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A multiplex real-time PCR assay enables simultaneous rapid detection and quantification of bacteria associated with acute oak decline

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Acute oak decline (AOD) is a syndrome affecting mature oak trees and is characterized by stem bleeds from vertical fissures on trunks, and inner bark necrosis caused by a polybacterial consortium, in which *Gibbsiella quercinecans* and *Brenneria goodwinii*, and to

a lesser extent *Rahnella victoriana* and *Lonsdalea britannica*, play key roles. Here we report a novel multiplex real-time PCR assay that enables simultaneous and rapid detection and quantification of these four bacterial species from stem bleed swabs. Experiments with axenic cultures were performed to determine specificity and sensitivity of the multiplex quantitative PCR (qPCR). Whilst the primer/probe set for *B. goodwinii* was species-specific, primer/probe sets for the other three species were able to identify other members of their respective genera. There was no cross detection of genera within the multiplex qPCR, and non-target bacteria were not detected. The multiplex AOD assay had differential sensitivity for each bacterial species. The assay was evaluated on swab samples collected from stem bleeds of declining oak trees at a site in south-east England and was able to detect all four bacterial species. Absolute quantification of the bacteria from swab samples was possible through the inclusion of a standard curve prepared from dilutions of gene copy standards. This diagnostic tool will facilitate rapid detection of AOD-associated bacteria from samples that can easily be taken by non-specialists without specific training, and will also find application in other experimental work such as pathogenicity and control trials.

Key words

acute oak decline, *Brenneria*, *Gibbsiella*, *Lonsdalea*, multiplex real-time PCR, *Rahnella*

1. Introduction

Acute oak decline (AOD) is a recently described syndrome affecting mature oak trees in England (Denman and Webber, 2009). *Quercus robur* (pedunculate oak) and *Q. petraea* (sessile oak) are particularly susceptible to AOD, which is characterized by typical symptoms

of stem bleeds from vertical fissures on oak trunks, irregular lesions in the inner bark beneath the bleed area, and presence of larval galleries of the buprestid *Agrilus biguttatus* close to the lesions (Denman *et al*., 2014; Brady *et al*., 2017; Brown *et al*., 2017). Oak trees are thought to be predisposed to AOD attacks through local biotic and environmental factors (Brown *et al*., 2016, 2018) and often die within four to five years following the onset of symptom development.

Marker gene amplicon sequencing, metagenomics, and isolation of bacteria from stem bleeds and necrotic inner bark lesions identified two key, consistently occurring, bacteria species, *Gibbsiella quercinecans* and *Brenneria goodwinii* (Denman *et al*., 2012, 2018; Sapp *et al*., 2016), which were shown to cause lesions in subsequent oak log inoculation trials (Denman *et al*., 2018). Causation by bacteria was further confirmed through multi-omic analysis of microbiota associated with AOD (Broberg *et al*., 2018). In addition, isolation and log inoculation studies implicated a secondary role for *Rahnella* spp. and *Lonsdalea britannica* (Brady *et al*., 2014b; Li *et al*., 2017) in AOD development (Denman *et al*., 2018; C. Brady, University of the West of England, UK, personal communication). Recent genome comparisons of AOD bacteria with other phytopathogens suggest that *B*. *goodwinii* and *L*. *britannica* are primary pathogens containing suites of pathogenicity and virulence genes encoding T3SS, harpins, and effectors, and that *G*. *quercinecans* and *Rahnella* sp. contribute to tissue necrosis through the release of plant cell wall-degrading enzymes (PCWDEs) (Doonan *et al*., 2019). Furthermore, a recent population study of *B*. *goodwinii* isolates from oak in the UK demonstrated that the population of this bacterium in the UK appears to be primarily clonal and may have an endemic form with high levels of recombinant evolution taking place (Kaczmarek *et al*., 2017), which is typical to native bacterial pathogens (Smith *et al*., 2000; Vinatzer *et al*., 2014).

Real-time PCR applications have enabled plant pathologists to develop assays for rapid detection of pathogens without the need for their isolation from diseased plant material (Schaad and Frederick, 2002). This rapid detection enables early, swift management through containment, control, and/or eradication of pathogens before they spread. Two real-time PCR methods can be effectively applied to detect multiple plant pathogens in a sample, namely high-resolution melt (HRM) and TaqMan assays. Although HRM analysis based on the amplification product of the *atpD* gene has been developed for detection of *G. quercinecans* and *B. goodwinii* (Brady *et al*., 2016), this method currently requires the additional step of isolation of pure cultures of bacteria from diseased material on which the HRM is run, thereby adding further time and expense to the process. Furthermore, HRM is not able to quantify the number of individual bacterial species in a sample that are associated with AOD.

TaqMan assays offer the advantage of improved specificity over other real-time PCR assays through the hybridization of a species-specific probe designed to the amplification template (Schaad and Frederick, 2002). The assay exploits the 5′ nuclease activity of *Taq* DNA polymerase together with the inclusion of template-specific fluorescent DNA probes. Amplification of the PCR product is thus directly related to the measured fluorescence (Weller *et al*., 2000). Detection of multiple pathogens within a sample is possible through the addition of different fluorescent dye molecules on the species-specific hybridization probes in a multiplex TaqMan assay. Whilst multiplex quantitative PCR (qPCR) applications are common in the detection of health and food related pathogens (Ibekwe *et al*., 2002; Liu *et al*., 2013), very few examples exist for the simultaneous detection of multiple plant pathogens. In 2000, Weller *et al*. (2000) applied a fluorogenic TaqMan PCR assay to detect *Ralstonia solanacearum* strains that infect potato. More recently, Enora *et al*. (2019) developed a tetraplex qPCR assay for simultaneous detection of *Xylella fastidiosa* subspecies in plant tissues. Probes for detection of the various *X. fastidiosa* subspecies were labelled with

different fluorogenic dye and quencher molecules allowing simultaneous detection within a sample in a single PCR. Additionally, a multiplex TaqMan assay was developed by Fonseca *et al*. (2019) to detect three different species of *Xanthomonas* sp. causing necrosis and bacterial spot in *Citrus* cultures.

