Rapid identification of bacteria associated with Acute Oak Decline by high resolution melt (HRM) analysis

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Running title: Identification of AOD bacteria

Significance and Impact of Study

Acute Oak Decline (AOD) is an increasing threat to Britain's native oak population. Two novel bacterial species both belonging to the family *Enterobacteriaceae*, *Gibbsiella quercinecans* and *Brenneria goodwinii*, are thought to play an important role in symptom development. Here we describe a rapid identification technique using high resolution melt (HRM) analysis of the *atpD* gene able to assign isolates to either *G. quercinecans* or *B. goodwinii* in a single assay, greatly reducing the time taken to identify if either or both of these species are present in symptomatic oak.

Abstract

Two Gram-negative *Enterobacteriaceae*, *Gibbsiella quercinecans* and *Brenneria goodwinii*, are frequently isolated from oak suffering from Acute Oak Decline. These two species are difficult to identify based on colony morphology, carbohydrate utilisation or 16S rRNA gene sequence, and identification using *gyrB* gene sequencing is time-consuming and laborious. A rapid identification technique, based on high resolution melt (HRM) analysis of the *atpD* gene, was designed to efficiently process numerous isolates from an increasing number of affected woodlands and parks. Principal component analysis of the resulting melt curves from strains of *G. quercinecans*, *B. goodwinii* and their close phylogenetic relatives allowed differentiation into distinct clusters based on species or subspecies identity.

Keywords

Gibbsiella quercinecans, *Brenneria goodwinii*, Acute Oak Decline, high resolution melt, HRM

48 **Introduction:**

49

50 Acute Oak Decline (AOD) is an increasingly serious problem in Britain, affecting the native
51 oak species *Quercus robur* and *Q. petraea* throughout the Midlands and south east of the
52 country. Symptoms appear on mature oaks and include weeping patches, cracks in the outer
53 bark, necrosis of the underlying tissue and larval galleries of the bark boring buprestid
54 *Agrilus biguttatus* (Denman *et al*., 2014). Investigations into the cause of AOD started in
55 2008/2009 and in the years following, numerous bacteria have been isolated from
56 symptomatic tissue and fluid exudates. A large proportion of these bacteria were found to
57 belong to the family *Enterobacteriaceae* and several were proposed as novel genera, species
58 or subspecies within this family (Brady *et al*., 2010, Brady *et al*., 2012, Denman *et al*., 2012,
59 Brady *et al*., 2014a, Brady *et al*., 2014b). It appears that the two most frequently isolated
60 species, *Gibbsiella quercinecans* and *Brenneria goodwinii*, play an important role in causing
61 symptoms of the decline (Denman *et al*., 2014). Other species belonging to the genera
62 *Brenneria*, *Lonsdalea*, *Rahnella*, *Erwinia* and *Ewingella* are often isolated in lower numbers
63 along with *G. quercinecans* and *B. goodwinii*, and could possibly contribute to lesion
64 formation and decay of the inner tissues.

65

66 Members of the *Enterobacteriaceae* are typically difficult to identify owing to a high degree
67 of similarity in both phenotype and 16S rRNA gene sequence. Sequencing and phylogenetic
68 analysis of protein-encoding genes such as *gyrB*, *rpoB* and *dnaJ* have greatly improved
69 identification and classification of enterobacteria (Dauga *et al*., 2002, Delmas *et al*., 2006,
70 Mollet *et al*., 1997, Pham *et al*., 2007). The majority of the novel species associated with
71 AOD were described using a multilocus sequence analysis (MLSA) scheme based on four

protein-encoding genes (*gyrB*, *rpoB*, *infB* and *atpD*), but routine identification of bacteria associated with AOD has been typically based on amplification and sequencing of the *gyrB* gene of cultured isolates. This approach, although time-consuming due to the large number of epi- and endophytes present in oak, has allowed researchers to gain insight into the composition of the culturable bacterial population of symptomatic native oak. However, with an increasing number of woodlands and parklands affected by AOD, new isolations from infected tissue are performed on a regular basis, highlighting the need for rapid methods of accurate identification and detection of both *G. quercinecans* and *B. goodwinii*.

High resolution melt (HRM) analysis is a real-time PCR-based molecular technique, whereby single nucleotide polymorphisms (SNPs) can be identified in amplification products without sequencing. A short stretch of the target gene is amplified in the presence of an intercalating dye that only fluoresces when bound to double-stranded DNA. Following amplification, samples are subjected to a melting step where the temperature increases with each cycle until 95 °C is reached. The loss of fluorescence is measured as the double-stranded amplification product denatures and a melt curve is generated. The rate of lost fluorescence correlates with the G+C content and sequence length of the amplification product. Therefore, samples differing by a single base pair can generate different melting curves. Genotyping by high-resolution melting has been in use for over 10 years (Gundry *et al*., 2003, Wittwer *et al*., 2003) and improvements in the chemistry of the saturating dyes and the system technology have made this an attractive alternative to standard techniques for SNP detection, mutant detection and methylation (Vossen *et al*., 2009). Typically HRM analysis is applied to identification of clinical bacteria (Pietska *et al*., 2009, Talmi-Frank *et al*., 2010, Sangal *et al*., 2013) and detection of mutations in cancer samples and markers (Boyd

et al., 2011, Takano *et al*., 2008). However, this technique is gaining popularity for SNP detection of plant and insect species (Mackay *et al*., 2008, Swisher *et al*., 2012, Wetten *et al*., 2015). Here we describe the application of HRM analysis of the *atpD* gene as a rapid identification technique for bacteria, particularly *G. quercinecans* and *B. goodwinii*, associated with AOD in Britain.

