Forestry Commission

Research Note

Horse chestnut bleeding canker

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Horse chestnut is an important amenity tree species which has been significantly affected over the past decade by a widespread outbreak of bleeding canker disease. Symptoms include rust-coloured or blackened bleeding cankers on the stem and branches, which can lead to tree mortality. The causal agent of this disease is the pathogenic bacterium *Pseudomonas syringae* pv. *aesculi*, which is believed to have originated in India on Indian horse chestnut. Development of a real-time polymerase chain reaction diagnostic test for *P. syringae* pv. *aesculi* has enabled its rapid detection in symptomatic trees and provides a useful tool for studying host infection and survival outside the host. The pathovar can survive in soil for up to one year and can tolerate lengthy periods of freezing. To better understand the evolutionary history and genetic make-up of this aggressive tree-infecting bacterium, draft genome sequences were generated for seven isolates of *P. syringae* pv. *aesculi* from Europe, and a type strain from India. Genomic comparisons suggest that this bacterium probably spread to Europe in the early 2000s via an unknown pathway, with the epidemic across several countries resulting from the introduction of a single bacterial strain. Future genomic comparisons with other *P. syringae* pathovars combined with functional analyses of genetic pathways should help unravel the key host–pathogen interactions that underlie bacterial diseases of trees.

Introduction

European horse chestnut *(Aesculus hippocastanum)* is an important amenity tree species throughout much of the UK. Native to northern Greece and Albania (Phillips, 1978), horse chestnut was introduced into the UK in the late 16th century and was planted widely in parks and gardens in both urban and rural areas, often in avenues bordering roads. Horse chestnut is highly regarded for its qualities as a shade tree, its showy white flowers in spring and the production of its fruits or 'conkers'.

Bleeding canker of horse chestnut is a disease that first appeared in 2002/03 and within just a few years became widespread in several countries of northwest Europe, including Britain, the Netherlands, Belgium, northern Germany and northern France. The causal agent is the bacterium *Pseudomonas syringae* pv. *aesculi*, originally isolated in 1969 from leaf lesions on Indian horse chestnut *(Aesculus indica)* in India (Durgapal, 1971; Durgapal and Singh, 1980; Webber *et al*., 2008; Green *et al*., 2009). Recent isolations of *P. syringae* pv. *aesculi* from diseased horse chestnut in Ireland, Norway (V. Talgø, personal communication), Czech Republic (I. Pánková, personal communication) and Saxony in eastern Germany (S. Hilgert, personal communication) suggest that the pathogen is extending its range in Europe.

This Research Note outlines disease symptoms, diagnostic techniques and epidemiological aspects of *P. syringae* pv. *aesculi* on horse chestnut, and provides an overview of how genomics will enable a greater understanding of the evolutionary background and genetic make-up of this important bacterial tree pathogen.

Symptoms and disease management

Symptoms of horse chestnut bleeding canker include rust-coloured liquid oozing from cracks in the bark located on the main stem and branches (Figure 1), necrotic phloem underlying the outer bark (Figure 2), and dieback often leading to tree death. Thousands of horse chestnut trees in Britain have exhibited these symptoms since 2003. A nationwide survey of horse chestnut trees conducted in 2007 found that over 70% of trees surveyed in parts of England had bleeding canker symptoms, with 36% and 42% of surveyed trees showing these symptoms in Wales and Scotland, respectively (Forestry Commission, 2008). Observations of the disease suggest that there may be variability in the horse chestnut population in terms of resistance to infection, with the most susceptible individuals killed outright during the initial wave of the epidemic

Figure 1 Horse chestnut with bark cracking and stem bleeding typical of bleeding canker disease.

