1    Rapid identification of bacteria associated with Acute Oak Decline by high resolution melt

2    (HRM) analysis

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4    Carrie Brady[1], Joël Allainguillaume[1], Sandra Denman[2] and Dawn Arnold[1]

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6    [1] Centre for Research in Bioscience, Faculty of Health and Applied Sciences, University of the

7    West of England, Frenchay Campus, Bristol, UK

8    [2] Forest Research, Centre for Ecosystems Society and Biosecurity, Alice Holt Lodge, Gravel

9    Hill Road, Farnham, UK

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11    Correspondence: Faculty of Health and Life Sciences, University of the West of England,

12    Coldharbour Lane, Bristol, BS16 1QY, United Kingdom

13    Tel: +44117 32 83647 email: carrie.brady@uwe.ac.uk

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15    Running title: Identification of AOD bacteria

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24 **Significance and Impact of Study**

25 Acute Oak Decline (AOD) is an increasing threat to Britain's native oak population. Two

26 novel bacterial species both belonging to the family *Enterobacteriaceae*, *Gibbsiella*

27 *quercinecans* and *Brenneria goodwinii*, are thought to play an important role in symptom

28 development. Here we describe a rapid identification technique using high resolution melt

29 (HRM) analysis of the *atpD* gene able to assign isolates to either *G. quercinecans* or *B.*

30 *goodwinii* in a single assay, greatly reducing the time taken to identify if either or both of

31 these species are present in symptomatic oak.

32

33 **Abstract**

34 Two Gram-negative *Enterobacteriaceae*, *Gibbsiella quercinecans* and *Brenneria goodwinii*,

35 are frequently isolated from oak suffering from Acute Oak Decline. These two species are

36 difficult to identify based on colony morphology, carbohydrate utilisation or 16S rRNA gene

37 sequence, and identification using *gyrB* gene sequencing is time-consuming and laborious. A

38 rapid identification technique, based on high resolution melt (HRM) analysis of the *atpD*

39 gene, was designed to efficiently process numerous isolates from an increasing number of

40 affected woodlands and parks. Principal component analysis of the resulting melt curves

41 from strains of *G. quercinecans*, *B. goodwinii* and their close phylogenetic relatives allowed

42 differentiation into distinct clusters based on species or subspecies identity.

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48    **Introduction:**

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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.

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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

72    protein-encoding genes (*gyrB*, *rpoB*, *infB* and *atpD*), but routine identification of bacteria

73    associated with AOD has been typically based on amplification and sequencing of the *gyrB*

74    gene of cultured isolates. This approach, although time-consuming due to the large number

75    of epi- and endophytes present in oak, has allowed researchers to gain insight into the

76    composition of the culturable bacterial population of symptomatic native oak. However,

77    with an increasing number of woodlands and parklands affected by AOD, new isolations

78    from infected tissue are performed on a regular basis, highlighting the need for rapid

79    methods of accurate identification and detection of both *G. quercinecans* and *B. goodwinii*.

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81    High resolution melt (HRM) analysis is a real-time PCR-based molecular technique, whereby

82    single nucleotide polymorphisms (SNPs) can be identified in amplification products without

83    sequencing. A short stretch of the target gene is amplified in the presence of an

84    intercalating dye that only fluoresces when bound to double-stranded DNA. Following

85    amplification, samples are subjected to a melting step where the temperature increases

86    with each cycle until 95 °C is reached. The loss of fluorescence is measured as the double-

87    stranded amplification product denatures and a melt curve is generated. The rate of lost

88    fluorescence correlates with the G+C content and sequence length of the amplification

89    product. Therefore, samples differing by a single base pair can generate different melting

90    curves. Genotyping by high-resolution melting has been in use for over 10 years (Gundry *et*

91    *al.*, 2003, Wittwer *et al.*, 2003) and improvements in the chemistry of the saturating dyes

92    and the system technology have made this an attractive alternative to standard techniques

93    for SNP detection, mutant detection and methylation (Vossen *et al.*, 2009). Typically HRM

94    analysis is applied to identification of clinical bacteria (Pietska *et al.*, 2009, Talmi-Frank *et al.*,

95    2010, Sangal *et al.*, 2013) and detection of mutations in cancer samples and markers (Boyd

96   *et al*., 2011, Takano *et al*., 2008). However, this technique is gaining popularity for SNP

97   detection of plant and insect species (Mackay *et al*., 2008, Swisher *et al*., 2012, Wetten *et*

98   *al*., 2015).  Here we describe the application of HRM analysis of the *atpD* gene as a rapid

99   identification technique for bacteria, particularly *G. quercinecans* and *B. goodwinii*,

100  associated with AOD in Britain.