Results and discussion:

Bacterial strains, isolated from native oak species in the UK, previously identified as either *G. quercinecans* or *B. goodwinii* were selected for this study. Additionally, strains belonging to the close phylogenetic relatives of *G. quercinecans* (*G. greigii*, isolated from oak in the USA, and *G. dentisursi*, not isolated from oak) and subspecies of both *B. roseae* and *L. quercina* (*B. roseae* ssp. *roseae*, *B. roseae* ssp. *americana*, *L. quercina* ssp. *quercina*, *L. quercina* ssp. *britannica* and *L. quercina*. ssp. *iberica*; all isolated from symptomatic oak in the UK, USA and Spain) were also included (Suppl. table 1). Sequence alignments of four housekeeping genes (*gyrB*, *rpoB*, *infB* and *atpD*), routinely used in a MLSA scheme to classify novel *Enterobacteriaceae* (Brady *et al*., 2008) were examined for regions of heterogeneity between species of *Gibbsiella*, *Brenneria* and *Lonsdalea*. The *atpD* gene (encoding the β subunit of ATP synthase) was found to be a good candidate target gene as several regions contain SNPs which could potentially differentiate between strains of *G. quercinecans* and *B. goodwinii*. Several different primer pairs targeting short sequences of the *atpD* gene were designed and tested against strains of *G. quercinecans*, *B. goodwinii*, their close phylogenetic relatives and species of *Rahnella* (often isolated from symptomatic oak in

lower numbers). The optimal primer pair should be able to amplify all strains tested and reveal enough diversity in the target gene for differentiation of species.

The *atpD* primer pair designed and selected for this study (Gq6bF and Gq6bR) could amplify all species tested belonging to the genera *Gibbsiella*, *Brenneria* and *Lonsdalea* (Suppl. table 1). A unique melting curve was generated for each bacterial species examined in the HRM analysis indicating the presence of SNPs. While the melting curve generated per strain was specific for that species, it was still difficult to definitively distinguish several strains of *Gibbsiella quercinecans* and *Brenneria goodwinii* based solely on the shape of the melt curve. This could possibly be due to a high degree of intraspecies relatedness within each of these species and the short gene region examined. To counteract this, each individual PCR product was combined ('spiked') with an *atpD* PCR probe of the *G. quercinecans* reference strain (FRB 97^{T}), prior to the melting step. The *atpD* gene of the *G*. *quercinecans* reference strain was amplified separately on a large scale to create the PCR probe. This maximised the differentiation between each species by forcing amplification products to create heteroduplexes on which the melting analysis was performed. Ideally, strains which are less related to the *G. quercinecans* reference strain will form a less stable heteroduplex, dissociate and lose fluorescence more easily than strains which are closely related to *G. quercinecans* and form stable heteroduplexes.

Fig. 1 shows the distinct melt curves of four species frequently associated with AOD in Britain (*Gibbsiella quercinecans*, *Brenneria goodwinii*, *Brenneria roseae* ssp. *roseae* and *Lonsdalea quercina* ssp. *britannica*), based on the mean of 9-10 strains of *G. quercinecans* and *B*. *goodwinii*, and 2-5 strains for *B. roseae* ssp. *roseae* and *L. quercina* ssp. *britannica* (all

143 HRM PCRs performed in duplicate). Principal component analysis performed on the data
144 generated by HRM in ScreenClust (Qiagen), reveals that strains belonging to each of these
145 four species are easily separated into four distinct species specific clusters, each containing
146 the relevant type strain (Fig. 2). Each dot represents a single HRM reaction, with all strains
147 performed in duplicate. Strains identified as *G. quercinecans* form a densely populated
148 cluster, suggesting little sequence variation in the 119 bp *atpD* region among these isolates.
149 When the *atpD* sequence alignment for these strains is examined, only one SNP (at position
150 92) is observed for two strains of *G. quercinecans* compared to the reference strain (FRB
151 97^{T}) (Table 1). Slightly more variation is evident within *B. goodwinii*, with six out of nine
152 strains exhibiting a SNP (at position 81) compared to the reference strain (FRB 141^{T}) (Suppl.
153 Fig. 1). This is reflected in the scatter plot where *B. goodwinii* strains are more dispersed
154 within their cluster. Both *B. roseae* ssp. *roseae* and *L. quercina* ssp. *britannica* strains are
155 contained in discrete clusters easily distinguishable from the two most frequently isolated
156 species associated with AOD (Fig. 2).
157
158 HRM analysis allows not only the differentiation between species belonging to different
159 genera, but also between closely related species belonging to the same genus and even
160 between different subspecies. A scatter plot, focussed only on *Gibbsiella*, demonstrates that
161 the three species belonging to this genus can be easily separated based on SNPs revealed by
162 their melt curve, despite their close phylogenetic relationship (Fig. 3a). *G. greigii* has, so far,
163 only been isolated from California black oak in the USA and *G. dentisursi* has been isolated
164 from the oral cavity of a bear in Japan and the intestinal tract of a butterfly in Korea. To
165 date, neither of these *Gibbsiella* species have been isolated from symptomatic oak in
166 Britain. However as these three species are so closely related (Suppl. Fig. 2), it was

167 necessary to ensure that the rapid identification technique could distinguish strains
168 belonging to all *Gibbsiella* species in the event of a host jump. The resolution which HRM
169 analysis can provide below the species level is evident within *B. roseae* and *L. quercina*.
170 Although strains belonging to both subspecies of *B. roseae* differ by a single SNP, they form
171 two distinct clusters in a scatter plot following principal component analysis, whereas
172 subspecies of *L. quercina* are more diverse with a range of SNPs amongst them and form
173 three specific clusters corresponding to *L. quercina* ssp. *quercina*, *L. quercina* ssp. *britannica*
174 and *L. quercina* ssp. *iberica* (Fig. 3b). In Britain, *B. goodwinii* is one of the most frequently
175 isolated bacteria from symptomatic oak, followed by *B. roseae* ssp. *roseae* and *L. quercina*
176 ssp. *britannica*. To our knowledge, *B. roseae* ssp. *americana* and *L. quercina* ssp. *quercina*
177 have been isolated from symptomatic oak only in the USA, and *L. quercina* ssp. *iberica* from
178 symptomatic oak only in Spain. Despite more diversity existing between species of *Brenneria*
179 and *Lonsdalea* (Fig. 2), it is crucial that strains belonging to any of these species found on
180 symptomatic oak can be distinguished from each other in the rapid identification assay.
181
182 To test whether the HRM assay is sensitive enough to detect lower quantities or
183 concentrations of *G. quercinecans*, the reference strain FRB 97^T was serially diluted from 10^0
184 to 10^{-10}. Each dilution was subjected to HRM analysis using the same primers and conditions
185 with an undiluted sample (a single colony in 750 µl sterile water) as a positive control. The
186 melt curve for each diluted sample was visually compared to the undiluted control and the
187 shape of the curves remain consistent up to 10^{-4} (4.4×10^6 CFU ml^{-1}) (data not shown). With
188 HRM analysis able to detect *G. quercinecans* in a 10 000 fold dilution, it's possible that with
189 some modifications, the assay could be applied to future samples swabbed directly from
190 infected oak tissue broadening the application of this identification technique.