In order to better understand the origin and spread of bacterial species associated with AOD, there is a need for a reliable and rapid molecular diagnostic assay, which will enable the detection and quantification of multiple species. The apparent emerging nature of this disease (Moradi-Amirabad *et al*., 2019; González and Ciordia, 2020) necessitates urgent provision of a diagnostic tool. Therefore, the aim of this study was to develop a sensitive multiplex TaqMan PCR assay that is capable of simultaneous detection and quantification of four bacterial species involved in AOD syndrome in the UK, *B*. *goodwinii*, *G*. *quercinecans*, *R*. *victoriana*, and *L*. *britannica*. An important additional requirement and aim was to develop a diagnostic protocol that was nondestructive, due to the intrinsic value of mature and veteran oak trees that are affected by AOD.

2. Materials and methods

2.1 Bacterial strains and growth

All bacteria used to validate the detection assay are shown in Table 1 and Table S1. Bacteria were cultured on nutrient agar (NA; Oxoid) at 22 °C in the dark for 5 days. For real-time PCR assays, one colony of bacteria was resuspended in 100 µl phosphate-buffered saline pH 7.4 (PBS), vortexed and heated at 95 °C for 10 min. Bacterial extracts were frozen at −20 °C until use.

2.2 Primer and probe design

DNA sequences of the gyrase B (*gyrB*) gene from various *Brenneria* sp., *Lonsdalea* sp., and *Rahnella* sp. were downloaded from GenBank (Table S2) and sequences belonging to each genus were aligned with Geneious v. 9.0.2 (Biomatters Ltd) to identify single nucleotide polymorphisms (SNPs) between species. Primer 3 (www.Primer3.com) was applied to design primers and probes to gene regions spanning SNPs between species. A primer set and a probe were designed to a DNA-directed RNA polymerase gene (*rpo*) from *G. quercinecans* strain FRB97. This gene is directly upstream of the *rpoB* gene used in barcoding studies (Brady *et al*., 2017). Primer and probe dimer formation was assessed in Primer 3 (Koressaar and Remm, 2007; Untergasser *et al*., 2012) to ensure there was no interaction between any of the oligonucleotides in the multiplex PCR mix. Prior to ordering the primers and probes, the reaction was tested in silico with the Multiple Primer Analyzer (www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html).

Primers and probes binding to *rpo* from *G. quercinecans* and the *gyrB* gene from *B. goodwinii*, *L. britannica*, and *R. victoriana* are indicated in Table 2. All primers and probes were synthesized by Integrated DNA Technologies.

2.3 Real-time PCR detection of AOD-associated bacteria

Real-time PCR reactions were set up in 96-well plates, and each 10 µl reaction contained 1 × LightCycler 480 Probes Master Mix (Roche), 0.1 µM Gq284F and Gq418R primers, 0.25 µM each of Bg99F, Bg179R, Rv15F, Rv134R, Lb503F, Lb634R primers, 0.1 µM of each probe, and 2 µl bacterial sample. Each reaction was made up to 10 µl with sterile distilled water. PCRs and fluorescence detections were performed on a LightCycler480 II instrument (Roche). Thermal cycling conditions included an initial denaturation step at 95 °C for 10 min;

followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 10 s. Fluorescence at emission wavelengths of 510 nm (FAM), 580 nm (JOE), 610 nm (ROX), and 660 nm (Cy5) was measured once per cycle at the end of the 65 °C segment. The Crossing point (Cp) values (the PCR cycle number at which the fluorescence generated through TaqMan probe cleavage exceeded the threshold; Chandelier *et al*., 2019) were determined by applying an absolute quantification/fit points method (Pfaffl, 2004), with the inclusion of colour compensation to account for overlap in emission spectra between fluorescent dyes.

2.4 Preparation of DNA standards

DNA standards for *B*. *goodwinii* (FRB171), *L*. *britannica* (FRB18), and *R*. *victoriana* (141a) *gyrB* and *G*. *quercinecans* (FRB97) *rpo* were prepared by generating amplification products with respective forward and reverse primers. Products were purified with a DNA Clean and Concentrator Kit (Zymo Research) and concentration of the purified products was measured on a Qubit 2.0 fluorimeter (Invitrogen) using a Qubit dsDNA HS Assay Kit (Invitrogen). As both the *gyrB* and *rpo* genes occur as single copies in bacteria, dilutions of the standards were made to represent 10–10^{11} gene copies. These copy number standards were used to quantify bacterial numbers in environmental samples with one gene copy representing one bacterium in a sample.

2.5 Determining sensitivity of detection of TaqMan real-time PCR assays

Single colonies of *B*. *goodwinii* (FRB171), *L*. *britannica* (FRB18), *R*. *victoriana* (141a), and *G*. *quercinecans* (FRB97T) were inoculated separately into 10 ml Luria broth and grown for approximately 20 hr (mid log phase) at 25 °C with shaking on an orbital shaker (SciQuip) at 150 rpm. Optical densities ($OD_{595\ nm}$; LAXCO Spectrophotometer) were adjusted to 0.6, and serial dilutions of the cultures were made (10^{-1} to 10^{-6}). Each dilution was plated (2 µl in 98

µl PBS) onto NA in triplicate and left to grow for 4 days at 22 °C. An equivalent volume (2 µl) of each dilution was subjected to the TaqMan multiplex PCR assay for AOD-associated bacteria to determine the minimum number of detectable bacteria.

