Development of a real-time PCR assay for the identification of *Brenneria goodwinii*, associated with Acute Oak Decline (AOD) of *Quercus* spp. in the United Kingdom

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Introduction

- Acute Oak Decline (AOD) is a disorder of Oak species (*Quercus robur, Q. petrea*) in England (Denman & Webber, 2009).
- Symptoms of AOD include stem bleeds, necrotic tissue underlying stem bleeds and sometimes the presence of larval galleries (Denman & Webber, 2009).
- Intensive surveys in England have found that AOD is increasing in distribution.
- Research carried out by Forest Research has identified two Enterobacterial species, *Gibbsiella quercinecans* and *Brenneria goodwinii*, and the buprestid *Agrilus biguttatus* that are associated with the development of AOD.
- The bacteria are generally identified using culturing techniques and DNA sequencing of informative gene regions. However, these techniques are expensive and labour intensive.
- The aim of this study was to develop a rapid and reliable diagnostic assay to identify *Brenneria goodwinii*.

Materials and Methods

- A DNA sequence dataset for the gyraseB gene region for species of *Brenneria*, *Gibbsiella*, *Lonsdalea*, *Dickeya* and several related genera from the Enterobacteriaceae was constructed by downloading sequences from the NCBI and by sequencing field isolates collected in the UK from AOD lesions on oak trees.
- GyraseB PCR and DNA sequencing followed the protocol of Brady *et al.*, (2008).
- Oligonucleotide primers and hydrolysis probes specific for *B. goodwinii* were identified and developed by the company PrimerDesign (www.primerdesign.co.uk/home).
- Developed primers and hydrolysis probes were used in initial validation experiments on the LightCycler® 480 II System (Roche Applied Science) in order to:
  - Determine sensitivity
  - Specificity testing
  - Generate reaction standard curves
  - Determine reaction efficiency

Results and Discussion

- Oligonucleotide primers and a hydrolysis probe were successfully designed for the gyraseB gene region of *Brenneria goodwinii* by PrimerDesign (Table 1). The PCR product was 89 bp. long with a Tm of 74.4°C.
- A specificity reaction was undertaken against many bacterial species and genera frquently isolated from AOD affected trees using the designed primers and probe and these proved specific for *B. goodwinii* (Table 2).
- A Standard curve for the *B. goodwinii* real-time PCR reaction was established showing the reaction had acceptable efficiency (Figure 1) and was able to reliably detect the *B. goodwinii* gyraseB gene down to 0.1 pg/µl (figure 2, Table3).

Conclusions

- A real-time PCR reaction has been developed for the bacterium *Brenneria goodwinii* associated with AOD.
- Further validation reactions will be carried out against additional Brenneria species and a broader reange of Enterobacterial species to ensure absolute specifity.
- This assay will eventually be upscaled and placed into routine diagnostic use at Forest Research.

Primer/ Probe	Sequence (5'-3')	Tm	GC %	Position
BG_F	CTGGCCGAGCCTGGAAAC	58.2	66.7	99
BG_R	AGTTCAGGAAGGAGAGTTCGC	58.0	52.4	187
BG_P	CCAGAATCTCATATTCGAACTCCACCATGTT	-	-	-

Table 1: Details of primers and probe used for the *Brenneria goodwinii* real-timehydrolysis PCR reaction.

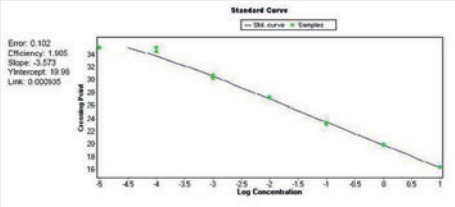

Figure 1: Standard curve of the *B. goodwinii* real-time hydrolysis PCR reaction. The reaction produced an efficiency of 1.905 and an error of 0.102 (acceptable values should be <0.2).

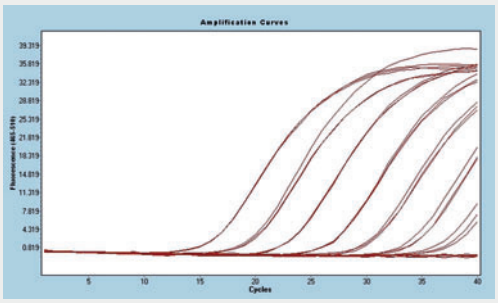

Figure 2: DNA concentration dilution and reaction amplification curve of *B. goodwinii* FRB141 DNA. Refer to Table 2 for Cp values.

Species	Isolate	Hydrolysis probe reaction
Brenneria alni	NCPPB3835	Negative
Brenneria goodwinii	FRB171	Positive
Brenneria nigrifluens	NCPPB564	Negative
Erwinia billingiae	NCPPB661	Negative
Erwinia toletana	LMG24162	Negative
Gibbsiella dentisursi	DSM23818	Negative
Gibbsiella quercinecans	FRB91	Negative
Gibbsiella quercinecans	FRB61	Negative
Gibbsiella quercinecans	FRB97	Negative
Kluyvera intermedia	LMG3019	Negative
Pantoea agglomerans	LMG1286	Negative
Pectobacterium rhapontici	NCPPB1578	Negative
Rahnella genomo sp. 2	CIP105588	Negative
Rahnella genomo sp. 3	DSM30078	Negative
Rahnella sp.	FRB165B	Negative
Serratia proteomaculans	FRB132	Negative
Serratia entomophila	LMG8456	Negative
Serratia fonticola	LMG7882	Negative
Serratia marcescens	LMG2792	Negative

Table 2: Initial specificity tests using the *B. goodwinii* primers and hydrolysis probe against phytogenetically related bacterial species and bacterial species commonly encountered in AOD affected oak trees.

Dilution	Isolate	Concentration	Mean Cp.	Std Cp.
10^{1}	FRB141	10.0 ng/µl	15.70	0.10
10^{0}	FRB141	1.0 ng/µl	16.31	0.01
10^{-1}	FRB141	0.1 ng/µl	19.78	0.05
10^{-2}	FRB141	10.0 pg/µl	23.13	0.10
10^{-3}	FRB141	1.0 pg/µl	27.14	0.01
10^{-4}	FRB141	0.1 pg/µl	30.43	0.21
10^{-5}	FRB141	10.0 fg/µl	34.77	0.40
10^{-6}	FRB141	1.0 fg/µl	35.00	0.00

Table 3: Serial distribution of *B. goodwinii* DNA and the associated Cp values. Each dilution was run in triplicate on the LightCycler® 480 II real-time PCR system.

References

Brady C, Cleenwerck I, Venter S, Vancanneyt M, Swings J, Coutinho T (2008). Phylogeny and identification of Pantoea species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). *Systematic and Applied Microbiology* **31**: 447-460.

Denman S, Webber J (2009). Oak Declines, new definitions and new episodes in Britain. *Quarterly Journal of Forestry* **103**: 285-290.