Host records for *Phytophthora multivora* associated with dieback in Victoria, Australia

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Received: 15 September 2015 / Accepted: 11 February 2016 / Published online: 24 February 2016
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Abstract *Phytophthora multivora* was detected from several soil samples collected from the rhizospheres of dieback-affected plants between 2009 and 2014 in the Melbourne Gardens, Royal Botanic Gardens Victoria and the Melbourne Museum, both located in inner city Melbourne, Victoria.

Keywords *Agapanthus* · *Eucalyptus* · *Grevillea* · *Howea* · *Sassafras* · *Xanthorrhoea*

Between February 2009 and June 2014, samples of rhizosphere soil from several dieback-affected plants in the Melbourne Gardens, Royal Botanic Gardens Victoria (RBG), Australia, were submitted to the Victorian government's plant diagnostic laboratory, Crop Health Services (CHS), to investigate the cause of the dieback. During this period, another sample was submitted by Victoria's Biosecurity surveillance team to investigate the cause of a dying *Sassafras* sp. in the Living Rainforest exhibit of the Melbourne Museum. There was a diverse range of 11 hosts represented (Table 1). Typical symptoms included wilting, necrosis, rotted roots and internal wood discolouration and necrosis (e.g. *Howea forsteriana*, Fig. 1).

Using the laboratory's routine pear baiting method based on Tsao (1983), soft rot lesions developed within 1–3 weeks in the pears used to bait soil from the rhizosphere of the diseased plants. The lesion surfaces were sterilised with 80 % ethanol,

internal pear tissue was excised from the diseased-healthy interface of the soft rot and plated onto 1.5 % water agar (Oxoid, Agar Technical, Agar No. 3) and 4 % V8 juice agar (Original Campbell's Soups Australia, pH adjusted to 6.5 prior to adding 1.5 % agar) media. The plates were incubated at 24 °C with a 12 h light/dark cycle, inspected daily and any subsequent growth was subcultured by excising hyphal tips onto fresh Potato Dextrose Agar (PDA) or V8 media plates.

A *Phytophthora*-like pathogen was routinely isolated from the rot-affected baiting pear tissues. To confirm the genus, 1 cm² agar blocks were cut from the colonies growing on V8 juice media and floated in sterile Petri dishes containing non-sterile soil-water extract. Within 5 days, typical *Phytophthora*-shaped sporangia had formed and zoospore discharge was observed. The sporangia were semipapillate and either limoniform, ellipsoid or obpyriform (Fig. 2a), the oogonia were globose and borne terminally, and the antheridia were irregular and paragynous (Fig. 2b). Morphology and size were consistent with the range reported for *P. multivora* (Scott et al. 2009). Koch's postulates were not undertaken due to the diversity and maturity of the hosts. The consistent association with symptoms known to be attributable to *P. multivora*, plus the lack of detection of other potential causal agents, was considered to be sufficient evidence of causality.

To confirm the identification, clean mycelia obtained from hyphal tip culturing were used for genomic DNA extraction. Mycelia were collected from cultures grown on potato dextrose agar, and macerated in a tissue lyser (QIAGEN). Genomic DNA was extracted using DNeasy Plant Mini Kits (QIAGEN) according to the manufacturers' instructions. The regions spanning the ribosomal DNA internal transcribed spacer region (ITS) and mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene were amplified using primers ITS5 and

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Table 1 Isolates of *Phytophthora multivora* obtained from rhizosphere soil of plants showing dieback in Melbourne

Isolate	Date	Origin ^a	Associated plant	Common names of plants	GenBank accession no.	
					ITS	cox1
VPRI 41501	Feb 2009	RBG	<i>Drimys winteri</i>	Winter's bark	KU593618	–
CHS 1	May 2009	RBG	<i>Podocarpus</i> sp.	Podocarp	KU146526	–
CHS 2	May 2009	RBG	<i>Quercus robur</i>	Oak	KU530160	–
CHS 3	Dec 2009	RBG	<i>Cinnamomum camphora</i>	Camphor laurel	KU593611	–
CHS 4	July 2010	RBG	<i>Agapanthus praecox</i>	Agapanthus	KU593612	–
CHS 5	Dec 2010	MM	<i>Sassafras</i> sp.	Sassafras	KU593613	–
CHS 6	Feb 2012	RBG	<i>Eucalyptus victrix</i>	Smooth-barked coolibah	KU593614	–
CHS 7	March 2014	RBG	<i>Podocarpus nivalis</i>	Alpine totara	KU593615	KU593620
CHS 8	March 2014	RBG	<i>Xanthorrhoea australis</i>	Grass-tree	KU593616	–
CHS 9	March 2014	RBG	<i>Grevillea shiressii</i>	–	KU593617	KU593621
VPRI 42425	June 2014	RBG	<i>Howea forsteriana</i>	Kentia palm	KU593619	KU593622

