacterium *Xanthomonas citri* subsp. *citri*, which causes the disease citrus canker, has been confirmed and reported for the first time in Timor-Leste. Herbarium collections indicate citrus canker has been present since at least 2006. The identity of *X. citri* subsp. *citri* has been confirmed using biochemical, and molecular tests, and by completion of Koch's postulates.

Keywords Citrus canker · *Citrus x aurantifolia* · *Xanthomonas citri* subsp. *citri* · Timor-Leste · East Timor

Citrus canker is a serious bacterial disease that produces characteristic symptoms consisting of erupting light brown pustules, which become rough, cracked and corky, surrounded by a water soaked margin that usually has a characteristic yellow halo. The disease infects all above ground plant parts making trees less productive and fruit unmarketable, serious infections can also lead to defoliation and premature fruit drop (Anon. 1997).

The citrus canker bacterium has been re-classified several times and was previously grouped into five strains of *Xanthomonas campestris* pv. *citri* (A,B,C,D,E). The A strain (Asiatic) is now known as *Xanthomonas citri* subsp. *citri*. The other strains have been reclassified as *X. fuscans* subsp. *aurantifolii* (groups B, C and D) and *X. alfalfa* subsp. *citrumelonis* (group E) (Schaad et al. 2006; Vauterin et al. 1995). *X. citri* subsp. *citri* infects *Citrus* spp., some other Rutaceae and one non Rutaceae host *Lansium domesticum* (Anon. 1997). *X. citri* subsp. *citri* has been recorded in many parts of the world including Africa, South America and Asia including the neighboring country of Indonesia although it probably originated from South East Asia (Anon. 1997). There are also variants of *X. citri* subsp. *citri* that show a restricted host range, A* strains causes a disease on Mexican lime in parts of the middle east and Asia and the A^w strains attacks only Key or Mexican limes and *Citrus macrophylla* in Florida (Cubero and Graham 2002; Verniere et al. 1998).

Plant health surveys were conducted over several years by the Timor-Leste Ministry of Agriculture and Fisheries and the Australian Government Department of Agriculture and Water Resources in Timor-Leste (East Timor). These surveys aimed to identify potential disease threats to Timor-Leste and Australia and to increase the knowledge of plant pests in Timor-Leste. During surveillance from 2006 to 2016 the leaves of citrus plants showing characteristic symptoms of citrus canker were collected (Table 1). These specimens were dried in a plant press, imported into Australia under permit and irradiated at 25 kGray upon arrival. DNA was extracted from the lesions using the DNeasy Plant Mini Kit (QIAGEN) and amplified with J-pth1/J-pth2 primers following the methods of Cubero and Graham (2002). This assay is known to detect the citrus canker pathogens of *X. citri* subsp. *citri* and *X. fuscans* subsp. *aurantifolii* (Cubero and Graham 2002). Samples that produced a diagnostic PCR amplicon of

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Table 1 Citrus leaves showing characteristic canker symptoms were collected during plant health surveillance in Timor-Leste. These herbarium specimens tested positive for *Xanthomonas citri* subsp. *citri* using a molecular assay with the J-pth1/J-pth2 primers described in Cubero and Graham (2002)

| Sample | Host | Date collected | Location / District |
|--------------------|------------------------------|----------------|------------------------------|
| PS1169 | <i>Citrus</i> sp. | 27/03/2006 | Sau village, Manatuto |
| JR1063 | <i>Citrus x aurantifolia</i> | 24/03/2011 | Wailacama, Vemase, Baucau |
| JR2811 | <i>Citrus hystrix</i> | 10/05/2013 | Watubo, Baucau |
| JR2819 | <i>Citrus x aurantifolia</i> | 12/05/2013 | Lospalos, Lautem |
| GS238 | <i>Citrus x aurantifolia</i> | 16/05/2013 | Watubo, Baucau |
| LMJ648 (T15_05499) | <i>Citrus x aurantifolia</i> | 06/05/2015 | Manleuana, Dili |
| JR3224 | <i>Citrus x aurantifolia</i> | 17/03/2016 | Aelafa, Daudere, Lautem |
| JR3265 | <i>Citrus x aurantifolia</i> | 21/03/2016 | Sabuli, Metinaro, Dili |
| GS16-146 | <i>Citrus</i> sp. | 23/03/2016 | Manleuana, lisbutac II, Dili |



Fig. 1 Symptoms on detached *Citrus* leaves inoculated with *Xanthomonas citri* subsp. *citri* isolate from Timor Leste (ICMP 22013). **a.** Infected *Citrus sinensis* leaf; **b.** Infected *Citrus aurantifolia* leaf; **c.** Uninfected *Citrus aurantifolia* leaf (negative control)

approximately 197 base pairs were considered positive for citrus canker (Table 1).