101

102  **Results and discussion:**

103

104  Bacterial strains, isolated from native oak species in the UK, previously identified as either *G.*

105  *quercinecans* or *B. goodwinii* were selected for this study. Additionally, strains belonging to

106  the close phylogenetic relatives of *G. quercinecans* (*G. greigii*, isolated from oak in the USA,

107  and *G. dentisursi*, not isolated from oak) and subspecies of both *B. roseae* and *L. quercina* (*B.*

108  *roseae* ssp. *roseae*, *B. roseae* ssp. *americana*, *L. quercina* ssp. *quercina*, *L. quercina* ssp.

109  *britannica* and *L. quercina*. ssp. *iberica*; all isolated from symptomatic oak in the UK, USA

110  and Spain) were also included (Suppl. table 1). Sequence alignments of four housekeeping

111  genes (*gyrB*, *rpoB*, *infB* and *atpD*), routinely used in a MLSA scheme to classify novel

112  *Enterobacteriaceae* (Brady *et al*., 2008) were examined for regions of heterogeneity

113  between species of *Gibbsiella*, *Brenneria* and *Lonsdalea*. The *atpD* gene (encoding the β

114  subunit of ATP synthase) was found to be a good candidate target gene as several regions

115  contain SNPs which could potentially differentiate between strains of *G. quercinecans* and *B.*

116  *goodwinii*. Several different primer pairs targeting short sequences of the *atpD* gene were

117  designed and tested against strains of *G. quercinecans*, *B. goodwinii*, their close

118  phylogenetic relatives and species of *Rahnella* (often isolated from symptomatic oak in

5

119    lower numbers). The optimal primer pair should be able to amplify all strains tested and

120    reveal enough diversity in the target gene for differentiation of species.

121

122    The *atpD* primer pair designed and selected for this study (Gq6bF and Gq6bR) could amplify

123    all species tested belonging to the genera *Gibbsiella*, *Brenneria* and *Lonsdalea* (Suppl. table

124    1). A unique melting curve was generated for each bacterial species examined in the HRM

125    analysis indicating the presence of SNPs. While the melting curve generated per strain was

126    specific for that species, it was still difficult to definitively distinguish several strains of

127    *Gibbsiella quercinecans* and *Brenneria goodwinii* based solely on the shape of the melt

128    curve. This could possibly be due to a high degree of intraspecies relatedness within each of

129    these species and the short gene region examined. To counteract this, each individual PCR

130    product was combined ('spiked') with an *atpD* PCR probe of the *G. quercinecans* reference

131    strain (FRB 97$^T$), prior to the melting step. The *atpD* gene of the *G*. *quercinecans* reference

132    strain was amplified separately on a large scale to create the PCR probe. This maximised the

133    differentiation between each species by forcing amplification products to create

134    heteroduplexes on which the melting analysis was performed. Ideally, strains which are less

135    related to the *G. quercinecans* reference strain will form a less stable heteroduplex,

136    dissociate and lose fluorescence more easily than strains which are closely related to *G.*

137    *quercinecans* and form stable heteroduplexes.

138

139    Fig. 1 shows the distinct melt curves of four species frequently associated with AOD in

140    Britain (*Gibbsiella quercinecans*, *Brenneria goodwinii*, *Brenneria roseae* ssp. *roseae* and

141    *Lonsdalea quercina* ssp. *britannica*), based on the mean of 9-10 strains of *G. quercinecans*

142    and *B. goodwinii*, and 2-5 strains for *B. roseae* ssp. *roseae* and *L. quercina* ssp. *britannica* (all

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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 $10^{-4}$ ($4.4 \times 10^6$ 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.