Figure 2 Stem of diseased horse chestnut with outer bark removed to show longitudinally spreading canker (black arrow) and laterally spreading lesions in the phloem (white arrows).

and the remaining population exhibiting a range of disease symptoms from mild to severe, which may appear worse in some years than others. Typically, moderately infected trees have bleeds and cracks on one or more branches or on part of the stem, and partial crown dieback. Such trees often exhibit premature leaf fall in the autumn as a result of damage to the phloem in the stem and branches (Figure 3). Since the pathogen is now considered to be widespread across Britain as far north as central Scotland and may be spread by wind-borne rain, little can be done to prevent a tree from becoming infected and there is no known cure for the disease. Trees which show partial infection can be monitored and any severely diseased branches removed as necessary.

Diagnosing *Pseudomonas syringae* pv. *aesculi* on horse chestnut

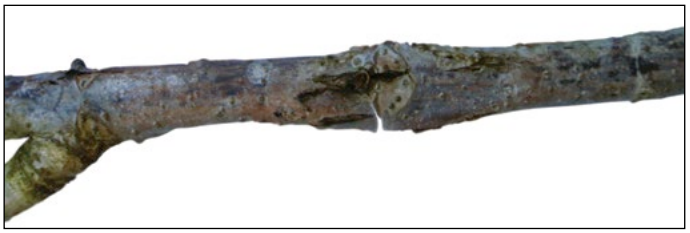
During the 1970s, a bleeding canker disease of horse chestnut was reported in southern parts of Britain and subsequently found to be caused by species of *Phytophthora* (Brasier and Strouts, 1976). Symptoms of *Phytophthora* disease also include foliage thinning, crown dieback and oozing lesions on stems and branches, but only a small number of horse chestnut trees are affected by *Phytophthora* compared with the more recent epidemic of bleeding canker caused by *P. syringae* pv. *aesculi*. Nonetheless, one of the obstacles to diagnosing *P. syringae* pv. *aesculi* on horse chestnut in the first years of the recent epidemic was the lengthy procedure associated with confirming its presence on the host, given that *Phytophthora* pathogens cause similar symptoms and also because damaged tree bark can be colonised by a large number of non-pathogenic bacterial species. Therefore, a quantitative real-time polymerase chain reaction (PCR) assay was developed to detect *P. syringae* pv. *aesculi* on horse chestnut (Green *et al*., 2009). This is a molecular test that uses DNA primers to recognise and amplify a short section of target pathogen DNA with a sequence of base pairs specific to *P. syringae* pv. *aesculi*. Rapid diagnosis of the pathogen in infected trees can now be achieved by sampling necrotic inner bark, incubating small bark fragments overnight on nutrient agar and testing bacterial growth the next day for the presence of *P. syringae* pv. *aesculi* using the real-time PCR assay. In addition to providing the international plant health community with a useful diagnostic tool for screening horse chestnuts for the bacterium, this assay was used to study several important aspects of the biology of *P. syringae* pv. *aesculi*, including routes of infection and longevity of survival outside the host.

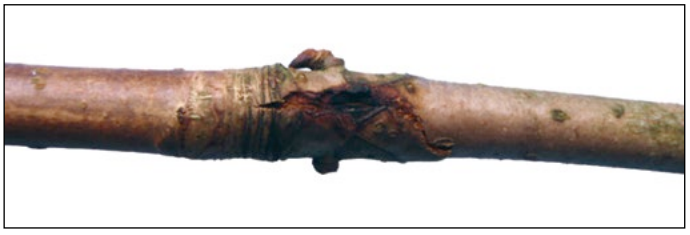
Figure 3 Mature horse chestnut with sub-lethal disease symptoms, showing dieback of scaffold branches and premature autumn colouration of leaves.