2.6 Field sample analysis

Sterile swabs (Sterilin) were used to collect exudate from stem bleeds of four *Quercus robur* trees (two swabs per tree) displaying decline symptoms at a field site in south-east England in the UK (51°40′37″N, 00°16′49″W). The sterile cotton tip of the swab was either dipped directly into the fluid seeping from the tree, or placed in a bark crack so that the cotton tip made contact with the decaying tissue. The swab was then placed back in the tube and taken back to the laboratory where it was stored at 4 °C until processing. Swabs were thoroughly rinsed in 1 ml sterile PBS to remove bacteria, after which the PBS containing bacteria cells was then taken up into a 1 ml syringe and filtered with constant pressure through a 5 µm pore hydrophilic filter (Sartorius) into a clean 1.5 ml microfuge tube. Filtered bacterial suspensions were centrifuged (Eppendorf 5804R) at 9,000 × g for 4 min, and subsequently the supernatant was discarded without dislodging the bacterial pellet. Bacterial pellets were resuspended and washed with 1 ml PBS, briefly vortexed, then centrifuged at 9,000 × g for 4 min and the supernatant pipetted off. The process was repeated three times before finally adding 50 µl PBS to yield bacterial cell suspensions that were then stored at −20 °C until use.

In addition, bark panels were collected from two bleed areas of *Q. robur* Tree 2 at the field site. Bark samples were surface sterilized for 1 min in 70% ethanol, 1 min in 0.5% sodium hypochlorite (Sigma), and thoroughly rinsed in sterile distilled water. Phloem tissue that spanned the healthy/lesion interface was plated onto PYGA bacterial culture medium (Denman *et al*., 2016). Emergent bacteria were streaked onto NA plates to obtain single colonies. These were resuspended in 100 µl PBS and subjected to PCR amplification of the

gyrB fragment (Brady *et al*., 2008). BlastN analysis (Altschul *et al*., 1990) was applied to query sequences against GenBank to identify bacterial species.

3. Results

3.1 TaqMan primer and probe design and specificity of the assay

Multiple sequence alignments of *gyrB* sequences from *Brenneria* sp., *Lonsdalea* sp., and *Rahnella* sp. (Figure 1) indicated polymorphisms between closely related species in the gene regions to which probes and primers were designed (Figure 1). However, primers and probes had identical sequences to the species from which they were designed. Multiple sequence alignment of the *gyrB* gene sequences from *B*. *goodwinii* were compared with closely related *B*. *roseae* subsp. *americana* and *B*. *roseae* subsp. *roseae* indicating that there is a single polymorphism between the *gyrB* sequence from *B*. *roseae* subsp. *americana* and the forward primer, while there are two mismatches between both *B*. *roseae* subspecies and the reverse primer. Similarly, the two *B*. *roseae* subspecies exhibit two base mismatches with the *gyrB* gene probe designed to *B*. *goodwinii*. These differences were enough for the multiplex assay to identify *B*. *goodwinii* at an early Cp in the qPCR, whilst related *Brenneria* sp. only amplified at Cps of greater than 35 cycles. We therefore only positively identified *B*. *goodwinii* if Cp values of less than 35 were obtained.

Primers and probes designed to *G*. *quercinecans rpo* were able to amplify and detect closely related *Gibbsiella* species: *G*. *acetica*, *G*. *dentisursi*, and *G*. *greigii*. Similarly, the qPCR TaqMan assay for *R*. *victoriana* was able to detect *R*. *variigena* (Table 1). No cross reaction between *B*. *goodwinii*, *L*. *britannica*, *R*. *victoriana*, and *G*. *quercinecans* was observed. Amplification was not observed in closely related species or non-target species

isolated from plant lesions and cankers including *Erwinia billingae*, *Klebsiella michiganensis*, *Lelliottia* sp. nov., *Ochrobactum* sp., *Panotea rodasii*, *Raoultella* spp., and various *Serratia* species (Table S1).

3.2 Sensitivity of multiplex TaqMan PCR

Purified *gyrB* amplicons from *B. goodwinii*, *L. britannica* and *R victoriana* and the *rpoI* amplicon from *G. quercinecans* were quantified, and serial dilutions were made for each of the products ranging from 10^{11} copies to 10^{2} copies. Plots of Cp versus $\log_{10}$ gene copy number indicated that reaction efficiencies of amplicons from all four bacteria were above 80% and $R^2 > .99$ (Figure 2). The sensitivity of the multiplex TaqMan assay was determined using serial dilutions of AOD bacteria and the minimum number of bacterial cells detected were 14 for *G. quercinecans*, 76 for *B. goodwinii*, 75 for *L. britannica*, and 25 for *R. victoriana* (Table 3).

3.3 Multiplex PCR amplification from swab samples

Bacterial suspensions obtained from processed swabs from *Q. robur* trees collected at a field site in south-east England (Figure 3) were assessed with the multiplex TaqMan assay for AOD-associated bacteria, and gene copy standards were included on the reaction plate in order to quantify the numbers of bacteria isolated from each swab sample. *B. goodwinii* and *Gibbsiella* sp. were detected in three of the four trees. *B. goodwinii* was present at higher levels in the exudates than *Gibbsiella* sp., and Tree 4 had the highest numbers of *B. goodwinii* (2.7×10^{7}) and *Gibbsiella* sp. (2.36×10^{3}) cells. *Rahnella* sp. was only present in Tree 2 (Figure 4). Although Tree 1 had a single dry stem bleed, no AOD bacteria were detected and the cause of the bleed was probably due to a wound and not further diagnosed.