AOD is an increasing threat to the native oak population in Britain and as such, a large number of samples from symptomatic tissue are processed on a regular basis. The aim of this study was to create a single rapid identification technique for both *G. quercinecans* and *B. goodwinii*, the two species most frequently isolated from symptomatic oak. HRM analysis of a short region of the *atpD* gene has achieved this and also shows great future promise for identification of non-cultured samples. Including a reference strain of *G. quercinecans* and *B. goodwinii* in each run of the assay allows for comparison of isolates to both species, greatly reducing the time taken to identify bacteria isolated from oak tissue.

Materials and methods:

Published sequences of the *atpD* gene (ATP synthase β subunit) from *Enterobacteriaceae* isolated from symptomatic oak were aligned and examined for a region containing heterogeneity between species. Primers were designed to amplify a short stretch (119 bp) of the *atpD* gene demonstrating sufficient sequence divergence between species of *Gibbsiella*, *Brenneria* and *Lonsdalea* (Gq6bF: 5'GGC AAC CCA TCG ACA TGA A 3'; Gq6bR: 5' CTT GAT ACC GGT TTC CAG CAG 3') (Suppl. Tables 1 and 2). For all bacterial strains, a single colony was re-suspended in 750 µl sterile distilled water and 2 µl was used in a 20 µl PCR mixture containing 2x SensiFAST HRM mix (Bioline) and 10 pmol of each primer. HRM analysis was performed on 2 – 10 strains of each of the following species in duplicate: *G. quercinecans*, *G. greigii*, *G. dentisursi*, *B. goodwinii*, *B. roseae* ssp. *roseae* and ssp. *americana*, *Lonsdalea quercina* ssp. *quercina*, ssp. *britannica* and ssp. *iberica* (Suppl. Table 1) in a Rotor-Gene Q cycler (Qiagen). Following initial denaturation for 10 min at 95 °C and 45 amplification cycles of 5 sec denaturation at 95 °C and 30 sec annealing at 58 °C, each PCR

reaction was ‘spiked’ with 20 µl of the *G. quercinecans* probe (the *atpD* amplification product of reference strain FRB 97^T which was amplified separately using the primers and conditions listed above). The melting step was then performed with 2 min denaturation at 95 °C, 2 min hold at 55 °C and melting from 55 to 90 °C where the temperature was increased by 0.1 °C each cycle. The fluorescence was measured at the end of each cycle and a melt curve was generated by the Rotor-Gene software (Qiagen).

To assess the statistical differences between each of the species tested, melting curves (normalised between 80-81 and 87-88 °C) were converted to residual plots in ScreenClust v 1.10.1.2 (Qiagen). A preliminary analysis was run with the number of possible clusters fixed to the number of expected species (supervised mode) and then compared to an analysis with the optimal number of clusters generated automatically by the software (unsupervised mode). Screenclust allocates strains to the most appropriate cluster, indicating which SNPs generate significantly different melt curves.

Amplification and sequencing of the *atpD* gene for each strain examined by HRM analysis was performed as previously described (Brady *et al*., 2008). *atpD* sequences were aligned and trimmed in BioEdit v 7.2.5 (Hall, 1999) to reflect the 119 bp fragment amplified used in the HRM analysis. A maximum likelihood tree was constructed in MEGA v 6 (Tamura *et al*., 2006) using the general time reversible model with proportion of invariable sites and gamma distribution and with 1000 bootstrap replicates.

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Conflict of interests:

The authors declare no conflict of interests.

