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Bleeding canker of horse chestnut (*Aesculus hippocastanum*) in Ireland: incidence, severity and characterization using DNA sequences and real-time PCR

A. McEvoy[a], F. O'Regan[a], C. C. Fleming[bc], B. P. Moreland[b], J. A. Pollock[b], B. W. McGuinness[d] and T. R. Hodkinson[e]*

[a]Our Lady's College, Greenhills, Drogheda Co., Louth, Ireland; [b]Agri-Food and Biosciences Institute, Newforge Lane, Belfast, BT9 5PX; [c]Molecular Bioscience-Parasitology, Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, BT9 7BL, UK; [d]Teagasc, Ashtown Research Centre, Dublin 15; and [e]Botany, School of Natural Sciences, Trinity College Dublin, The University of Dublin, Ireland

A survey of bleeding canker disease, caused by *Pseudomonas syringae* pv. *aesculi*, was undertaken across Ireland. Incidence has become severe and can be considered epidemic, as 61% of the 1587 horse chestnut trees surveyed showed symptoms of the disease. Bacteria were isolated from a sample of trees and characterized using *gyrB* DNA sequencing. DNA was also extracted directly from wound tissue. The Irish *P. syringae* pv. *aesculi* genotype was identical to genotypes previously sequenced with *gyrB* from the UK and some other locations in Europe. Real-time PCR, using existing primers and a newly designed, more pathovar-specific primer set, was assessed for use in disease screening. With molecular screening, a total of 11 trees from a sample of 55 tested positive for *P. syringae* pv. *aesculi* in Ireland. It was more efficient to extract DNA directly from wound tissue, especially fresh bark, for disease detection than to undertake bacterial isolation with subsequent molecular analysis. A further set of sequencing primers was developed for the amplification of the *gyrB* gene from *P. syringae* pv. *aesculi* and their specificity was shown using a diverse sample of bacterial isolate DNAs. The study also isolated and identified other bacterial species from diseased material; some of these are known pathogens (*Brenneria nigrifluens*, *P. marginalis* and *P. syringae*) or have previously been identified as potentially beneficial endophytes of host trees (*Erwinia billingiae*, *E. tolentana*, *P. fluorescens*, *P. putida* and *Raoultella*).

Keywords: *Aesculus hippocastanum*, bleeding canker, *gyrB*, *Pseudomonas syringae* pv. *aesculi*, real-time PCR, specific primers

Introduction

Bleeding canker is an epidemic disease of horse chestnut (*Aesculus hippocastanum*) in many countries of northwest Europe (Green *et al.*, 2009, 2010) that induces bark to exude a dark sticky fluid (Webber *et al.*, 2007; Schmidt *et al.*, 2009). The horse chestnut tree is native to northern Greece and Albania and was introduced into northern Europe in the late 16th century as an amenity tree and to Britain and Ireland *c.* 1616 (Philips, 1978). During the 1970s it was reported that bleeding canker disease in southern parts of the UK was caused by a species of *Phytophthora* (Brasier & Strouts, 1976), but attempts to isolate the oomycete from infected trees were largely unsuccessful. In retrospect this is not surprising, as the disease is now known to be caused by the bacterium *Pseudomonas syringae* pv. *aesculi* (Webber *et al.*, 2007, 2008). Bleeding canker also affects the leaves of the Indian horse chestnut (*Aesculus indica*) in the Himalayan mountains of northern India (Brasier & Strouts, 1976; Durgapal & Singh, 1980). The red-flowered horse chestnut (*Aesculus* × *carnea*) is equally susceptible (Sullivan, 2011).

Early symptoms tend to be limited to bleeding lesions, consisting of scattered drops of rusty-red, yellow-brown or almost black gummy liquid ooze from small or large patches of bark on the trunk or branches (Sullivan, 2011). Less severe symptoms include rusty-brown spots or a mottled orange-brown colour encircling the trunk. If a tree is heavily infected with bleeding areas on all sides of the trunk, the dead phloem and cambium beneath the bleeding areas can merge and extend until they surround the whole trunk or branch (Sullivan, 2011). Following this, the crown may be affected with brittle branches, chlorotic foliage, premature leaf drop and leaf distortion; in some cases tree mortality occurs (Sullivan, 2011). Nevertheless, the UK Forestry Commission (Webber *et al.*, 2007; Webber, 2015) reported that many trees with trunk infections can survive and show signs of recovery. Young trees (10–30 years old) have been reported as being at greater risk of death by the disease than larger trees (Green *et al.*, 2009). Studies of internal symptoms have shown that the inner bark, including phloem, under the oozing patches is usually dead, and

*E-mail: trevor.hodkinson@tcd.ie

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has a watery orange-brown colour that is often visibly mottled. The edges of the lesion margin tend to be pale with a watersoaked appearance and the wood may be stained blue/black (Forest Research, 2015). Although little is known about the infection biology of the disease, it is believed to enter trees via lenticels, leaf scars, nodes, cracks and other bark weaknesses (Steele *et al.*, 2010). It infects the cortex, phloem and cambium, but there is no evidence of spread in the xylem (Green *et al.*, 2014).

Since the early 2000s, the reported incidence of horse chestnut bleeding canker has dramatically increased and the disease has become severe in most areas of England, Wales and parts of Scotland. A similar increase in bleeding canker recordings has been found in the Netherlands (de Keijzer *et al.*, 2012), Belgium (Bultreys *et al.*, 2008), the Czech Republic (Mertelik *et al.*, 2013) and Germany (Schmidt *et al.*, 2009). In 2007, surveys showed 70% of horse chestnut trees in parts of England exhibited some signs of the disease, with 36% and 42% of the surveyed trees showing signs in Wales and Scotland, respectively (Forestry Commission, 2008). The first official report of the disease in Ireland was made by the National Plant Protection Organisation (NPPO) in 2010 from trees infected in Phoenix Park, Dublin. The disease was placed on the European and Mediterranean Plant Protection Organisation (EPPO) alert list in 2011 and deleted from the list in 2014. The UK Forest Research Centre identified these trees as being infected by *P. syringae* pv. *aesculi*. However, apart from this initial characterization, little is known about the severity and geographical spread of the disease in Ireland or the distribution and diversity of bacterial strains. It has possibly spread from Britain, but, until now, no genotypic data were available to investigate this hypothesis.

The aim of this study was to determine the extent of bleeding canker in Irish horse chestnut, to characterize the bacterial strains present and improve the molecular tools available for its diagnosis.