191 AOD is an increasing threat to the native oak population in Britain and as such, a large

192 number of samples from symptomatic tissue are processed on a regular basis. The aim of

193 this study was to create a single rapid identification technique for both *G. quercinecans* and

194 *B. goodwinii*, the two species most frequently isolated from symptomatic oak. HRM analysis

195 of a short region of the *atpD* gene has achieved this and also shows great future promise for

196 identification of non-cultured samples. Including a reference strain of *G. quercinecans* and *B.*

197 *goodwinii* in each run of the assay allows for comparison of isolates to both species, greatly

198 reducing the time taken to identify bacteria isolated from oak tissue.

199

200 **Materials and methods:**

201

202 Published sequences of the *atpD* gene (ATP synthase β subunit) from *Enterobacteriaceae*

203 isolated from symptomatic oak were aligned and examined for a region containing

204 heterogeneity between species. Primers were designed to amplify a short stretch (119 bp)

205 of the *atpD* gene demonstrating sufficient sequence divergence between species of

206 *Gibbsiella*, *Brenneria* and *Lonsdalea* (Gq6bF: 5'GGC AAC CCA TCG ACA TGA A 3'; Gq6bR: 5'

207 CTT GAT ACC GGT TTC AG CAG 3') (Suppl. Tables 1 and 2). For all bacterial strains, a single

208 colony was re-suspended in 750 µl sterile distilled water and 2 µl was used in a 20 µl PCR

209 mixture containing 2x SensiFAST HRM mix (Bioline) and 10 pmol of each primer. HRM

210 analysis was performed on 2 – 10 strains of each of the following species in duplicate: *G.*

211 *quercinecans*, *G. greigii*, *G. dentisursi*, *B. goodwinii*, *B. roseae* ssp. *roseae* and ssp.

212 *americana*, *Lonsdalea quercina* ssp. *quercina*, ssp. *britannica* and ssp. *iberica* (Suppl. Table 1)

213 in a Rotor-Gene Q cycler (Qiagen). Following initial denaturation for 10 min at 95 °C and 45

214 amplification cycles of 5 sec denaturation at 95 °C and 30 sec annealing at 58 °C, each PCR

215    reaction was 'spiked' with 20 µl of the *G. quercinecans* probe (the *atpD* amplification

216    product of reference strain FRB 97[T] which was amplified separately using the primers and

217    conditions listed above). The melting step was then performed with 2 min denaturation at

218    95 °C, 2 min hold at 55 °C and melting from 55 to 90 °C where the temperature was

219    increased by 0.1 °C each cycle. The fluorescence was measured at the end of each cycle and

220    a melt curve was generated by the Rotor-Gene software (Qiagen).

221

222    To assess the statistical differences between each of the species tested, melting curves

223    (normalised between 80-81 and 87-88 °C) were converted to residual plots in ScreenClust v

224    1.10.1.2 (Qiagen). A preliminary analysis was run with the number of possible clusters fixed

225    to the number of expected species (supervised mode) and then compared to an analysis

226    with the optimal number of clusters generated automatically by the software (unsupervised

227    mode). Screenclust allocates strains to the most appropriate cluster, indicating which SNPs

228    generate significantly different melt curves.

229

230    Amplification and sequencing of the *atpD* gene for each strain examined by HRM analysis

231    was performed as previously described (Brady *et al*., 2008). *atpD* sequences were aligned

232    and trimmed in BioEdit v 7.2.5 (Hall, 1999) to reflect the 119 bp fragment amplified used in

233    the HRM analysis. A maximum likelihood tree was constructed in MEGA v 6 (Tamura *et al*.,

234    2006) using the general time reversible model with proportion of invariable sites and

235    gamma distribution and with 1000 bootstrap replicates.

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246     **Conflict of interests:**

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248     The authors declare no conflict of interests.