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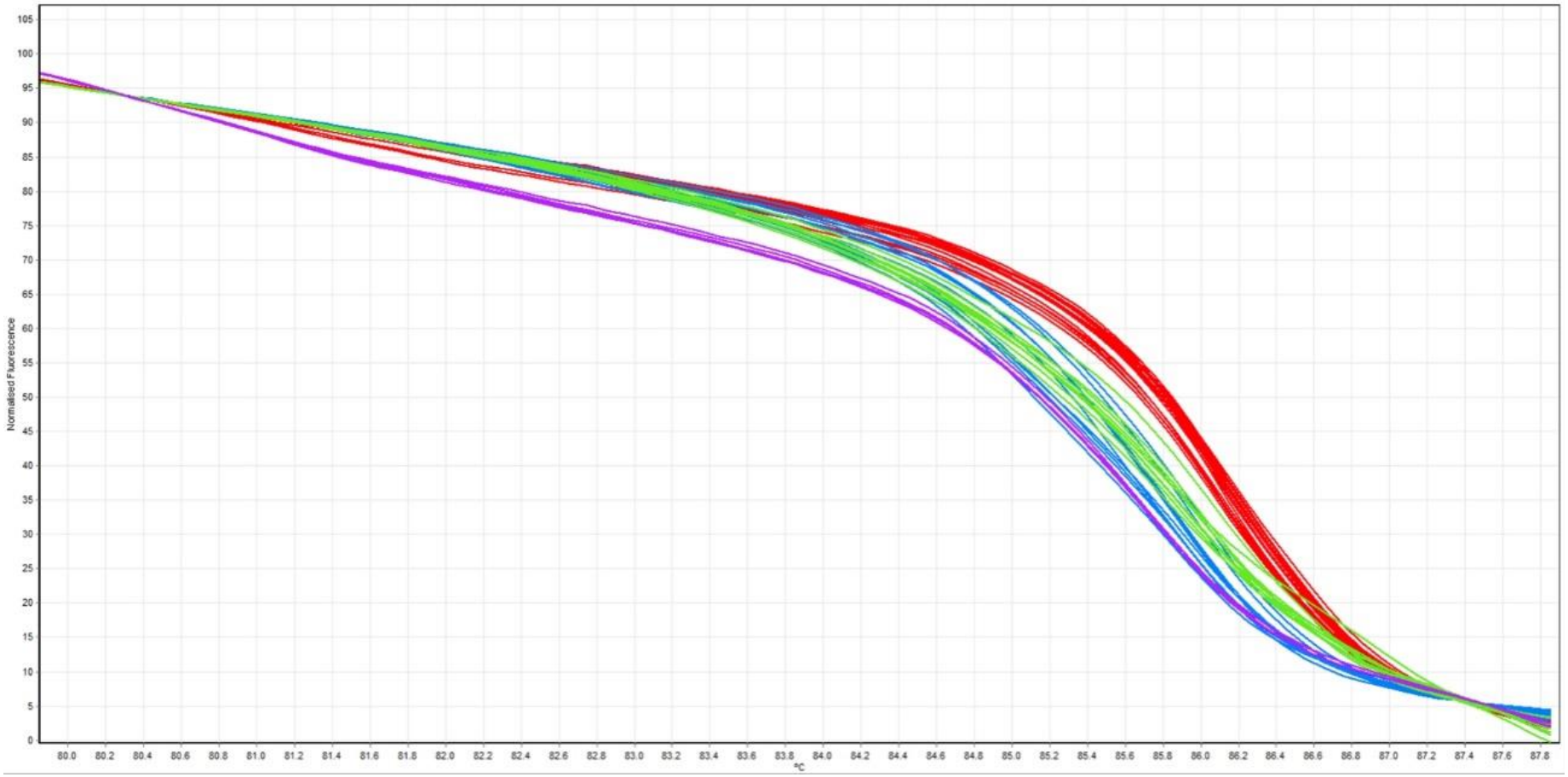
Table 1: Sequence variation within the partial *atpD* gene (119 bp) of *Gibbsiella quercinecans*, *Brenneria goodwinii*, *Brenneria roseae* subsp. *roseae* and *Lonsdalea quercina* subsp. *britannica* associated with Acute Oak Decline in Britain. Single nucleotide polymorphisms are indicated as compared against the *Gibbsiella quercinecans* FRB 97^{T} reference sequence.

	Single nucleotide polymorphism position																														
	2	3	5	8	9	20	23	24	25	26	32	41	44	50	53	59	60	61	62	68	69	70	74	80	81	86	88	92	95	98	110	113
Gq reference FRB 97^{T}	G	C	A	C	A	G	T	G	A	C	C	T	C	G	T	C	G	C	G	A	A	G	C	G	T	C	A	C	G	A	C	T
Gq variation																																
n = 10	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•
Bg																																
n = 9	T	G	C	G	G	A	C	•	•	•	T	G	T	T	•	•	•	•	T	•	•	•	•	A	•/C	•	G	G	A	C	•	C
Brr																																
n = 5	C	G	•	A	G	A	•	C	C	G	•	C	•	•/A	•	T	C	•	•	•	•	C	T	A	C	A	•	A	•	•	•	C
Lqb																																
n = 2	C	G	G	G	G	A	C	•	•	T	•	G	T	T	C	•	•	A	A	G	G	C	•	A	C	•	•	•	•	•	G	•

Gq = *Gibbsiella quercinecans*, *Bg* = *Brenneria goodwinii*, *Brr* = *Brenneria roseae* ssp. *roseae*, *Lqb* = *Lonsdalea quercina* ssp. b*ritannica*

• = nucleotide identical to the *Gq* reference sequence at that nucleotide position

Figure 1: HRM curves generated for species associated with Acute Oak Decline (AOD) in Britain by amplification of partial *atpD* genes, followed by 'spiking' with the FRB 97^{T} probe and a high resolution melting step. The assay was performed on each strain in duplicate. *Gibbsiella quercinecans* n = 10 (—), *Brenneria goodwinii* n = 9 (—), *Brenneria roseae* subsp. *roseae* n = 5 (—) and *Lonsdalea quercina* subsp. *britannica* n = 2 (—).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°C

Figure 2: Scatter plot generated by ScreenClust v 1.10.1.2 (Qiagen) from HRM curve data. Each dot represents a single strain (in duplicate) of species associated with Acute Oak Decline (AOD) in Britain: *Gibbsiella quercinecans* n = 10 (●), *Brenneria goodwinii* n = 9 (●), *Brenneria roseae* subsp. *roseae* n = 5 (●) and *Lonsdalea quercina* subsp. *britannica* n = 2 (●). Cluster plot was produced from principal components 1 and 2 in an unsupervised mode.