Materials and methods

Survey and bacterial isolation

A total of 1587 trees were surveyed in Ireland (50 from South/Southwest, 50 from West, 63 from North, 52 from Southeast, 70 from Northwest, 70 from Midlands, 49 from East, 504 from Northeast and 679 from Co. Dublin). Tree age was estimated, based on girth measured at a height of 1.5 m (diameter at breast height; Read, 2000; Gallagher *et al.*, 2013) and tree height (recorded using a clinometer). The extent of the symptoms on bark (of trunk and branches) was recorded on a scale of 0–4, based on recommendations of the UK Forestry Research Commission (Forestry Commission, 2008): 0 indicates no symptoms; 1, light symptom expression; 2, moderate symptom expression; 3, heavy symptom expression; 4, canker symptoms observed at many points surrounding the whole tree – the bleeding areas may have coalesced and encircled the entire trunk or branch. Severity of crown symptoms were recorded on the same scale, and burrs and vertical or horizontal splits/cracks on branches and trunk were noted. These scales were based on descriptions of the disease made by Webber *et al.* (2007). Soil pH and moisture content were recorded using indicator paper/solution and a Tenax moisture meter (scale 1–10), respectively. *Phytophthora* presence was tested using a *Phytophthora* Pocket Diagnostic Test Kit (Forsite Diagnostics).

Bacteria were collected, isolated and cultured from lesions found at the necrotic phloem of 17 diseased horse chestnut trees (Table 1a). A sterilized 3 cm chisel was used to remove the outer bark to reveal the dead/live junction and active lesions in the inner bark phloem. A sterilized small trowel was used to take multiple samples from the borders of the junction. Samples were placed on sterile swabs in sterile plastic bags. A pea-sized extract (*c.* 1 g) was then transferred to bottles containing 50 mL sterile buffered water (pH 7), mixed and then subjected to a five-step serial dilution to achieve a 10^{-5} dilution. Serial dilutions were then spread onto amended nutrient agar (following Green *et al.*, 2009), tested for fluorescence under UV light and cells examined under a microscope with oil immersion (×1000) for length (μm) and shape. Isolated colonies were then streaked onto King's B agar (King *et al.*, 1954) and characterized further using Gram stain testing with 3% aqueous KOH on a subsample of the colony, oxidase testing and catalase testing (following Green *et al.*, 2009). Samples tentatively assigned the category of potential *Pseudomonas* were kept for further DNA analysis. Samples were taken from a further 39 trees using the previous method for bark sampling, but isolation was also attempted from large and small tree branches.

DNA extraction from bacterial isolates and bark lesions

DNA was extracted from bacterial cultures using two different approaches. In the first method, DNA was extracted directly from wound tissue following the tissue dissociation protocol for the MagCore Genomic DNA Plant kit. Fresh plant tissue (50 mg) was cut from each sample and homogenized by grinding the sample to a fine powder with liquid nitrogen using a pestle and mortar. Samples were transferred into a 1.4 mL microcentrifuge tube for extraction with the kit following the manufacturer's protocol. In the second method, used for bacteria in culture, a small bacterial colony was picked from the culture plate using a sterile pipette tip and placed into a 1.5 mL microcentrifuge tube containing 1 mL sterile ultra-pure water at 95 °C for 10 min. The sample was then immediately placed on ice and either used directly for PCR or kept frozen at −80 °C for further use. The former method was most suitable for long-term DNA storage but the latter method was also found ideal for rapid and cost-effective screening.

Sequencing of DNA using universal *gyrB* primers and a newly designed set of *P. syringae* pv. *aesculi*-specific PCR primers

PCR was carried out on 47 samples of DNA extracted from bacterial cultures (Table 1a) using *gyrB* primers from Sarkar & Guttman (2004). The DNA sample was centrifuged for 1 min at 20 000 *g* to pellet solid cellular material and DNA was sampled from the upper section of the tube. The PCR reaction contained 1 μL DNA extract (*c.* 20 ng μL^{-1}), 5 μL of 5× buffer (Promega), 0.5 μL of 10 mm dNTPs, 0.25 μL of 20 pmol μL^{-1} forward primer (5′-MGGCGGYAAGTTCGATGACAAYTC-3′), 0.25 μL of 20 pmol μL^{-1} reverse primer (5′-TRATBKCAGTCARACCTTCRCGSGC-3′; Sarkar & Guttman, 2004), 2 μL of

Table 1 Results of *gyrB* sequencing and real-time PCR for (a) samples and results from bacterial cultures, (b) samples and results from plant disease lesions