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250     **References:**

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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 (——).
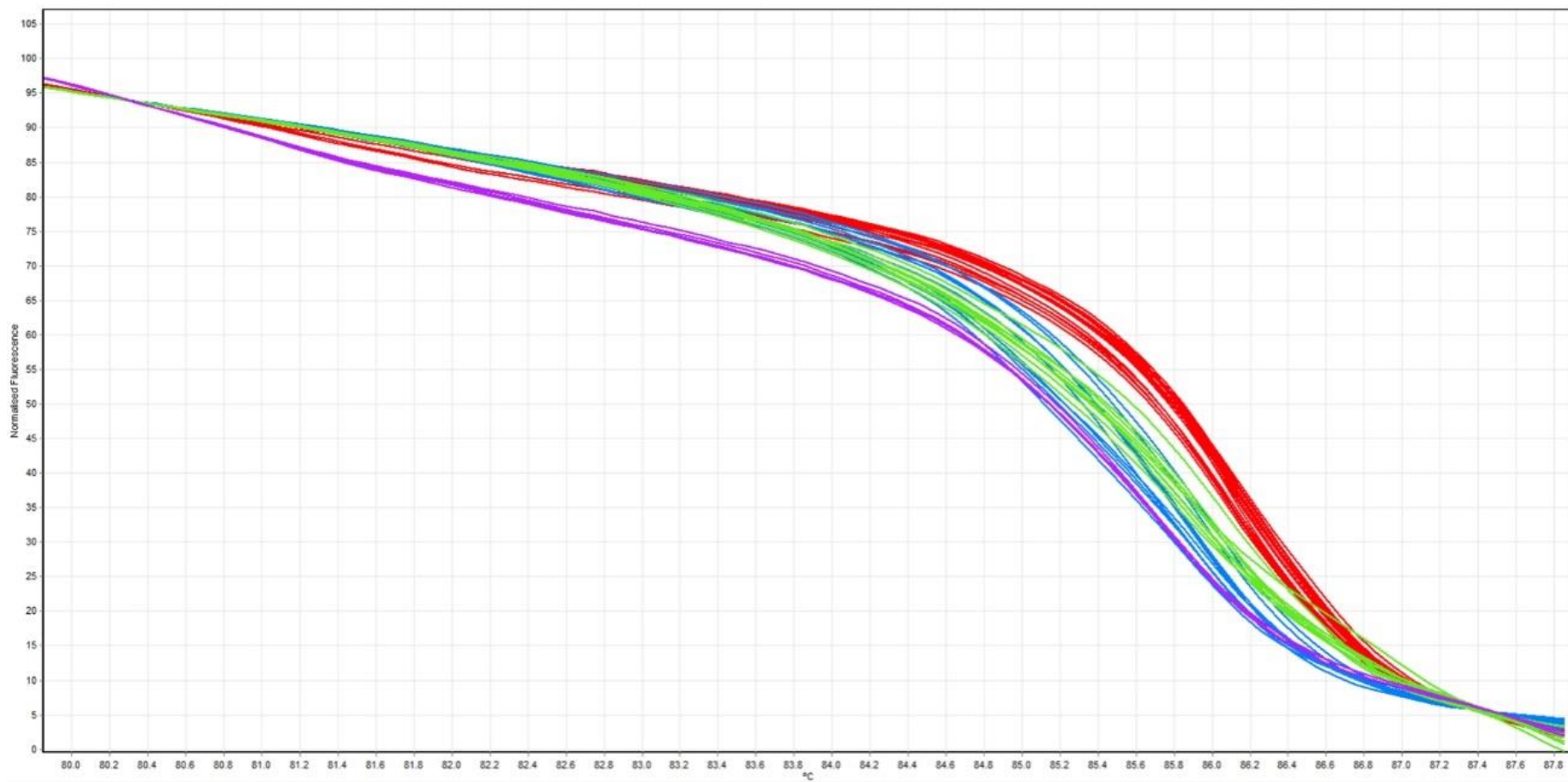
Figure 2:  Scatter plot generated by ScreenClust v 1.10.1.2 (Qiagen) from HRM curve data.