To confirm the identity of citrus canker a sample of *Citrus aurantifolia* (LMJ 648) was collected from Manleuana, Dili, Timor-Leste in May 2015 and sent to New Zealand for further testing, under relevant biosecurity protocols. Isolations of the bacterium from infected leaf material were conducted in a containment laboratory (Physical containment level 2 with additional controls) by streaking suspensions of macerated diseased leaf tissue onto semi-selective media developed by Pruvost et al. (2005) and Yeast peptone glucose agar media. Mucoid pale yellow bacterial colonies were consistently produced following 48 h incubation at 25 °C. All colonies were gram negative, oxidase negative, catalase positive. Testing of DNA extracted from plant tissue and cultures with specific real-time PCR assays, developed by Mavrodieva et al. (2004) and Park et al. (2006) confirmed that these isolates were *X. citri* subsp. *citri*. The genetic diversity of these strains was assessed by an analysis of *Irp* gene sequences as described by Cubero and Graham (2004). A phylogenetic analysis of *Irp* sequences of *X. citri* subsp. *citri* and other *Xanthomonas* strains that occur on citrus show that the Timor-Leste strains cluster with the *X. citri* subsp. *citri* type strain and distinguish them from the atypical milder *X. citri* subsp. *citri* pathotypes (A* and A^W) (Fig. 1). Representative cultures have been stored in the International Collection of Micro-organisms from Plants (ICMP), Landcare Research, Auckland, New Zealand, under ICMP 22013, 22,014 and 22,015.

Pathogenicity tests were performed with three different isolates (ICMP 22013, 22,014, 22,015) on young detached

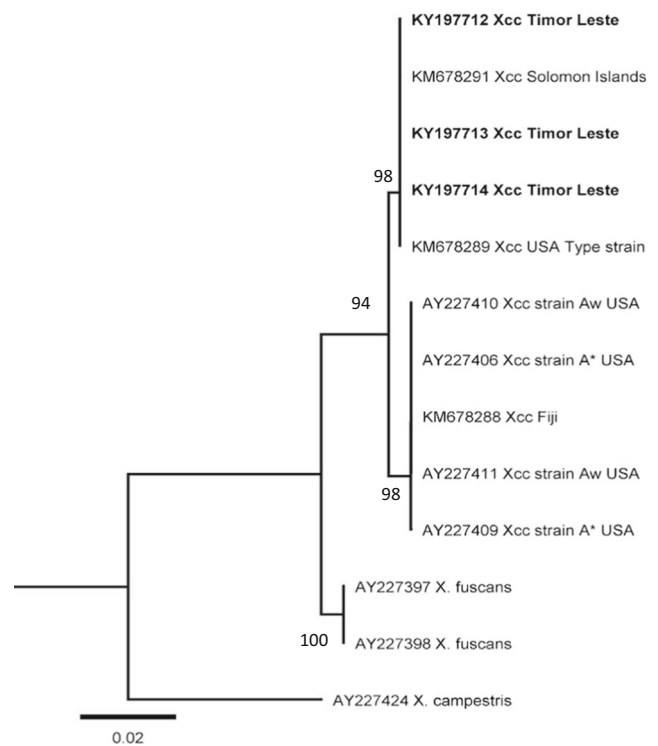


Fig. 2 Maximum likelihood tree illustrating the phylogenetic relationships between the Timor-Leste *Xanthomonas citri* subsp. *citri* (Xcc) strains (in bold type) and other Xcc strains. Phylogenetic analyses of the *Irp* gene was performed via Geneious Pro 9.5.1 (Biomatters, Auckland, New Zealand) using RAxML 7.2.8 for tree construction. Topology was rooted with *X. campestris* and bootstrap values from 10,000 replicates are given at the nodes. Sequences produced in this study are deposited in Genbank under accession numbers KY197712 – KY197714

leaves of orange and lime. Twenty μl of bacterial suspension (10^7 cfu/ml) from each isolate was inoculated on the surface of healthy leaves by pricking through the drops using sterile needles. Leaves were incubated in humid conditions at 25 °C. Pustule-like symptoms developed after 10 days (Fig. 2). Bacterial colonies identified as *X. citri* subsp. *citri* were re-isolated from the lesion fulfilling Koch's postulates.

Citrus canker caused by the virulent bacteria *X. citri* subsp. *citri* (broad host range) has been confirmed as present in Timor-Leste. Citrus canker has been present since at least 2006 and is fairly widely distributed (Table 1.). However, unconfirmed survey records indicate the bacterium was present in 2002. The majority of Timor-Leste is comprised of subsistence farmers who experience a 'hungry season' each year while waiting for maize and rice crops to mature (Costa et al. 2013). Citrus is a relatively minor crop but important as it provides nutrition and generates some income. Citrus canker can cause substantial economic losses to trees grown in commercial orchards; however, the impact on trees grown in isolated subsistence or domestic situations like much of Timor-Leste can be much lower (Gottwald et al. 2002). Canker free nurseries providing disease free planting materials and grove sanitation are recommended for reducing the prevalence of the disease in Timor-Leste.

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