Sample	Location (tree extract no.)	County	Source	Age (years)	RT-PCR[a] (+/–)	*gyrB* sequence
(a)						
1, 2	Control – Beaulieu (1)	Louth	Fresh, bleeding trunk canker, necrotic phloem	140	+	Psa
3	Control – Beaulieu (1)	Louth	Fresh, bleeding trunk canker, necrotic phloem	140	+	Psa
4	Control – Beaulieu (1)	Louth	Fresh, bleeding trunk canker, necrotic phloem	140	+	Psa
5	Control – Beaulieu (1)	Louth	Fresh, bleeding trunk canker, necrotic phloem	140	+	Psa
6, 7	Tara (2)	Meath	Fresh, bleeding trunk canker, necrotic phloem	144	+	Psa
8, 9	Tara (2)	Meath	Fresh, bleeding trunk canker, necrotic phloem	144	–	ds
10, 11	Tara (2)	Meath	Fresh, bleeding trunk canker, necrotic phloem	144	–	ds
12, 13	Greenhills (3)	Louth	Fresh, bleeding trunk canker, necrotic phloem	146	–	ds
14	Greenhills (3)	Louth	Fresh, bleeding trunk canker, necrotic phloem	146	–	ds
15	Greenhills (3)	Louth	Fresh, bleeding trunk canker, necrotic phloem	146	–	ds
16, 17	Townley Hall (4)	Louth	Fresh, blackened branch canker, nectrotic phloem	30	–	ds
18, 19	Townley Hall 4)	Louth	Fresh, blackened branch canker, nectrotic phloem	30	–	ds
20, 21	Control – Townley Hall (5)	Louth	Healthy trunk, phloem	30	–	ds
22, 23	Control – Townley Hall (5)	Louth	Healthy trunk, phloem	30	–	ds
24, 25	Balrath (6)	Meath	Fresh, bleeding trunk canker, necrotic phloem	121	–	ds
26, 27	Balrath (6)	Meath	Fresh, bleeding trunk canker, necrotic phloem	121	–	ds
28, 29, 30	Westport (7)	Mayo	Ooze (dry), black exude at cambium on trunk	56	–	ds
31	Phoenix Park (8)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	112	–	Not sequenced
32	Phoenix Park (8)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	112	–	Not sequenced
33	Phoenix Park (8)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	112	–	Not sequenced
34	Phoenix Park (9)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	60	–	Not sequenced
35	Phoenix Park (10)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	26	–	Not sequenced
36	Phoenix Park (10)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	26	–	Not sequenced
37	Phoenix Park (11)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	78	–	Not sequenced
38	Phoenix Park (11)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	78	–	Not sequenced
39	Phoenix Park (12)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	120	–	Not sequenced
40	Phoenix Park (13)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	105	–	Not sequenced
41	Phoenix Park (13)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	105	–	Not sequenced
42	Pheonix Park (14)	Dublin	Fresh, bleeding trunk canker, necrotic phloem	98	–	Not sequenced
43	Townley Hall (15)	Louth	Fresh, bleeding lenticel trunk canker, necrotic phloem	16	–	Not sequenced
44	Greenhills branch (16)	Louth	Fresh, blackened branch canker, nectrotic phloem	146	–	Not sequenced
45	Greenhills branch (16)	Louth	Fresh, blackened branch canker, nectrotic phloem	146	–	Not sequenced
46	Old Mellifont (17)	Louth	Fresh, bleeding trunk canker, necrotic phloem	69	–	Not sequenced
(b)						
47	Greenhills (18)	Louth	Fresh, bleeding trunk canker, necrotic phloem	146	–	Not sequenced
48	Tara (18)	Meath	Fresh, bleeding trunk canker, necrotic phloem	139	–	Not sequenced
49	Tara (19)	Meath	Fresh, bleeding trunk canker, necrotic phloem	120	–	Not sequenced
50	Tara (20)	Meath	Fresh, bleeding trunk canker, necrotic phloem	108	–	Not sequenced
51	Belvedere (21)	Westmeath	Fresh, bleeding trunk canker, necrotic phloem	80	–	Not sequenced
52	Donore (Lake)(22)	Westmeath	Fresh, bleeding trunk canker, necrotic phloem	102	–	Not sequenced
53	Control – Kilpeader (23)	Co. Wicklow	Healthy trunk, phloem	80	–	Not sequenced
54	Greenhills (24)	Louth	Fresh, bleeding trunk canker, necrotic phloem	146	–	Not sequenced
55	Townley Hall (25)	Louth	Fresh, bleeding trunk canker, necrotic phloem	30	–	Not sequenced
56	Townley Hall (26)	Louth	Fresh, bleeding trunk canker, necrotic phloem	30	–	Not sequenced
57	Tara Branch 27	Meath	Fresh, blackened branch canker, nectrotic phloem	106	–	Not sequenced
58	Waterford (28)	Waterford	Fresh, bleeding trunk canker, necrotic phloem	34	–	Not sequenced
59	Waterford (29)	Waterford	Fresh, bleeding trunk canker, necrotic phloem	19	–	Not sequenced

(continued)

Table 1 (continued)

Sample	Location (tree extract no.)	County	Source	Age (years)	RT-PCR[a] (+/–)	*gyrB* sequence
60	Balrath (30)	Meath	Fresh, bleeding trunk canker, necrotic phloem	89	–	Not sequenced
61	Balrath (31)	Meath	Fresh, bleeding trunk canker, necrotic phloem	59	–	Not sequenced
62	Balrath (32)	Meath	Fresh, bleeding trunk canker, necrotic phloem	93	–	Not sequenced
63	Westmeath (33)	Westmeath	Fresh, bleeding trunk canker, necrotic phloem	53	–	Not sequenced
64	Westmeath (34)	Westmeath	Fresh, bleeding trunk canker, necrotic phloem	56	–	Not sequenced
65	Dunleer (35)	Louth	Fresh, bleeding trunk canker, necrotic phloem	60	–	Not sequenced
66	Dunleer (36)	Louth	Fresh, bleeding trunk canker, necrotic phloem	65	–	Not sequenced
67	Dunleer (37)	Louth	Fresh, bleeding trunk canker, necrotic phloem	62	–	Not sequenced
68	Termonfeckin (38)	Louth	Fresh, bleeding trunk canker, necrotic phloem	74	+	Psa
69	Greenhills Branch (39)	Louth	Fresh, blackened branch canker, nectrotic phloem	146	+	Psa
70	Greenhills (40)	Louth	Fresh, bleeding trunk canker, necrotic phloem	181	+	Psa
71	Greenhills (40)	Louth	Fresh, bleeding trunk canker, necrotic phloem	181	+	Psa
72	Courtown Wood (41)	Wexford	Fresh, bleeding trunk canker, necrotic phloem	80	–	Not sequenced
73	Market Square (Gorey) (42)	Wexford	Fresh, bleeding trunk canker, necrotic phloem	100	–	Not sequenced
74	Dalgan (43)	Meath	Fresh, bleeding trunk canker, necrotic phloem	60	+	Psa
75	Balrath (44)	Meath	Fresh, bleeding trunk canker, necrotic phloem	62	–	Not sequenced
76	Gosford (45)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	118	–	Not sequenced
77	Gosford (45)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	118	–	Not sequenced
78	Gosford (46)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	44	–	Not sequenced
79	Gosford (46)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	44	–	Not sequenced
80	Gosford (47)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	60	–	Not sequenced
81	Gosford (47)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	60	+	Psa
82	Gosford (48)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	48	+	Psa
83	Gosford (48)	Armagh, NI	Fresh, bleeding trunk canker, necrotic phloem	48	+	Psa
84	Gortin Wood (49)	Tyrone, NI	Fresh, bleeding lenticel trunk canker, necrotic phloem	40	–	Not sequenced
85	Gortin Wood (49)	Tyrone, NI	Fresh, bleeding lenticel trunk canker, necrotic phloem	40	+	Psa
86	Gortin Wood (50)	Tyrone, NI	Fresh, bleeding lenticel trunk canker, necrotic phloem	37	–	Not sequenced
87	Gortin Wood (50)	Tyrone, NI	Fresh, bleeding lenticel trunk canker, necrotic phloem	37	+	Psa
88	Parkanaur (51)	Tyrone, NI	Fresh, bleeding trunk canker, necrotic phloem	108	–	Not sequenced
89	Parkanaur (52)	Tyrone, NI	Fresh, bleeding trunk canker, necrotic phloem	36	+	Psa
90	Parkanaur (53)	Tyrone, NI	Fresh, bleeding trunk canker, necrotic phloem	58	–	Not sequenced
91	Slieve Gullion (54)	Armagh, NI	Fresh, blackened branch canker, nectrotic phloem	116	–	Not sequenced
92	Slane (55)	Meath	Fresh, bleeding trunk canker, necrotic phloem	50	–	Not sequenced

[a]RT-PCR was performed using primers specific to *Pseudomonas syringae* pv. *aesculi*. Results are recorded as presence (+) or absence (–) of *P. syringae* pv. *aesculi*.