In order to validate the results obtained with the AOD multiplex TaqMan assay, two bark panels were taken from Tree 2. A total of 29 bark pieces were extracted from two bark panels from Tree 2 and were plated onto PYGA from which bacteria were isolated from 19 bark pieces. BlastN analysis of the sequenced *gyrB* (Table 4) indicated that *B*. *goodwinii* (one isolate), *G*. *quercinecans* (seven isolates), and *R*. *variigena* (five isolates) were all present in necrotic tissue, confirming real-time PCR results from swab samples.

4. Discussion

Diagnosis of AOD in oak trees is based on the presence of *A*. *biguttatus* larval galleries in the inner bark, and often the presence of exit holes, stem bleeds, and occurrence of AOD-associated bacteria in the necrotic tissue beneath the bleeds. Until recently, identification of AOD bacteria has relied heavily on destructive sampling of the inner bark tissue, bacterial isolation, strain purification, DNA extraction, PCR, sequencing, and BLAST identification. Here we present a multiplex TaqMan method for the rapid and simultaneous detection of four bacteria present in stem bleeds associated with AOD that can be applied to raw samples eliminating a culturing step, saving time and labour costs. The method can distinguish *B*. *goodwinii* from other *Brenneria* sp., and can detect *Gibbsiella* sp., *Lonsdalea* sp., and *Rahnella* sp. to genus level. Importantly, there was no cross detection of AOD bacterial species in the assay. Inclusion of gene copy number standards for each of the genes amplified from the four species enabled quantification of bacteria from the stem swab bleed sample. The multiplex TaqMan assay was effectively applied to identify and quantify bacteria in stem bleeds from oak trees showing decline symptoms in south-east England.

The multiplex TaqMan assay for AOD bacteria was effectively able to discriminate *B*. *goodwinii* from other *Brenneria* sp. at Cp values lower than 35. This distinction is important

as occasionally *B*. *roseae* subsp. *roseae* is also found in necrotic tissue associated with bleeding tree cankers. However, it is possible that this subspecies has a virulence gene repertoire similar to that of *B*. *goodwinii* and can perform the same necrotic function as *B*. *goodwinii* in bleeding stem cankers. This hypothesis is currently being tested through genome comparison studies to examine the virulence gene complement in both species (author's unpublished data). *Gibbsiella* spp. could only be detected to the genus level, but *G*. *greigii* has so far only been isolated from California black oak in the USA (Brady *et al*., 2014a), and *G*. *dentisursi* was isolated from the oral cavity of a bear in Japan and the intestine of a butterfly in Korea (Brady *et al*., 2015). Although Geider and co-workers unified all known *Gibbsiella* species in *G*. *acetica* (Geider *et al*., 2015), ongoing research in our laboratories indicates that this grouping is incorrect, and isolate BKI used in this study belongs to *G*. *quercinecans* (C. Brady, University of the West of England, UK, personal communication). Thus, as shown here, application of the TaqMan assay should only detect *G*. *quercinecans* on European oak trees. The assay could detect both *R*. *variigena* and *R*. *victoriana* in oak stem bleeds; both bacteria have previously been isolated from necrotic lesions on oak trees (Doonan *et al*., 2019).

The multiplex TaqMan protocol was applied to rapidly identify AOD-associated bacteria present on swab samples collected from oak stem bleeds at a field site in south-east England. Trees with prolific stem bleeds all contained both *B*. *goodwinii* and *G*. *quercinecans*. This was expected as *B*. *goodwinii* was highly abundant and dominated the AOD lesion microbiome in previous studies; G. *quercinecans* was consistently present in the AOD lesion microbiome (Broberg *et al*., 2018; Denman *et al*., 2018). Although the assay indicated that *B*. *goodwinii* was the most abundant bacteria in stem bleeds, only one isolate of this bacteria was obtained from bark isolations. *B*. *goodwinii* is facultatively anaerobic (Denman *et al*., 2012) and is outcompeted by other bacteria under aerobic growth conditions,

thus yielding low numbers of isolates. Only bleeds from Tree 2 harboured *Rahnella* sp., but this genus is more ubiquitous than *G. quercinecans*, and has been shown to be associated with oak trees both with and without symptoms (Broberg *et al*., 2018).

Acute oak decline symptoms can develop rapidly, resulting in high levels of tree mortality (Denman *et al*., 2010). It is therefore necessary to implement management strategies to help curb the spread of the disease. Such strategies include surveying woodlands for AOD symptoms on *Quercus* sp., identification of disease-causing agents including AOD-associated bacteria and *A. biguttatus*, and if necessary, felling diseased oaks and appropriate disposal of diseased tissue (Denman *et al*., 2010; Brown *et al*., 2016). As other pathogens such as *Phytophthora* sp. can cause stem bleeds on oak (Denman *et al*., 2019), it is necessary to ensure that bleeds are indeed associated with AOD bacteria. The multiplex real-time PCR assay described in this paper provides a rapid diagnostic tool to identify bacteria, which can be applied to monitoring programmes to minimize the spread of the disease to other trees.