^a RBG Royal Botanic Gardens Melbourne, MM Melbourne Museum

ITS4 (White et al. 1990) and FM84 and FM83 (Martin and Tooley 2003), respectively. The PCR products were purified using QIAquick PCR Purification Kits (QIAGEN), and sequenced using the full cycle Sanger DNA sequencing service offered by Micromon, Monash University, Melbourne (<https://platforms.monash.edu/micromon/>). ITS sequences were generated for all eleven isolates, but *cox1* sequences were only generated for three isolates (CHS 7, CHS 9, VPRI 42425). The sequence data were edited using Geneious software version 7 created by Biomatters (www.geneious.com). The sequences were then subjected to BLAST searches on GenBank (<http://www.ncbi.nlm.nih.gov>) and the Phytophthora Database (<http://phytophthora-id.org/>) to find the closest sequence matches, and aligned in Geneious to confirm identity. They were all 99.9–100 % similar to the ex-type sequence of *P. multivora*. Using Geneious, neighbour-joining

inference phylogenetic trees were built to show phylogenetic relationships between our isolates and representative isolates from the *P. multivora* - *P. citricola* complex (Figs. 3 and 4). Most isolates were lost after the work was finished but two cultures were still viable and deposited in the Victorian Plant Pathology Herbarium (VPRI) as accessions VPRI 41501 and VPRI 42425, respectively.

Fig. 1 *Howea forsteriana*: Disease symptoms on flowering parts and leaves and stem bases of plant growing on soil infested with *Phytophthora multivora*. Note internal pocket rots indicated by white arrows

Fig. 2 *Phytophthora multivora* ex. *Howea forsteriana*: **a** antheridia and oogonia; **b** sporangia; scale bar = 25 µm

Fig. 3 Neighbour-joining inference phylogenetic tree (built using Geneious 7) comparing ITS gene sequence data of the Crop Health Service isolates with reference sequences from GenBank including the ex-type specimens of *Phytophthora multivora* and *Phytophthora citricola*. The respective associated plants are provided in parentheses

Phytophthora species are well known for causing rots, dieback, defoliation and death in numerous host plants worldwide. *Phytophthora multivora* was first described from declining *Eucalyptus*, *Banksia*, *Agonis* and other plant species in Western Australia, recovered during extensive testing for *P. cinnamomi* (Scott et al. 2009). In Australia, disease in natural ecosystems (Jarrah dieback, *Phytophthora* dieback, dieback) has been attributed to *P. cinnamomi*, and is considered a key threatening process to Australia's natural environment under the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act 1999). In pathogenicity trials, infesting the rhizospheres of young trees with *P. multivora* isolates significantly reduced the fine root surface area and the number of root tips on *Eucalyptus gomphocephala* and *E. marginata*, and

Fig. 4 Neighbour-joining inference phylogenetic tree (built using Geneious 7) comparing *cox1* gene sequence data of the Crop Health Service isolates with reference sequences from GenBank including the ex-type specimens of *Phytophthora multivora* and *Phytophthora citricola*. The respective associated plants are provided in parentheses

under-bark inoculation caused significantly expanded rot lesions (Scott et al. 2012). According to Callaghan and Guest (2015), *P. multivora* is now considered the second-most widespread *Phytophthora* species in Western Australia after *P. cinnamomi*, and at many sites it is *P. multivora* rather than *P. cinnamomi* that causes tree mortality. Since first described, it has been reported from declining *Wollemia nobilis* (Wollemi pine) in New South Wales (Puno et al. 2015), lucerne (Bezuidenhout et al. 2010) and citrus (Meitz-Hopkins et al. 2014) in South Africa, soybean in Japan (Rahman et al. 2014, 2015), oaks in Hungary (Szabó et al. 2013), *Viburnum* spp. in Switzerland (Prospero et al. 2013), and from soil in the UK (Henricot et al. 2014).

Morphologically *P. multivora* resembles *P. citricola*, and many earlier collections of *P. citricola* in WA were later determined to belong to *P. multivora* (Scott et al. 2009). In the Victorian Plant Pathology Herbarium, there are several collections of *P. citricola* prior to 2009, but without molecular comparison it is not possible to know if these are in fact *P. multivora*. Therefore, to our knowledge, these are the first records of *P. multivora* from Victoria. All are on new hosts, and all are from two sites within inner metropolitan Melbourne. In some cases, the incidence of disease reported by the client (RBG) was severe. For example, 30 % of >20 year old Podocarp trees in the garden bed of the RBG were affected (CHS 1), 75 % of the >20 year old oaks (CHS 2) in the oak lawn were affected by sporadic dieback, 100 % of the *Agapanthus* plants (CHS 4) and smooth-barked coolibah trees (CHS 6) in their respective beds were affected. In Western Australia, this pathogen has now been linked with decline and death of urban trees in Perth (Barber et al. 2013).

Although all of the new hosts reported here were growing as ornamental plants in the city, *Grevillea shiressii* and *H. forsteriana* are considered vulnerable in their natural habitats (Gosford, NSW; Lord Howe and Norfolk Islands, respectively). *P. multivora* has been recently found causing disease within the Gondwana Rainforests of Australia World Heritage Area (Scarlett et al. 2015) and the Wollemi National Park, NSW (Puno et al. 2015), both eastern coastal areas of Australia. These new host reports provide support to concerns raised about the impact that *P. multivora* could have if spread through the Australian natural environment (Callaghan and Guest 2015; Scarlett et al. 2015).

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