Psa = *P. syringae* pv. *aesculi*; ds = different species, not Psa; not sequenced = not sequenced due to negative PCR or RT-PCR.

25 mM $MgCl_2$, and 0.125 μL Go Taq Flexi DNA polymerase (Promega). Thermal cycling in an Applied Biosystems Veriti thermal cycler comprised: a premelt of 94 °C for 30 s; 32 cycles of 94 °C for 2 min, 63 °C for 1 min and 72 °C for 1 min; and a final extension of 7 min at 72 °C. A further set of amplifications were made with the same PCR mix but with an alternative thermal cycling protocol performed in a Bioer thermal cycler with initial denaturation of one cycle at 95 °C for 3 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 7 min. Both protocols amplified products equally well (two protocols were used because research was conducted in two institutes, Trinity College Dublin and the Agri-Food and Biosciences Institute, Belfast).

Amplicons were assessed for quality by standard agarose gel electrophoresis (1.2% agarose in TBE buffer, 0.445 M Tris-HCl, 0.455 M boric acid, 0.055 M disodium EDTA) or a QIAxcel Advanced Capillary Electrophoresis System (QIAGEN). PCR products were cleaned using an ExoSAP kit (Affymetrix) or ChargeSwitch PCR Clean-up kit (Invitrogen) and then sequenced using Applied Biosystems Big Dye Terminator v. 3.1 cycle sequencing kits and run on an Applied Biosystems 3100xl automated DNA sequencer. Sequences were edited, assembled and analysed in GENEIOUS v. 7 (Biomatters Ltd). Assembled consensus sequences were analysed using BLAST searches. The nucleotide sequences were aligned in MEGA 6 (Tamura *et al.*, 2013) using the MUSCLE algorithm and analysed using neighbour joining with the Tamura-Nei+G model of evolution determined optimal for

the matrix using the MODEL.TEST function in MEGA 6. The matrix was also subjected to a bootstrap analysis with 500 bootstrap replicates to test support for the resulting groups with the same settings used in the individual neighbour joining analysis (following guidelines in Salamin *et al*., 2005).

A new set of PCR and sequencing primers was designed using PRIMER3 within GENEIOUS v. 7 to improve specificity of amplification, using the aligned DNA matrix of *P. syringae* pv. *aesculi* and using conserved regions in this species relative to other *Pseudomonas* spp. in the sequence matrix. The forward primer AM60F had a sequence of 5′-TGTCGGTTGTTAACGCCCTT-3′ and reverse primer AM602R a sequence of 5′-GACCTTCCTGCTCGATGTAGT-3′ with melting temperatures of 60.2 and 59.2 °C respectively. This primer pair amplified a DNA fragment of 543 bp long. The newly designed primers were tested on all the bacterial isolates, and amplicons were checked on agarose gels for predicted length. The successfully amplified samples were sequenced using the same protocol as above to confirm that they were *P. syringae* pv. *aesculi*.

Real-time PCR

Real-time PCR was conducted on samples extracted from bacterial cultures (Table 1a) and also samples taken directly from bark tissue without a bacterial isolation step (Table 1b). A new set of primers was developed to improve the specificity of the real-time PCR. The real-time PCR primers of Green *et al*. (2009) known as Psa2F (5′-CAAAGACGAGCGCAGCGGGA-3′) and Psa2R (5′-CATTGGTGTCGAGATTGCGCTG-3′) were compared to the new set of primers designed here. The new primers were designed using PRIMER3 within GENEIOUS v. 7 and verified by BLAST analysis for species specificity. The sequences of the new primers were AM-Aes1F 5′-CGTGCCTTCGTTGAA TACCT-3′ and AM-Aes1R 5′-CCGTCGTCGCGTTGAATA-3′ (Fig. S1). They amplify a hypervariable region of *gyrB* with a predicted amplicon size of 80 bp. An annealing temperature of 63 °C was found to be optimal.

For all reactions, real-time PCR amplification of *P. syringae* pv. *aesculi* DNA was performed using qPCR Mastermix-SYBR (Promega) in 12.5 μL reaction volumes with a final primer concentration of 600 nM and a total of 1 μL template DNA. PCR reactions were performed in an ECO Illumina Real-time PCR thermal cycler using cycling parameters of 95 °C for 10 min for initial denaturation; then 40 cycles of 95 °C for 15 s denaturation, 60 or 63 °C (depending on primer combination used) for 1 min for annealing/extension, with signal thresholds set automatically. A melting curve analysis was carried out for each real-time PCR test to confirm specific amplification of the target product, and rule out nonspecific amplification and false positive reactions.

Results

Table 2 shows the survey results of bleeding canker incidence across Ireland. The results show that of the 1587 trees assessed in the various regions, 61% displayed disease symptoms. None of the geographical regions had less than 48% of trees affected by the disease symptoms (Table 2) and the southeast of Ireland had the highest incidence of trees with symptoms (89%). Approximately a quarter of the infected trees showed the highest severity of symptoms (categories 3 and 4 of disease; Fig. 1a) and over two-thirds of infected trees were less than 80 years old (Fig. 1b). Regional differences in the severity of damage/disease are shown in Table 2. One area seemed free of disease (County Sligo) but this was based on a sample

Table 2 Incidence of bleeding canker disease on horse chestnut in regions of Ireland

Region	Total no. of trees	% of trees diseased	% of diseased trees with cracked branches and bark
South/Southwest	50	54.0	73.3
West	50	58.0	0
North	63	47.6	90.9
Southeast	52	88.5	94.3
Northwest	70	51.4	83.3
Midlands	70	71.4	75.6
East	49	55.1	56.0
Northeast	504	60.7	77.9
Dublin	679	65.5	–[a]
	Total = 1587	Mean = 61.4	Mean = 68.9

[a]The Dublin sampling was carried out in 2010 when cracked branches and bark were not recorded. The rest of the sampling was performed in 2012/13.