In 2019 alone, Forest Research processed 208 swab samples from oak trees sent in by the public and scientists both in the UK and in Europe. Presence of AOD bacteria in combination with photographic evidence of tree decline, bark cracking and, if present, *A. biguttatus* exit holes are used to diagnose AOD. If photographic evidence strongly suggests that an oak tree has AOD, but swab samples are negative for presence of associated bacteria, we recommend that a further swab is taken when the bleed is more active (e.g., spring or early summer) or in some cases we apply a selective enrichment culturing procedure using eosin methylene-blue (EMB) culture media (Moradi-Amirabad *et al*., 2019). An important application of the rapid diagnostic assay would be to detect seasonal changes in AOD bacterial dynamics as well as differences in AOD bacterial composition in lesions as they progress.

Future research will focus on improving the specificity of the multiplex TaqMan assay for specific detection of *G. quercinecans*. This could be achieved by designing primers and a fluorescent probe to a different gene which is conserved between *G. quercinecans* isolates but differs between *Gibbsiella* species. Ongoing sequencing projects to compare different *Gibbsiella* sp. genomes (author's unpublished data) will greatly assist in the selection of such a gene region. An alternative approach in the short term could be to include locked nucleic acids (LNA) bases at nucleotide positions that distinguish *G. quercinecans* from other *Gibbsiella* species. LNA bases are modified RNA analogues in which an oxymethylene bridge locks the ribose ring in the ideal conformation for specific binding to the complementary base. This increases hybridization stability and ensures differentiation between closely related sequences down to as little as one nucleotide difference (Kauppinen *et al*., 2005; Lumia *et al*., 2018). Furthermore, probes could be designed to include a minor groove binder (MGB) at their 3′ end. This would increase the probe-target duplex stability and enable the use of shorter target specific probes (Roussel *et al*., 2005). Roussel and co-workers (2005) designed probes with MGBs to detect fruit tree viruses that have high genome variability and it was thus necessary to target a small number of conserved nucleotides shared among different isolates.

In conclusion, we have presented a rapid TaqMan PCR method to simultaneously detect bacteria associated with tree stem bleeds. Moreover, the protocol presented here is nondestructive and allows for detection of bacteria directly from stem bleeds without the need for removal of inner bark panels from high commodity trees. This will greatly assist diagnostics and management of AOD in woodland areas.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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.

Table S1 Non-target bacteria tested for nonspecific detection with acute oak decline-associated bacteria primers and probes.

Table S2 GenBank accession numbers of sequences used to design primers and probes for *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Lonsdalea britannica*, and *Rahnella victoriana*.

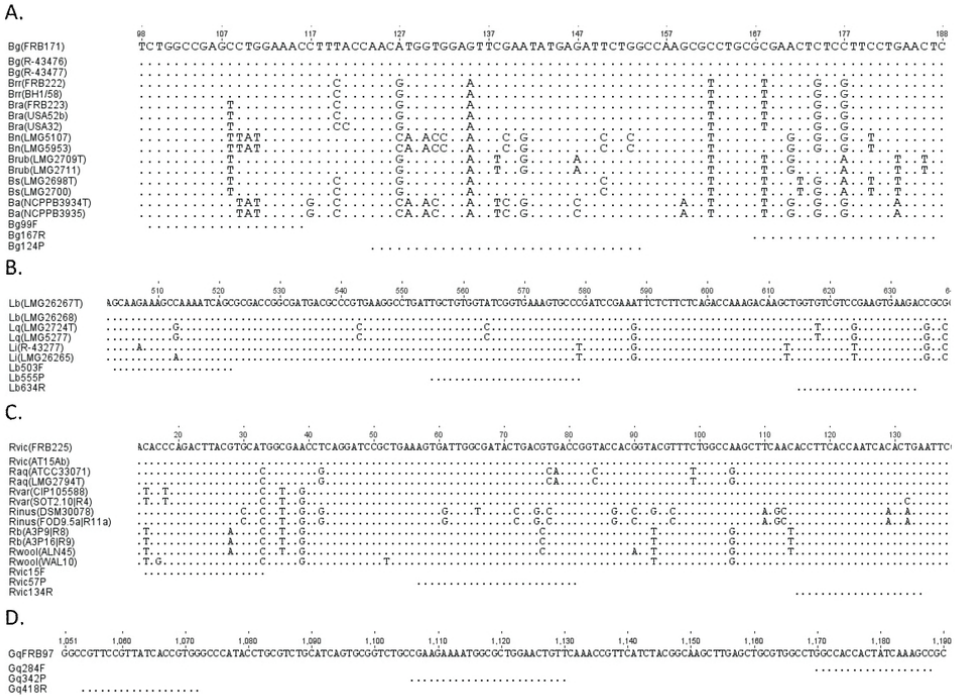
Figure legends

Figure 1 Multiple alignments of *gyraseB* (*gyrB*) gene fragments for detection of (a) *Brenneria goodwinii*, (b) *Lonsdalea britannica*, and (c) *Rahnella victoriana* and the *rpo* fragment for detection of (d) *Gibbsiella quercinecans*. The binding sites of respective primers and probes are indicated on the alignments. Genes for bacteria associated with acute oak decline were set as the reference and were aligned to homologs of closely related species within a genus to detect polymorphisms between species and determine optimal primer/probe positions. Bg, *Brenneria goodwinii*; Brr, *Brenneria roseae* subsp. *roseae*; Bra, *Brenneria roseae* subsp. *americana*; Bn, *Brenneria nigrifluens*; Brub, *Brenneria rubrifaciens*; Bs, *Brenneria salicis*; Ba, *Brenneria alni*; Lb, *Lonsdalea britannica*; Lq, *Lonsdalea quercina*; Li, *Lonsdalea iberica*; Rvic, *Rahnella victoriana*; Raq, *Rahnella aquatilis*; Rvar, *Rahnella variigena*; Rinus, *Rahnella inusitata*; Rb, *Rahnella bruchi*; Rwool, *Rahnella woolbedingensis;* Gq, *Gibbsiella quercinecans*. Isolate numbers are indicated in brackets after species abbreviations.