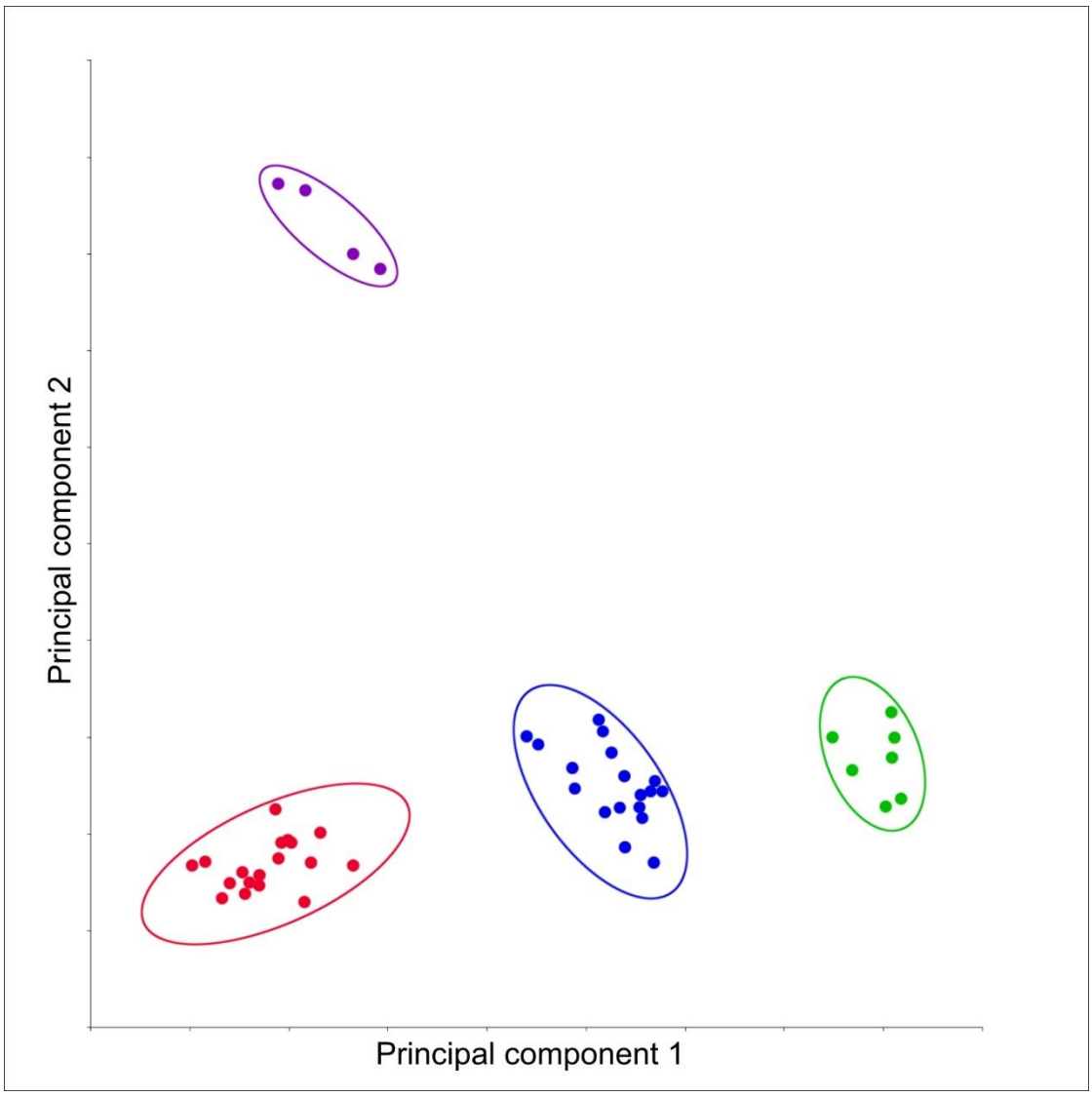
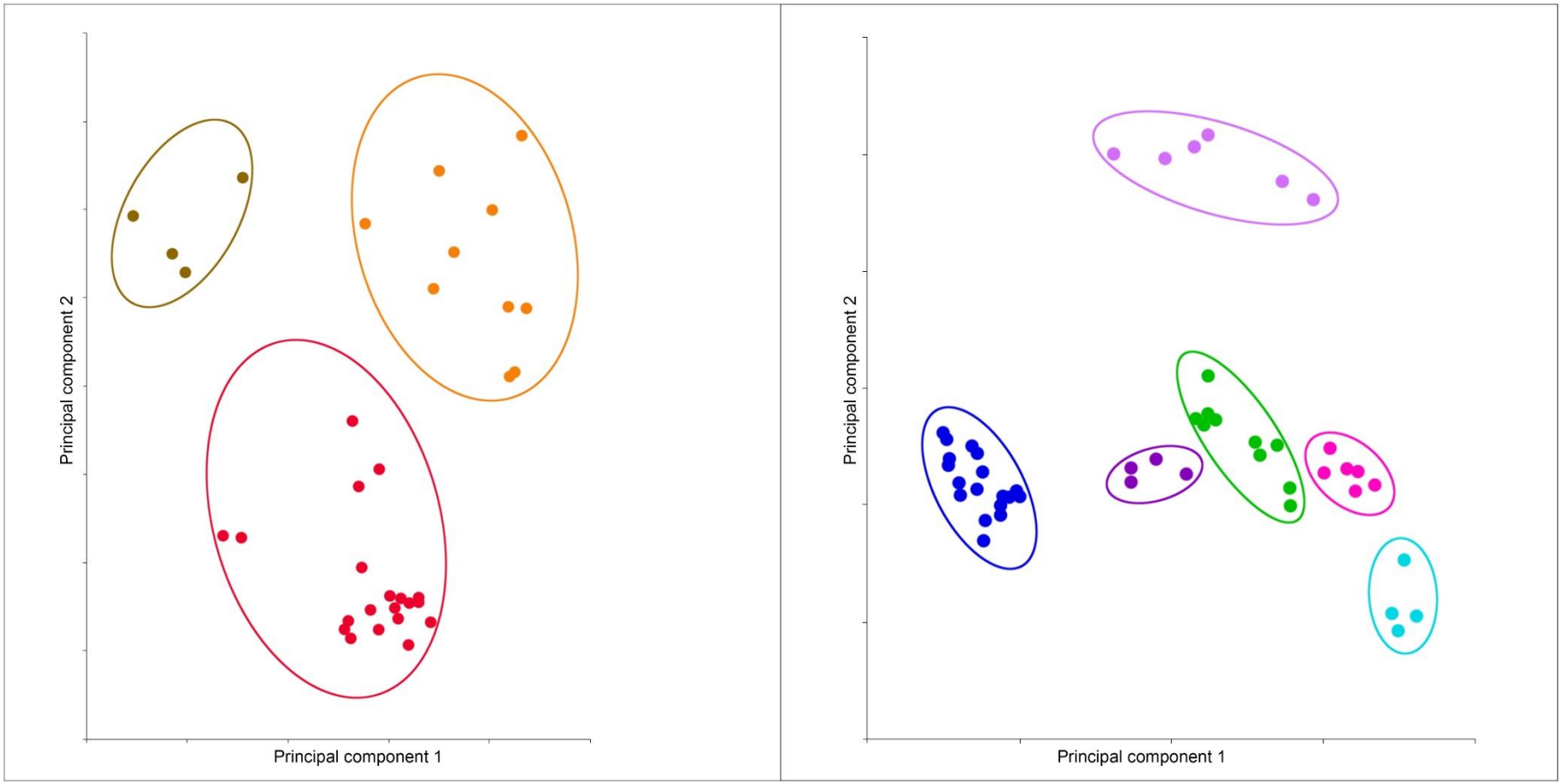

Principal component 2
Principal component 1

Figure 3a: Scatter plot generated by ScreenClust v 1.10.1.2 (Qiagen) from HRM curve data. Each dot represents a single strain (in duplicate) of *Gibbsiella quercinecans* n = 10 (●) and close phylogenetic relatives *Gibbsiella greigii* n = 5 (●) and *Gibbsiella dentisursi* n = 2 (●). Cluster plot was produced from principal components 1 and 2 in an unsupervised mode.