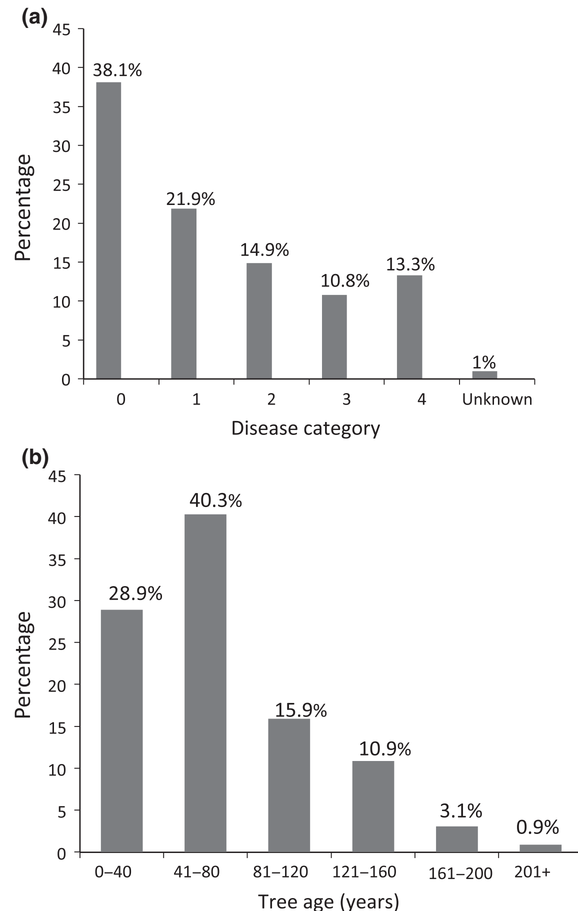

Figure 1 Incidence of bleeding canker disease in Irish horse chestnut and age class distribution of infected trees. (a) Percentage of trees diseased over all surveys, with symptoms of bleeding canker on a scale of 0–4, (b) estimated age of diseased trees.

of only 13 trees. None of these differences were associated with variations in soil pH, which ranged from 6 to 8 (data not shown). However, a higher level of disease was recorded on trees growing in wet and heavy clay soils (data not shown). Cracking on either trunk or branches was also very prevalent. Of the 441 trees that displayed symptoms of canker-related cracking or splits, 345 trees were diseased (Table 2). No *Phytophthora* was detected in any of the sampled trees.

Bacteria were isolated from 17 trees and tentatively identified as *Pseudomonas* or related bacteria using Gram staining, fluorescence, cell length and shape (Table 1a). Little variation in cell length and shape of the bacteria was seen. The rod shape was consistent with the morphology of *P. syringae* pv. *aesculi* and other *Pseudomonas* species. Cell length range was 1–3 μm, which can be regarded as within the normal range for this species. Unfortunately, on this occasion it was not possible to identify the presence of flagella.

Therefore, DNA testing using *gyrB* was required to accurately identify the isolates. The partial *gyrB* region was amplified and sequenced from 15 bacterial colonies and identified using BLAST searches (Table 2). The GenBank accession numbers of the new sequences are from KT879798 to KT879813. Eight isolates were revealed as *Pseudomonas* with six identified as *P. syringae* pv. *aesculi* based on sequence similarity in the BLAST search. These six *P. syringae* pv. *aesculi* cultures were isolated from two individual trees (Table 1a) from Beaulieu (1) and Tara (2). These *gyrB* sequences showed no differences from several isolates from the UK (FJ268847; Green *et al.*, 2008), India (DQ072677; Durgapal & Singh, 1980) and Germany (AM886133; Schmidt *et al.*, 2009) (Table 3).

DNA extracted directly from wound tissue was also successfully characterized using *gyrB* sequencing and real-time PCR (Table 1). This direct method of extraction and characterization proved more efficient for disease diagnosis than the experiments using isolated culturable bacteria from the trees with subsequent molecular analysis. Efforts to isolate the disease from necrotic exudate that dries to form a black crust were not successful.

The newly designed real-time PCR primers (AM-Aes1F and AM-Aes1R) proved ideal for specific detection of *P. syringae* pv. *aesculi*. An annealing temperature in PCR of 63 °C was found to be optimal. There was a single peak in the melting profile indicating a single amplification product. The new AM primers were found to be more efficient for identifying *P. syringae* pv. *aesculi* than the Psa primers of Green *et al.* (2009). They provided more reliable amplification and showed higher specificity in the melt curve analyses in the tests undertaken (Fig. S2).

New PCR and sequencing primers were also designed from the *gyrB* sequences obtained in this study via the 'universal primers' of Sarkar & Guttman (2004). These were tested on the extracted DNA of the bacterial isolates characterized above. Only DNA extracted from *P. syringae* pv. *aesculi* amplified using these primers. The amplicons were of the predicted length and had the same sequence as the corresponding region of amplicons generated with the primers of Sarkar & Guttman (2004).

Table 3 Characterization of the bacterial isolates (cultures) and their identification using the *gyrB* gene

Sample	GenBank search (top hit)	Species (or closest taxon)	Identity (%)
1–6	FJ268847	*Pseudomonas syringae* pv. *aesculi*	100
	DQ072677	*P. syringae* pv. *aesculi*	100
	AM886133	*P. syringae* pv. *aesculi*	100
7	KC571240	*Brenneria nigrifluens*[a]	97
	KC571241	*B. nigrifluens*	97
9	KC571240	*B. nigrifluens*	96
	KC571241	*B. nigrifluens*	96
11	KC571240	*B. nigrifluens*	96
	KC571241	*B. nigrifluens*	96
13	AB039465	*Pseudomonas marginalis*	98
	KC834089	*P. orientalis*	97
14	FJ268869	*Pseudomonas* sp.	98
	FJ012231	*P. fluorescens*	98
	AM293563	*P. koreensis*	97
	AB39403	*P. marginalis*	97
15	JF311587	*Erwinia toletana*	94
	JF311586	*E. toletana*	94
17	JX000477	*Kluyvera georgiana*	90
18	AY370862	*Raoultella terrigena*	92
	JX425102	*R. terrigena*	92
19	JX000477	*K. georgiana*	89
	GQ426101	*Citrobacter* sp.	89
21	FJ268854	*Erwinia billingiae*	99
	JF311564	*E. billingiae*	99
23	FP236843	*E. billingiae*	99
	FJ268857	*E. billingiae*	99
25	GQ426105	*R. terrigena*	98
	GQ426106	*R. terrigena*	98
	AY370862	*R. terrigena*	99
27	JX425102	*R. terrigena*	100
	GQ426105	*R. terrigena*	99

[a]Syn. *Erwinia nigrifluens*.