Figure 2 Linear relationship between crossing point (Cp) and gene copy number (log transformed) for *Brenneria goodwinii* (•), *Gibbsiella quercinecans* (△), *Rahnella victoriana* (+), and *Lonsdalea britannica* (×). Linear regression curves were calculated for each species and reaction efficiencies for each bacterial species was determined from the slope of the regression curve. Regression curves were as follows: *B. goodwinii*, $y = -3.8037x + 43.087$; *G. quercinecans*, $y = -3.5354x + 44.567$; *R. victoriana*, $y = -3.6141x + 44.42$; and *L. britannica*, $y = -3.6268x + 46.745$.

Figure 3 Decline symptoms on *Quercus robur* trees in a site in south-east England. (a) Tree 1 displaying dieback of upper branches. (b) Tree 2 and (c) Tree 3 with numerous bleeds along the length of the stem. (d) Tree 4 with a bleed at the base of the stem.

Figure 4 Detection and quantification of acute oak decline-associated bacteria on oak trees at a field site in south-east England. Tree 1 had a dry stem bleed and no acute oak decline-associated bacteria were detected.



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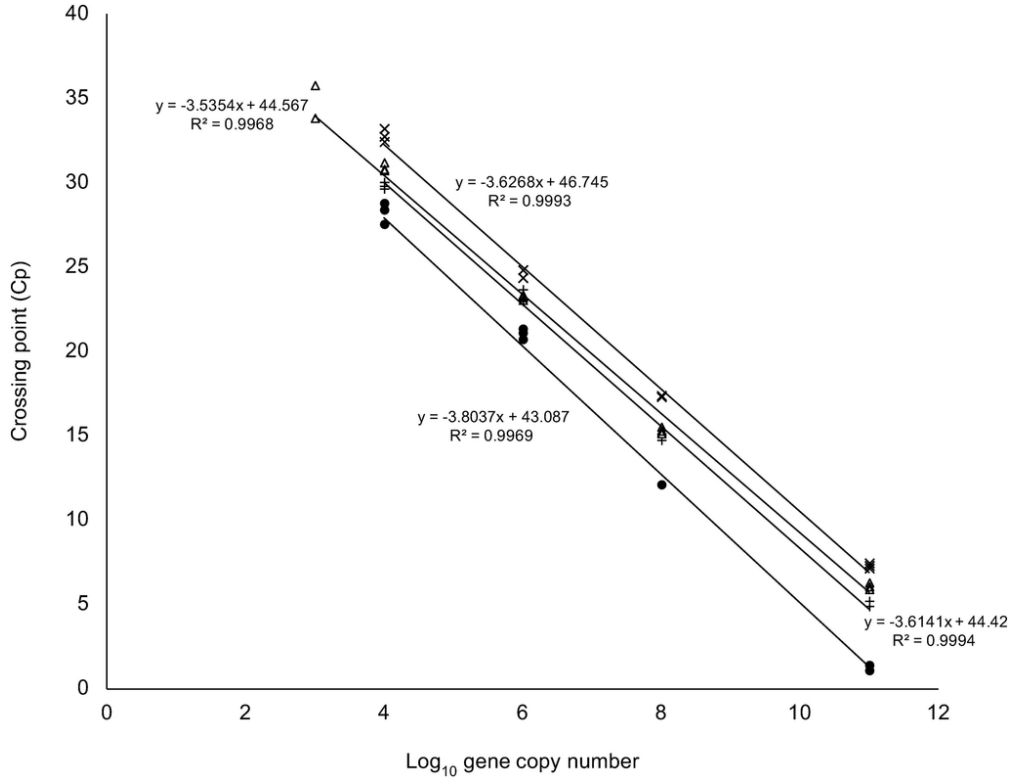