Infection of horse chestnut

A study was carried out in Scotland in 2009 in which branches and whole trees of horse chestnut naturally infected with *P. syringae* pv. *aesculi* were subjected to detailed morphological and histological examination to identify the primary infection sites, the time of year that infection had occurred, and the patterns of subsequent lesion expansion within the host (Steele *et al*., 2010). Real-time PCR was used to confirm the presence of *P. syringae* pv. *aesculi* in the host tissues. The study found that lesions developed on branches of various ages during the spring and summer and were centred mainly on lenticels (Figures 4a and 4b), leaf scars (Figure 4c) and nodes (Figure 4d). Lesions developed in the cortex and phloem, and extended into the cambium to form cankers in the period between the cessation of active host growth in late summer and the onset of host growth the following spring (Steele *et al*., 2010).

Figure 4 Young branches of horse chestnut with lesions caused by *Pseudomonas syringae* pv. *aesculi*. (a) Four lesions centred on lenticels. (b) Close view of a single lesion centred on a lenticel. (c) Lesion centred on a leaf scar. (d) Lesion centred on a node.





Primary infection of woody tree parts via lenticels has not been demonstrated for other *Pseudomonas* pathogens and may not be typical for these diseases. The fact that *P. syringae* pv. *aesculi* can invade horse chestnut via lenticels is of particular interest since lenticels provide a very high number of potential infection sites. Thus, the success of *P. syringae* pv. *aesculi* as a tree pathogen and the causal agent of a large-scale epidemic may in part reflect an ability to infect the aerial woody parts of its host directly.

Dispersal of *Pseudomonas syringae* pv. *aesculi*

In southern Scotland it was found that *P. syringae* pv. *aesculi* first started causing lesions on horse chestnut during the 2004/05 dormant season. Thereafter the number of new infections increased in each subsequent year, reflecting the arrival and establishment of the pathogen in the area during the mid-2000s and a subsequent increase in local inoculum levels (Steele *et al*., 2010). *Pseudomonas* diseases of trees are most prevalent in regions with cool, wet climates and the causal pathogens are thought to be spread mainly in wind-blown rain (Crosse, 1966; Kennelly *et al*., 2007). The *P. syringae* pv. *aesculi* epidemic on horse chestnut appears to be geographically centred on northwestern Europe, where cool, wet climatic conditions prevail, and the bacterium may well be disseminated in wind or rain since lesions on branches are almost certainly initiated by aerial inoculum. It has been suggested that plant pathogenic strains of *Pseudomonas syringae* can be disseminated across very long distances at high altitudes, with deposition in precipitation (Morris *et al*., 2007). A similar mode of dispersal by *P. syringae* pv. *aesculi* might explain its rapid spread across northern Europe (Steele *et al*., 2010), most recently into southern Norway. The ideal time for infection of horse chestnut in Britain is likely to occur during wet, stormy weather in early summer when shoots are actively extending and lenticels have greatest permeability (Langenfeld-Heyser, 1997). Premature defoliation by strong winds in spring and summer will also expose unprotected leaf traces and allow direct entry of bacteria into the shoots (Billing, 2011).

To understand more about how *P. syringae* pv. *aesculi* survives outside the host, an experiment was conducted in which cells of the pathogen were added to sterile and non-sterile soil and incubated at either 5 °C or 15 °C (Laue *et al*., 2014). Surprisingly, the bacterium was still present and pathogenic after 50 weeks of incubation in sterile soil and 41 weeks of incubation in non-sterile soil in the absence of host debris (Laue *et al*., 2014). These results raise the possibility that *P. syringae* pv. *aesculi* could be spread in soil, either in potted plants, or on boots, tools and

vehicle tyres. Another series of experiments showed that *P. syringae* pv. *aesculi* remained viable and pathogenic after one year's storage in broth culture at –20 °C and –80 °C, and was not killed by freeze–thaw treatments (Laue *et al*., 2014). The ability of *P. syringae* pv. *aesculi* to tolerate lengthy periods of freezing may also facilitate a greater understanding of the epidemiology and spread of the pathogen in northern Europe.