Two of the bacterial isolates from infected trees were identified as alternative *Pseudomonas* species (Table 3). The sequence of sample 13 was most similar to *P. marginalis* (AB039465, sequence similarity 98%) and sample 14 was most similar to *Pseudomonas* sp. (FJ268869, 98%) and *P. orientalis* (KC834089, KC834142, 97%). However, sample 13 was placed with *P. putida* and *P. fluorescens* in the neighbour-joining analysis with no clear grouping to a named species (Fig. 2). A range of unspecified *Pseudomonas* species were also included that had high percentage similarities (98/97/96%) to these sequences. Two bacterial culture isolates (*Betula* a & *Betula* b) from birch (*Betula pendula*) were included for comparison to horse chestnut and were found to be most similar to *P. fluorescens* (KJ158897, 99%) and *P. chlororaphis* (CP008696, 88%), but they did not group closely to any taxon in the phylogenetic tree (Fig. 2).

Several non-*Pseudomonas* bacteria were also identified using the *gyrB* sequences (Table 3). The sequences had

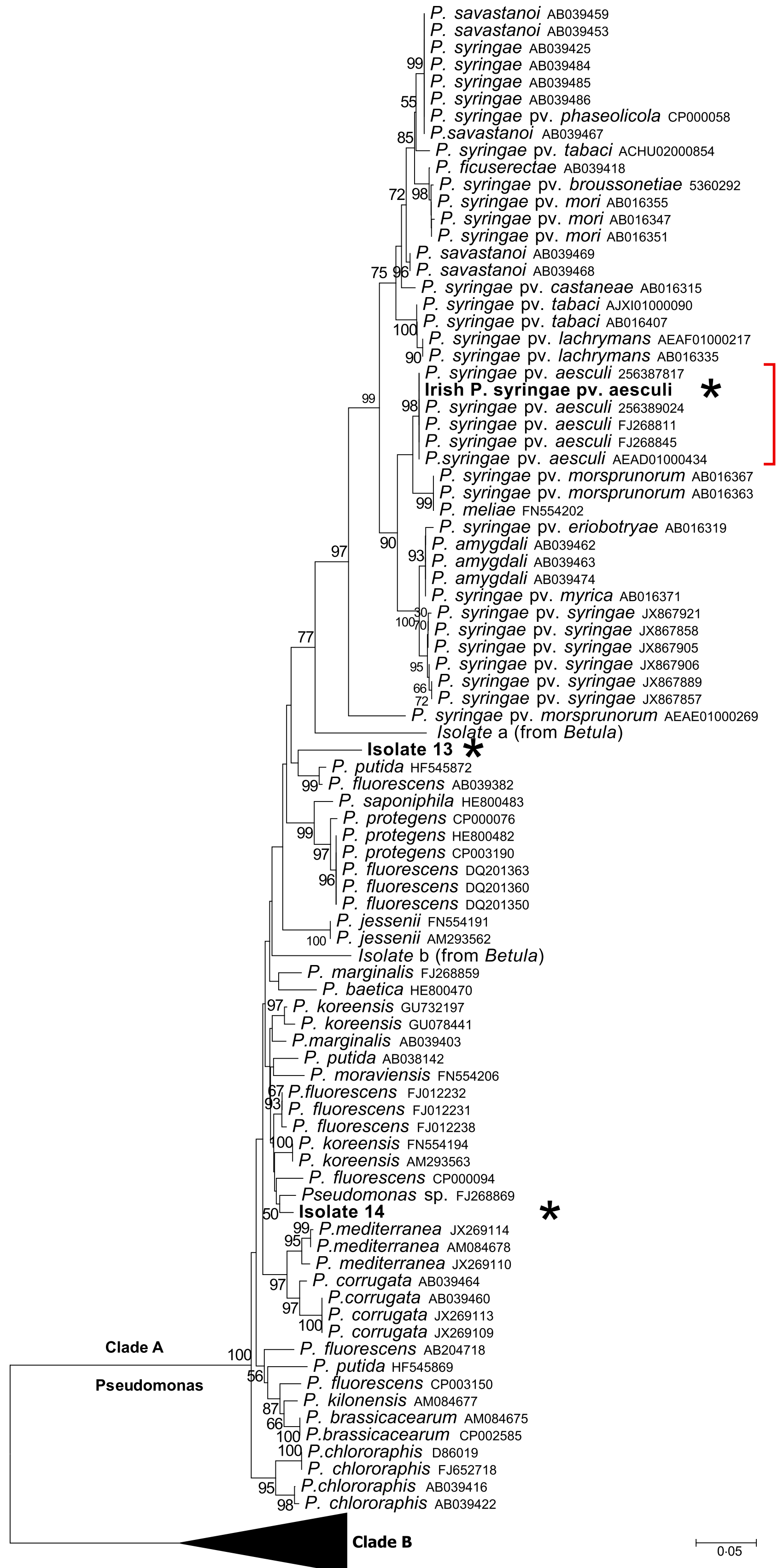

Figure 2 Neighbour-joining tree for *Pseudomonas*. Branch lengths are proportional to genetic distance based on the Tamura-Nei+G model of evolution. Bootstrap values are shown on branches. Samples isolated and sequenced from infected horse chestnut bleeding canker are in bold with an asterisk. Bracket indicates *P. syringae* pv. *aesculi* sequences. Note: *Betula* a and b are isolate sequences obtained from *Betula pendula* and were included for comparative purposes.