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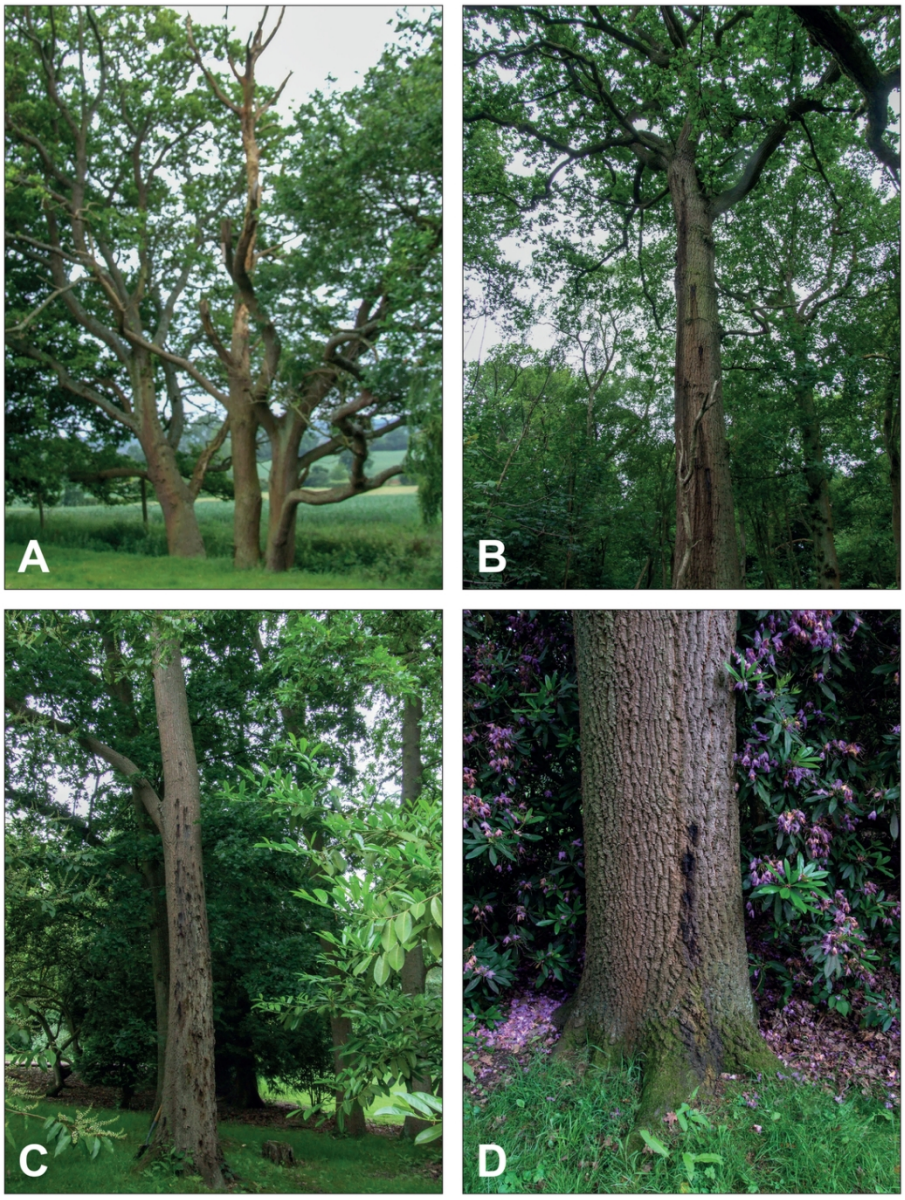

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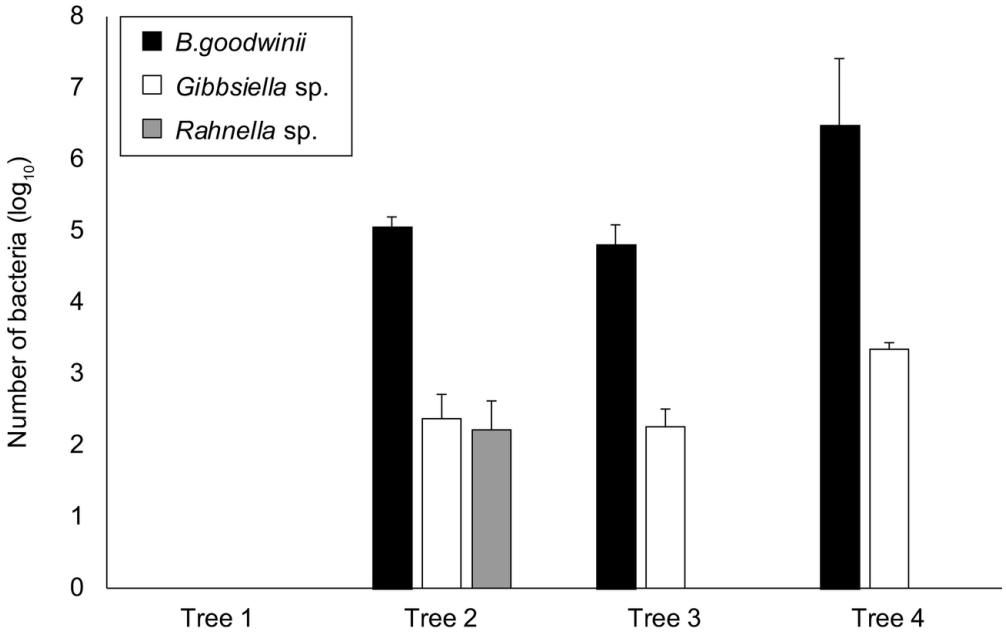

219x138mm (150 x 150 DPI)

Table 1 Bacterial reference strains used in this study and their associated real-time PCR detection result

Bacterial species	Isolate number	Host association	PCR detection[a]			
			Bg	Lb	Rvic	Gq
Brenneria alni	NCPPB934	*Alnus* sp.	−	−	−	−
Brenneria goodwinii	FRB171	*Quercus robur*	+	−	−	−
	FRB141^T	*Q. robur*	+	−	−	−
	FRB186	*Q. robur*	+	−	−	−
Brenneria roseae ssp.	FRB222^T	*Q. robur*	−	−	−	−
roseae	BH1/40b	*Q. robur*	−	−	−	−
	BH1/43c	*Q. robur*	−	−	−	−
	BH1/43d	*Q. robur*	−	−	−	−
	BH1/58	*Q. robur*	−	−	−	−
	BH1/82b	*Q. robur*	−	−	−	−
B. roseae ssp. *americana*	FRB223^T	*Quercus kelloggi*	−	−	−	−
	USA32	*Q. kelloggi*	−	−	−	−
	USA52b	*Q. kelloggi*	−	−	−	−
Brenneria rubrifaciens	NCPPB2020	*Jugulans regia*	−	−	−	−
Brenneria salicis	NCPPB447^T	*Salix* sp.	−	−	−	−
Gibbsiella acetica	BK1	Necrotic apple wood	−	−	−	+
Gibbsiella dentisursi	DSM23818^T	Oral cavity of a bear	−	−	−	+
	LEN33	Butterfly intestine	−	−	−	+
Gibbsiella greigii	FRB224T	*Q. kelloggi*	−	−	−	+
Gibbsiella quercinecans	FRB97^T	*Q. robur*	−	−	−	+
	FRB24	*Q. robur*	−	−	−	+
	BH1/19	*Q. robur*	−	−	−	+
Lonsdalea britannica	FRB18	*Q. robur*	−	+	−	−
	DUD5	*Q. robur*	−	+	−	−
Rahnella bruchi	FRB226T	*Agrilus biguttatus*	−	−	−	−
Rahnella inusitata	B1	Unknown	−	−	−	−
Rahnella variiega	CIP105588^T	Human burn	−	−	+	−