Figure 3b: Scatter plot generated by ScreenClust v 1.10.1.2 (Qiagen) from HRM curve data. Each dot represents a single strain (in duplicate) of *Brenneria goodwinii* n = 9 (●) and close phylogenetic relatives *Brenneria roseae* subsp*. roseae* n = 5 (●)*, Brenneria roseae* subsp*. americana* n = 3 (●)*, Lonsdalea quercina* subsp*. quercina* n = 3 (●)*, Lonsdalea quercina* subsp*. britannica* n = 2 (●) and *Lonsdalea quercina* subsp*. iberica* n = 1 (●). Cluster plot was produced from principal components 1 and 2 in an unsupervised mode.


Principal component 2
Principal component 1
Principal component 2
Principal component 1

Supporting information:

Supplementary table 1: Strains of *Gibbsiella quercinecans*, *Gibbsiella greigii*, *Gibbsiella dentisursi*, *Brenneria goodwinii*, *Brenneria roseae* and *Lonsdalea quercina* used in this study

Strain	Source	Location	Accession number
***Gibbsiella quercinecans*:**			
FRB 97^T	*Quercus petraea*	Hoddesdon Park, UK	KX083699
BER12	*Quercus robur*	Warwickshire, UK	KX083700
BW2/28	*Quercus robur*	Essex, UK	KX083701
FOD9/25	*Quercus robur*	Forest of Dean, UK	KX083702
BH1/65b	*Quercus robur*	Essex, UK	KX083703
BH1/44b	*Quercus robur*	Essex, UK	KX083704
CH214	*Quercus robur*	Charnwood, UK	KX083705
AT18b	*Quercus robur*	Attingham, UK	KX083706
BH1/86	*Quercus robur*	Essex, UK	KX083707
2134	*Aesculus hippocastanum*	York, UK	KX083708
***Gibbsiella dentisursi*:**			
DSM 23818^T	Oral cavity, bear	Japan	KX083709
JCM 18389^T	Intestinal tract, butterfly	Korea	KX083710
***Gibbsiella greigii*:**			
FRB 224^T	*Quercus kelloggii*	California, USA	KX083711
USA42	*Quercus kelloggii*	California, USA	KX083712
USA15	*Quercus kelloggii*	California, USA	KX083713
USA25	*Quercus kelloggii*	California, USA	KX083714
USA56	*Quercus kelloggii*	California, USA	KX083715

***Brenneria goodwinii*:**			
FRB 141^{T}	*Quercus robur*	Outwood, UK	KX083716
BH1/28a	*Quercus robur*	Essex, UK	KX083717
BH1/28b	*Quercus robur*	Essex, UK	KX083718
J10.1	*Quercus robur*	Unknown	KX083719
J11.1	*Quercus robur*	Unknown	KX083720
FRB 171	*Quercus robur*	Gorse Covert, UK	KX083721
FRB 186	*Quercus robur*	Gorse Covert, UK	KX083722
FRB 193	*Quercus robur*	Gorse Covert, UK	KX083723
BH4/25a	*Quercus robur*	Essex, UK	KX083724
***Brenneria roseae* ssp. *roseae*:**			
FRB 222^{T}	*Quercus cerris*	Suffolk, UK	KX083725
BH1/58	*Quercus robur*, *Agrilus biguttatus*	Essex, UK	KX083726
BH1/82b	*Quercus robur*	Essex, UK	KX083727
BH1/40b	*Quercus robur*	Essex, UK	KX083728
BH1/43f	*Quercus robur*	Essex, UK	KX083729
***Brenneria roseae* ssp. *americana*:**			
FRB 223^{T}	*Quercus kelloggii*	California, USA	KX083730
USA52b	*Quercus kelloggii*	California, USA	KX083731
USA32	*Quercus kelloggii*	California, USA	KX083732
***Lonsdalea quercina* ssp. *quercina*:**			
LMG 2724^{T}	*Quercus* sp.	USA	KX083733
USA44	*Quercus kelloggii*	California, USA	KX083734
USA1b	*Quercus kelloggii*	California, USA	KX083735
***Lonsdalea quercina* ssp. *britannica*:**			
FRB 18^{T}	*Quercus robur*	Booth Wood, UK	KX083736
J2.1	*Quercus robur*	Unknown	KX083737
***Lonsdalea quercina* ssp. *iberica*:**			
1914/14^{T}	*Quercus ilex*	Madrid, Spain	KX083738

Supplementary table 2: Sequence variation within the *atpD* gene of species associated with Acute Oak Decline in Britain and their close phylogenetic relatives. Single nucleotide polymorphisms are indicated as compared against the *Gibbsiella quercinecans* reference sequence.