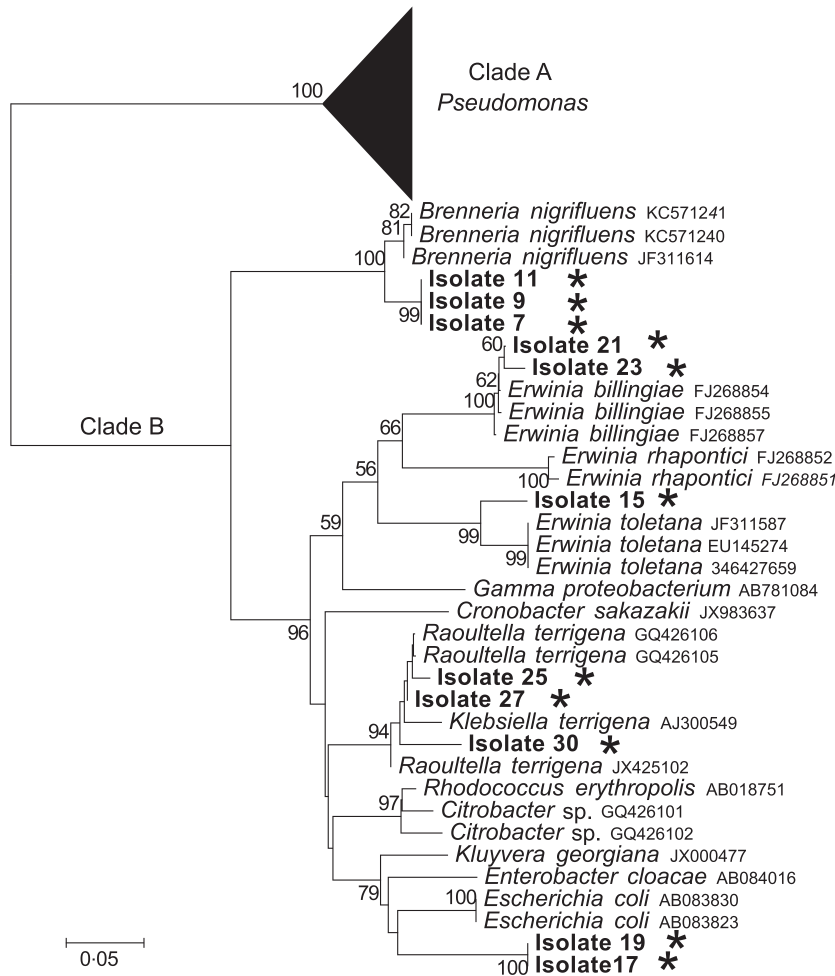

Figure 3 Neighbour-joining tree for non-*Pseudomonas* bacterial isolates associated with infected plant tissue. Branch lengths are proportional to genetic distance based on the Tamura-Nei+G model of evolution. Bootstrap values are shown on branches Samples isolated and sequenced from infected horse chestnut bleeding canker are in bold with an asterisk.

highest sequence similarities with *Erwinia billingiae* (FS268854, 99%), *Erwinia toletana* (JF311587, 94%), *Raoultella terrigena* (GQ426105, 99%) and *Brenneria nigrifluens* (KC571240, 97%) and for two of the samples (17 and 19), the highest similarity was with *Kluyvera georgiana* (JX000477), although this similarity was fairly low (90% and 89%, respectively). The neighbour-joining tree (Fig. 3) shows how these species are related and demonstrates the broad diversity of culturable bacteria that reside in the diseased plant tissue.

Discussion

The incidence and severity of bleeding canker of horse chestnut across Ireland was recorded, and it was clear that the disease is widespread and often severe. Of the 1587 trees assessed in the various regions, 61% displayed symptoms of the disease. Approximately a quarter of the infected trees showed the severest symptoms (3 and 4 category of disease). A tree recorded with a disease level of 3 or 4 in the survey is unlikely to survive for many years (Sullivan, 2011). This may lead to a high incidence of horse chestnut tree deaths within Ireland in the future. A tree (sample 46) with category 4 symptoms of disease was recorded in Mellifont Abbey (County Louth) while surveying in 2012. This tree has since died and has had to be cut down for safety. However, many infected trees have been shown to survive (Green *et al.*, 2014) and resistance of some Czech horse chestnuts has been demonstrated (Pánková *et al.*, 2015).

Little is known about the infection biology of bleeding canker disease (Laue *et al.*, 2014). Related canker pathogens such as *P. syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum* of *Prunus* fruit trees and *P. avellanae* of hazelnut typically initiate infections on leaves, young shoots and fruits and subsequently invade the woody perennial tissue of the trees (Steele *et al.*, 2010). This route does not seem to be the case for *P. syringae* pv. *aesculi* on horse chestnut and it is possible that the disease may be able to infect the aerial woody parts of the host tree directly (Steele *et al.*, 2010). A large number of the trees surveyed in the present study were in woodlands. There is a higher proportion of diseased trees in areas where they are located in close proximity to each other and research elsewhere indicates that bleeding canker is contagious (Sullivan, 2011). Some trunk infections

on trees last for many years and others spread rapidly and can cause death of the crown (Green *et al.*, 2014). In this study, a low incidence of crown symptoms was recorded; only 33% of diseased trees showed crown symptoms and these were mostly recorded on trees with severe bark infections. This suggests that bleeding canker on the trunk bark may eventually affect the trees' crown and foliage. Green *et al.* (2009) noted that initial infection might occur on woody parts of the tree as lesions and cankers occurred on the stem, branches and young, extending shoots (Green *et al.*, 2009). They also showed that it was able to infect the host directly when inoculated onto young unwounded branches, thus prompting the hypothesis that the disease enters tissues within stems and branches via natural openings of, for example, the outer bark such as leaf scars, growth cracks at branch junctions, dormant buds or wounds (Steele *et al.*, 2010).

Steele *et al.* (2010) also showed that lesions developed in the cortex and phloem and then extended into the cambium to cause cankers. There was no evidence of necrosis in the xylem. They found that lesions on the branches were discrete and apparently contained by a necrophylactic periderm. In the present investigation, cracks in bark on trees over 80 years of age were found to be common, so recording cracks in relation to bleeding canker may seem unnecessary. However, the cracks associated with bleeding canker indicate that the cambium ruptures outwards (authors' personal observation). Frequently, a tree was observed with a raised bulge and in some cases the bark may have cracked with the pressure from inside, with perhaps the cambium rupturing outwards. It is also a possibility that the damaged cambium is no longer able to produce a continuous layer of phloem, resulting in growth 'gaps'. Often the cracks are long vertical splits. Cracking on either trunk or branches was very prevalent. Of 441 trees that displayed symptoms of cracking or splits, 345 trees were diseased.

It is known that *P. syringae* pv. *aesculi* can survive independently for extended periods of time in soil and water, and can tolerate lengthy periods of freezing at very low temperatures (Laue *et al.*, 2014). However, a literature review indicates that little is known about other environmental factors. No studies have shown that soil moisture or soil pH have a direct connection with the disease, although trees growing in less favourable conditions could be more stressed and more susceptible to disease. Optimum growing conditions for horse chestnut trees tend to be moist, but well-drained, soil of pH 6–8. During the course of this research, it was observed that a higher level of disease incidence generally occurred on wet and heavy clay soils (data not shown), but this hypothesis would require further investigation.