Conversely, *P. syringae* pv. *aesculi* did not survive in soil exposed to temperatures greater than 30 °C (B. Laue, unpublished), and others found that a temperature of 39 °C for 48h was sufficient to kill the pathogen (de Keijzer *et al*., 2012), as were conditions of 35 °C or greater in liquid culture (Mullett and Webber, 2013). These observations might explain the apparent absence of horse chestnut bleeding canker disease in southern European regions where these temperatures can be reached.

Understanding *Pseudomonas syringae* pv. *aesculi* through genomics

The horse chestnut bleeding canker epidemic has highlighted gaps in the general understanding of the biology of bacterial diseases of trees. Currently, very little is known about the genetic basis for the association of bacterial pathogens with trees. Given that *P. syringae* pv. *aesculi* has only recently emerged in Europe, has spread rapidly, is highly virulent and is evidently capable of attacking branches directly, it is a useful model organism for further studies to address the questions of origin and biology of such newly emerging diseases. There are at least 50 pathovars of the species *Pseudomonas syringae*, which can be distinguished by host range and which infect a wide range of mostly herbaceous but also woody plants. Due to the economic importance of *P. syringae* pathovars and their value as models for studying plant pathogenesis, complete or draft genome sequence data are available for many pathovars including several from woody hosts. These *P. syringae* genome sequences have provided important reference sequences for a comparative genomic study of *P. syringae* pv. *aesculi*.

In this study, draft genome sequences were obtained for seven isolates of *P. syringae* pv. *aesculi* collected from symptomatic horse chestnut trees in Britain and continental Europe (The Netherlands, Belgium, Czech Republic and Norway) between 2002 and 2011, as well as the Indian type strain of *P. syringae* pv. *aesculi* that causes a leaf-spot disease on Indian horse chestnut *(Aesculus indica)* (Green *et al*., 2010). The aim of this study was to gain insights into the biology and recent evolution of *P. syringae* pv. *aesculi* isolates causing the current disease epidemic on horse chestnut in Europe. This was done by comparing the *P. syringae* pv. *aesculi* genomes with sequences from other *P. syringae* pathovars and by determining the genomic variation among the seven *P. syringae* pv. *aesculi* isolates from five European countries. The analysis showed that *P. syringae* pv. *aesculi* harbours unique sets of genes that are absent from other *P. syringae* pathovars that infect herbaceous hosts and which may be required for pathogenicity on a woody host. The study also revealed that the seven British and continental European isolates of *P. syringae* pv. *aesculi* were genetically almost identical, and very closely related to the Indian type strain. This strongly suggests that the epidemic in Europe has most likely resulted from the recent introduction of a single genotype of the bacterium, highlighting the risks posed by the accidental introduction of exotic pathogens to new geographical locations. Future work will elucidate the diversity of genomic mechanisms within *P. syringae*, and will aim to identify potential gene pathways that may underlie an ability to attack woody plants.

Conclusions

The causal agent of the recent epidemic of horse chestnut bleeding canker is the pathogenic bacterium *P. syringae* pv. *aesculi*, which is thought to have originated in India on Indian horse chestnut. Genomics has shown that the epidemic in Europe probably resulted from the introduction of a single bacterial strain via an unknown pathway. *Pseudomonas syringae* pv. *aesculi* is most likely spread by wind and rain, and can infect horse chestnut through discontinuities in the bark, including lenticels, leaf scars, nodes and artificial wounds. Lesions extend in the cortex and phloem and into the cambium, killing the cambium to cause cankers. Many horse chestnuts died as the epidemic spread across Britain in the early to mid-2000s; however, a good number of horse chestnuts still survive, albeit with varying severity of disease symptoms. Moderate to mildly affected trees should be monitored and badly cankered branches removed as necessary, particularly where they represent a safety risk. Tools, boots and vehicles should also be cleaned after working with symptomatic trees, since there is a risk that *P. syringae* pv. *aesculi* could be transmitted in soil, potentially over long distances. Ongoing comparative genomics work will illuminate the important mechanisms by which bacteria have evolved to infect trees.

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