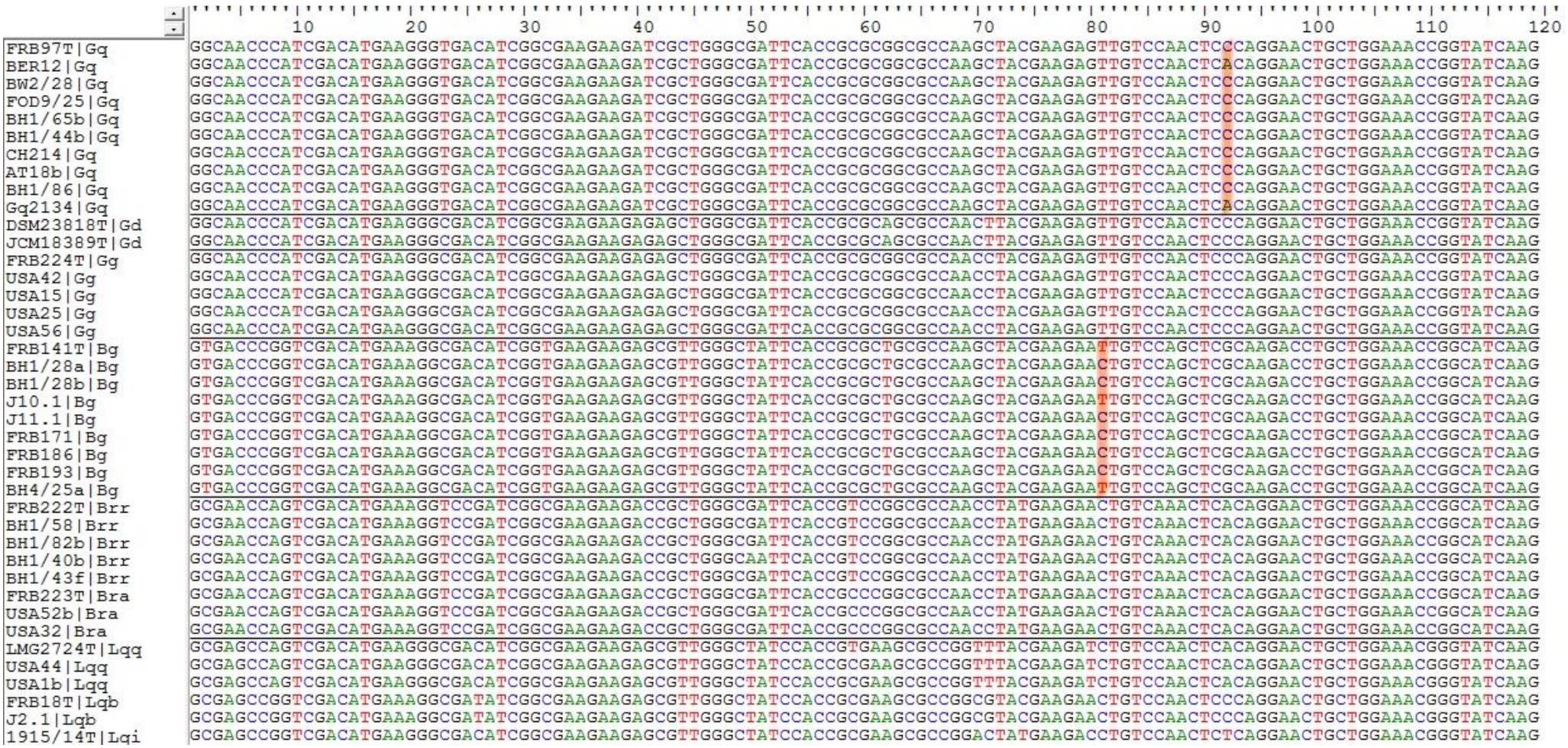
Single nucleotide polymorphism position

	2	3	5	8	9	20	23	24	25	26	32	41	42	44	50	53	59	60	61	62	68	69	70	71	74	80	81	86	88	92	95	98	110	113
Gq reference	G	C	A	C	A	G	T	G	A	C	C	T	C	C	G	T	C	G	C	G	A	A	G	C	C	G	T	C	A	C	G	A	C	T
Gq variation	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•
Gd	•	•	•	•	•	•	C	•	•	•	•	G	A	•	•	•	•	•	•	A	•	•	C	T	•	•	•	•	•	•	•	•	•	•
Gg	•	•	•	•	•	•	C	•	•	•	•	G	A	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
Bg	T	G	C	G	G	A	C	•	•	•	T	G	•	T	T	•	•	•	•	T	•	•	•	•	•	A	•/C	•	G	G	A	C	•	C
Brr	C	G	•	A	G	A	•	C	C	G	•	C	•	•	•/A	•	T	C	•	•	•	•	C	•	T	A	C	A	•	A	•	•	•	C
Bra	C	G	•	A	G	A	•	C	C	G	•	C	•	•	•	•	•	C	•	•	•	•	C	•	T	A	C	A	•	A	•	•	•	C
Lqq	C	G	G	A	G	•	C	•	•	•	•	G	•	T	T	C	•/T	•	A	A	G	G	T	T	•	T	C	•	•	A	•	•	G	•
Lqb	C	G	G	G	G	A	C	•	•	T	•	G	•	T	T	C	•	•	A	A	G	G	C	G	•	A	C	•	•	•	•	•	G	•
Lqi	C	G	G	G	G	•	C	•	•	•	•	G	•	T	T	C	•	•	A	A	G	G	A	•	T	C	C	•	•	T	•	•	G	•

Gq = *Gibbsiella quercinecans* n = 10, *Gd* = *Gibbsiella dentisursi* n = 2, *Gg* = *Gibbsiella greigii* n = 5, *Bg* = *Brenneria goodwinii* n = 9, *Brr* = *Brenneria roseae* ssp. *roseae* n = 5, *Bra* = *Brenneria roseae* ssp. *americana* n = 3, Lqq = *Lonsdalea quercina* ssp. *quercina* n = 3, *Lqb* = *Lonsdalea quercina* ssp. *britannica* n = 2, *Lonsdalea quercina* ssp. *iberica* n = 1

• = nucleotide identical to the *Gq* reference sequence at that nucleotide position