In this study, bacteria were isolated from infected tissue and characterized using Gram staining, fluorescence, high powered light microscopy and DNA sequencing of the partial *gyrase B* (*gyrB*) gene. The *gyrB* gene has been used successfully for species identification and classification in a wide range of bacteria (Sarkar & Guttman, 2004) and also for *P. syringae* pv. *aesculi* using real-time PCR (Green *et al.*, 2009). The universal *gyrB* PCR primers were found to be effective in amplifying and sequencing all bacteria isolated from Irish horse chestnut. *Pseudomonas syringae* pv. *aesculi* was isolated from fresh, bleeding trunk canker at the necrotic phloem, cultured, identified and sequenced. This is the first known report of successful identification and sequencing of *P. syringae* pv. *aesculi* strains from Ireland. Necrotic exudate dries to form a black crust (Brasier & Strouts, 1976) but efforts to isolate the disease from this type of material were not successful. Green *et al.* (2009) also report that older dried exudate yielded less target DNA and hence less detection possibilities. BLAST database searches revealed that the Irish isolates (samples 1–6, 68–71, 74, 81–83, 85, 87, 89) had 100% similarity with a *gyrB* sequence obtained from a *P. syringae* pv. *aesculi* strain (FJ268847) isolated by Green *et al.* (2008) from *A. hippocastanum* in the UK. The sequence also showed a 100% match to isolates from India (DQ072677; Durgapal & Singh, 1980) and Germany (AM886133; Schmidt *et al.*, 2009). This supports the hypothesis that it is the same pathogen that is destroying horse chestnut trees elsewhere in Europe as well as *A. indica* in India. It is probable that the disease has been in Ireland since 2002/3, as is the case in England; however, it was only identified in Ireland in 2010. Until 2003, it was believed that a *Phytophthora* species was the causal agent of bleeding canker disease. *Phytophthora* is a destructive parasitic fungus-like organism causing brown rot in plants (Brasier & Strouts, 1976). Each tree in the present study tested negative for *Phytophthora*, a finding consistent with several other authors (Bultreys *et al.*, 2008; Schmidt *et al.*, 2009; de Keijzer *et al.*, 2012; Mertelik *et al.*, 2013). However, *Phytophthora* is identified in one or two samples of horse chestnut a year (Forest Research, 2015).

DNA sequencing has also provided insight into the range of pathovars and endophytes that reside in horse chestnut trees. Another three *Pseudomonas* bacterial isolates, including *P. marginalis* and *P. fluorescens*, were identified by comparing the sequences obtained with those from GenBank. Several non-*Pseudomonas* bacteria were also identified from the sequenced samples, including *E. billingiae*, *E. toletana*, *R. terrigena*, *B. nigrifluens* and *K. georgiana*. Some of these bacteria have previously been found in horse chestnut by Green *et al.* (2008) and Bultreys *et al.* (2008) in Belgium.

Phylogenetic relationships were reconstructed from sequences from the present study and from those in GenBank. The Irish *P. syringae* pv. *aesculi* sequence type was embedded with other strains of the same species with high bootstrap support and all Irish samples had the same *gyrB* sequence. *Pseudomonas syringae* pv. *morsprunorum* and *P. meliae* group together and phylogenetically sister to *P. syringae* pv. *aesculi*. These bacteria are known to cause canker of stone fruits and bacterial gall of berry trees (Kennelly *et al.*, 2007; Steele *et al.*, 2010). Six further, closely related, bacteria in the phylogenetic tree are known canker-causing agents on various woody

hosts/trees. Among these are *P. syringae* pv. *castaneae*, *P. syringae* pv. *amygdali*, *P. syringae* pv. *eriobotrya*, *P. syringae* pv. *syringae*, *P. savastonoi* and *B. nigrifluens* (Ménard & Delort, 2004; Temsah *et al.*, 2008). The related *P. avellanae* causes bacterial canker of European hazelnut (Scortichini, 2002), but was not included in the phylogenetic tree.

Endophytic bacteria and fungi can confer multiple benefits to their host plants (Murphy *et al.*, 2015). Several potential plant protecting biocontrol bacteria were either the closest sequence match to some of the bleeding canker samples, or grouped most closely to them in the phylogenetic tree. These bacteria include *E. billingiae*, *E. tolentana*, *P. fluorescens*, *P. protegens* and *P. putida* (Espinosa-Urgel *et al.*, 2000; Rojas *et al.*, 2004; Couillerot *et al.*, 2008; Kube *et al.*, 2010). Their presence may help to reduce the severity of disease or indeed prevent pathogenic infection; however, little is known about endophytes in horse chestnut or its microbiome in general.

The universal *gyrB* primers of Sarkar & Guttman (2004) are highly useful for general species diagnosis in bacteria. However, the primers are not specific to *P. syringae* pv. *aesculi*. Therefore, a *gyrB* PCR primer pair was designed to allow more specific detection of *P. syringae* pv. *aesculi*. Such primers are needed to allow rapid detection and mass screening for the disease. The Irish *P. syringae* pv. *aesculi* sequence type was used to compare to other GenBank sequences of *Pseudomonas* so that pathovar-specific primers could be designed that amplify a 543 bp region. New real-time PCR primers were also developed in this study (AM-Aes1F and AM-Aes1R). The new AM primers were designed in the hypervariable region of *gyrB* and were found to be more specific for identifying *P. syringae* pv. *aesculi* than the ‘Psa’ primers of Green *et al.* (2009). They provided more reliable amplification and showed higher specificity in the melt curve analyses. The results therefore demonstrate the high utility of real-time PCR primers in combination with *gyrB* sequencing for *P. syringae* pv. *aesculi* detection and characterization. It has also been demonstrated that bacterial culture is not necessary for disease diagnosis, as DNA extraction directly from fresh potentially infected bark material and subsequent characterization (sequencing and real-time PCR) was found to be efficient.

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[a]Note: the project won first prize in the Senior Individual Biological and Ecological category and the Elan Award for excellence in biology at the BT Young Scientist & Technology Exhibition for secondary school students in 2014. It won Drogheda Young Innovator of the year 2014. It won the Sentinus Young Innovator of the year in 2014 also and qualified for entry into the Intel International Science and Engineering Fair 2015, where it won the Monsanto Company Award for Innovation in Plant Sciences and Sustainability and the Second Award in Plant Sciences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Sequence of Irish *Pseudomonas syringae* pv. *aesculi* with positions of sequencing and real-time PCR primers developed here. Sequencing and real-time primers developed here are bold or bold/boxed respectively. Those real-time PCR primers of Green *et al*. (2009) are underlined.

Figure S2 Real-time PCR melt curve comparison of Psa primers (Green *et al*., 2009) and new AM-Aes primers developed here. (a) Psa primer melt curve analysis, (b) new AM-Aes primer melt curve analysis with positive control and sample 83 (C0373).