Rahnella victoriana	FRB141a	*Q. robur*	−	−	+	−
Rahnella woolbedingensis	FRB227^{T}	*Alnus glutinosa*	−	−	−	−

Note. +, positive detection; −, negative detection.

[a]Probe for: Bg, *Brenneria goodwinii*; Lb, *Lonsdalea britannica*; Rvic, *Rahnella victoriana*; Gq, *Gibbsiella quercinecans.*

Table 2 Primer and TaqMan probe sequences for multiplex quantitative PCR detection of acute oak decline-associated bacteria

Species	Gene	Primer[a]	Sequence (5′–3′)	Product size (bp)
Brenneria goodwinii	*gyrB*	Bg99F	CTGGCCGAGCCTGGAAAC	88
		Bg179R	AGTTCAGGAAGGAGAGTTCGC	
		Bg124P	FAM-CCAGAATCTCATATTCGAACTCCACCATGTT-BHQ1	
Gibbsiella quercinecans	*rpo*	Gq284F	GGCTTTGATAGTGGTGGCC	134
		Gq418R	CGTTCCGTTATCACCGTGG	
		Gq342P	Cy5-AACAGTTCCAGCGCCATTTTCTTCG-BHQ3	
Lonsdalea britannica	*gyrB*	Lq503F	GCAAGAAAGCCAAAATCAGC	131
		Lq634R	TCTTCACTTCGGACGACACC	
		Lq555P	JOE-TGCTGTGGTATCGGTGAAAGTGCCC-BHQ1	
Rahnella victoriana	*gyrB*	Rv15F	CACCCAGACTTACGTGCAT	119
		Rv134R	TCAGTGTGATTGGTGAAGGT	
		Rv57P	ROX-AGTGATTGGCGATACTGACGTGACC-BHQ2	

[a]F, forward; R, reverse; P, probe.

Table 3 Assessment of the sensitivity of the acute oak decline (AOD) TaqMan assay to detect bacterial species

AOD-associated bacterial species	Dilution factor	Colony count[a]	Equivalent cfu/ml	TaqMan Cq value[a]
Brenneria goodwinii	10^{-1}	TNTC	—	16.07 ± 0.57
	10^{-2}	TNTC	—	23.97 ± 1.82
	10^{-3}	2203 ± 48	1.1×10^{6}	26.65 ± 1.02
	10^{-4}	417 ± 72	2.1×10^{5}	28.70 ± 1.87
	10^{-5}	76 ± 2	3.8×10^{4}	34.13 ± 0.84
Gibbsiella quercinecans	10^{-1}	TNTC	—	18.93 ± 0.14
	10^{-2}	TNTC	—	24.49 ± 0.08
	10^{-3}	1647 ± 97	8.2×10^{5}	27.67 ± 0.03
	10^{-4}	263 ± 34	1.3×10^{5}	31.36 ± 0.10
	10^{-5}	14 ± 4	7.0×10^{3}	35.72 ± 0.28
Lonsdalea britannica	10^{-1}	TNTC	—	21.47 ± 0.48
	10^{-2}	TNTC	—	25.48 ± 0.18
	10^{-3}	2107 ± 257	1.0×10^{6}	28.83 ± 0.18
	10^{-4}	557 ± 66	2.8×10^{5}	31.93 ± 0.01
	10^{-5}	75 ± 11	3.8×10^{4}	35.68 ± 0.02
Rahnella victoriana	10^{-1}	TNTC	—	25.47 ± 1.00
	10^{-2}	TNTC	—	28.84 ± 0.03
	10^{-3}	948 ± 128	4.7×10^{5}	31.51 ± 0.15
	10^{-4}	80 ± 14	4.0×10^{4}	35.13 ± 0.83
	10^{-5}	25 ± 2	1.2×10^{3}	36.85 ± 1.29

Note. AOD-associated bacteria were grown, diluted, and plated at a volume equivalent to the volume added to the TaqMan assay. Colony count is the number of bacteria added to TaqMan assay. Counts were performed and compared with Cq values obtained in TaqMan PCRs for each of the bacterial species. TNTC, too numerous to count.

[a]Each sample consisted of three replicates. Reported values represent the average of the three replicates.

Table 4 Bacteria isolated from lesions beneath stem bleeds of *Quercus robur* Tree 2 at a site in south-east England

Top BlastN hit	**No. of isolates**	**Accession no.**
Brenneria goodwinii FR141	1	CP014137.1
Gibbsiella quercinecans FRB97	7	CP014136.1
Rahnella variigena	5	FJ268864.1

Note. Bacteria were identified by sequencing the *gyrase B* gene and subsequent BlastN analysis (>99% query coverage; >98% maximum identity).