Supplementary figure 1: Sequence variation within the *atpD* gene of species associated with Acute Oak Decline in Britain and their close phylogenetic relatives. Single nucleotide polymorphisms are indicated as compared against the *Gibbsiella quercinecans* (FRB 97^{T}) and *Brenneria goodwinii* (FRB 141^{T}) reference sequences. *Gq* = *Gibbsiella quercinecans* n = 10, *Gd* = *Gibbsiella denstisursi* n = 2, *Gg* = *Gibbsiella greigii* n = 5, *Bg* = *Brenneria goodwinii* n = 9, *Brr* = *Brenneria roseae* subsp. *roseae* n = 5, *Bra* = *Brenneria roseae* subsp. *americana* n = 3*, Lqq* = *Lonsdalea quercina* subsp. *quercina* n = 3, *Lqb* = *Lonsdalea quercina* subsp. *britannica* n = 2, *Lqi* = *Lonsdalea quercina* subsp. *iberica* n = 1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BW2/28|Gq GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
FOD9/25|Gq GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
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BH1/28b|Bg GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
J10.1|Bg GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAATTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
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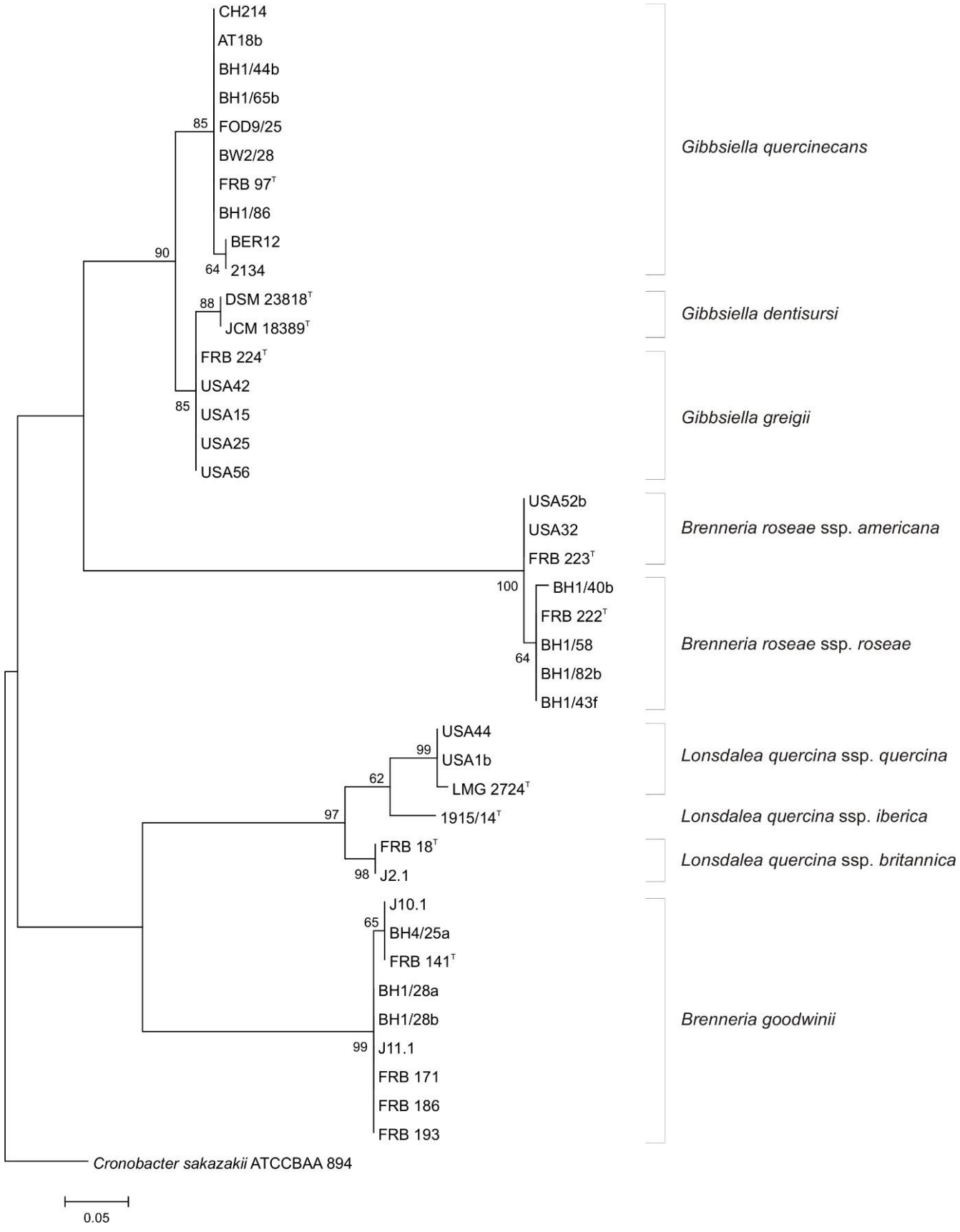
USA1b|Lqq GCGAGCCAGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGTTTACGAAGATCTGTCCAACTCACAGGAACTGCTGGAAACGGGTATCAAG
FRB18T|Lqb GCGAGCCGGTCGACATGAAAGGCGATATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGCGTACGAAGAACTGTCCAACTCCCAGGAACTGCTGGAAACGGGTATCAAG
J2.1|Lqb GCGAGCCGGTCGACATGAAAGGCGATATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGCGTACGAAGAACTGTCCAACTCCCAGGAACTGCTGGAAACGGGTATCAAG
1915/14T|Lqi GCGAGCCGGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGACTATGAAGACCTGTCCAACTCTCAGGAACTGCTGGAAACGGGTATCAAG

Supplementary figure 2: Maximum likelihood tree based on partial *atpD* gene sequences of species of *Gibbsiella*, *Brenneria* and *Lonsdalea*. Bootstrap values after 1000 replicates are expressed as percentages. *Cronobacter sakazakii* is included as an outgroup, gene sequence were obtained from the genome sequencing database (http://www.ncbi.nlm.nih.gov). The scale bar indicates the fraction of substitutions per site.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BH1/44b
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BW2/28
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BER12
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64
2134
Gibbsiella quercinecans
88
DSM 23818T
JCM 18389T
Gibbsiella dentisursi
FRB 224T
USA42
85
USA15
USA25
USA56
Gibbsiella greigii
USA52b
USA32
FRB 223T
Brenneria roseae ssp. americana
100
BH1/40b
FRB 222T
BH1/58
64
BH1/82b
BH1/43f
Brenneria roseae ssp. roseae
USA44
99
USA1b
62
LMG 2724T
Lonsdalea quercina ssp. quercina
97
1915/14T
Lonsdalea quercina ssp. iberica
FRB 18T
98
J2.1
Lonsdalea quercina ssp. britannica
J10.1
65
BH4/25a
FRB 141T
BH1/28a
BH1/28b
Brenneria goodwinii
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J11.1
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FRB 193
Cronobacter sakazakii ATCCBAA 894
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