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.
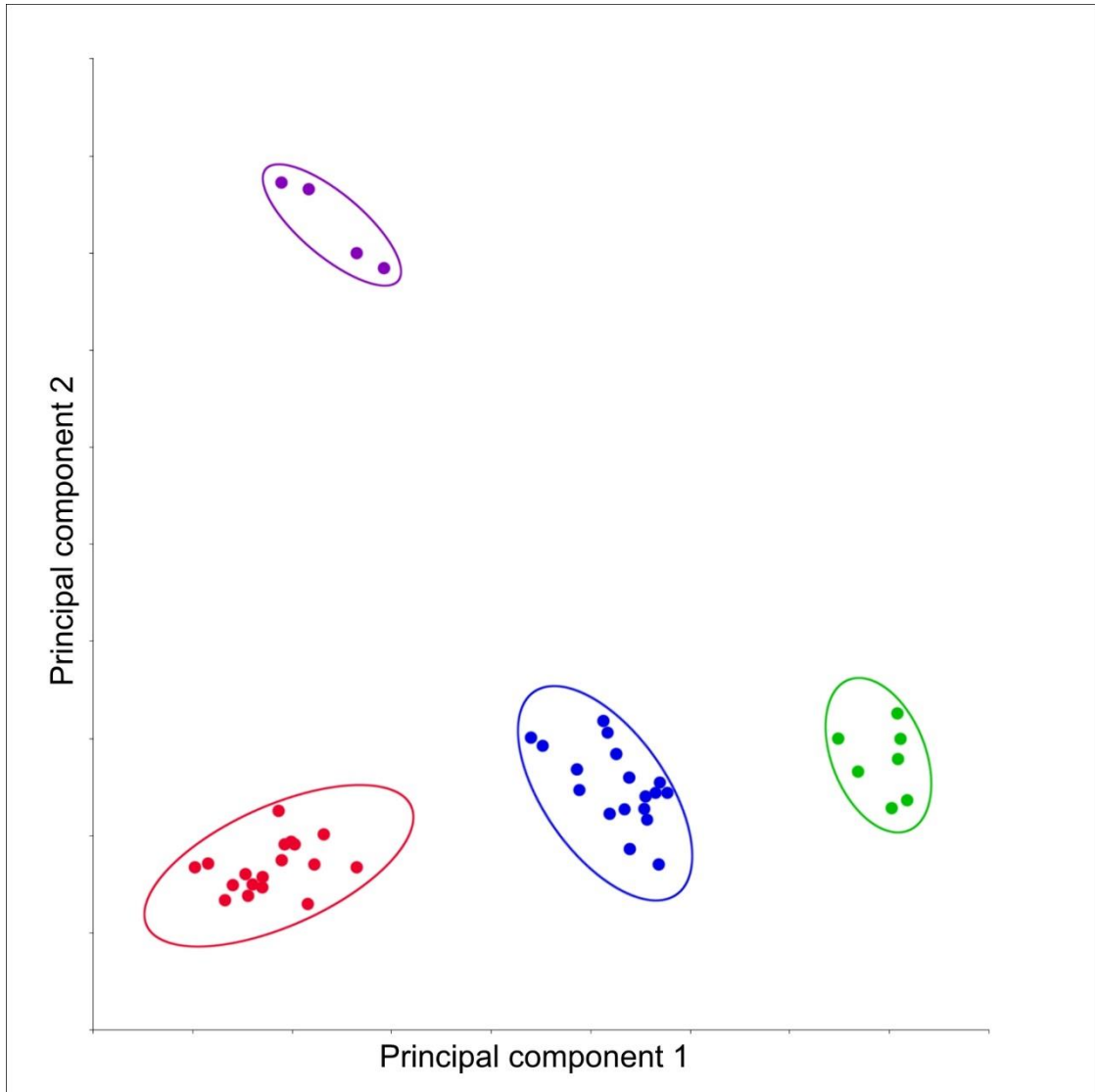
Figure 3a: Scatter plot generated by ScreenClust v 1.10.1.2 (Qiagen) from HRM curve data. Each dot represents a single strain (in duplicate) of *Gibbsiella quercinecans* n = 10 (•) and close phylogenetic relatives *Gibbsiella greigii* n = 5 (•) and *Gibbsiella dentisursi* n = 2 (•). Cluster plot was 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.
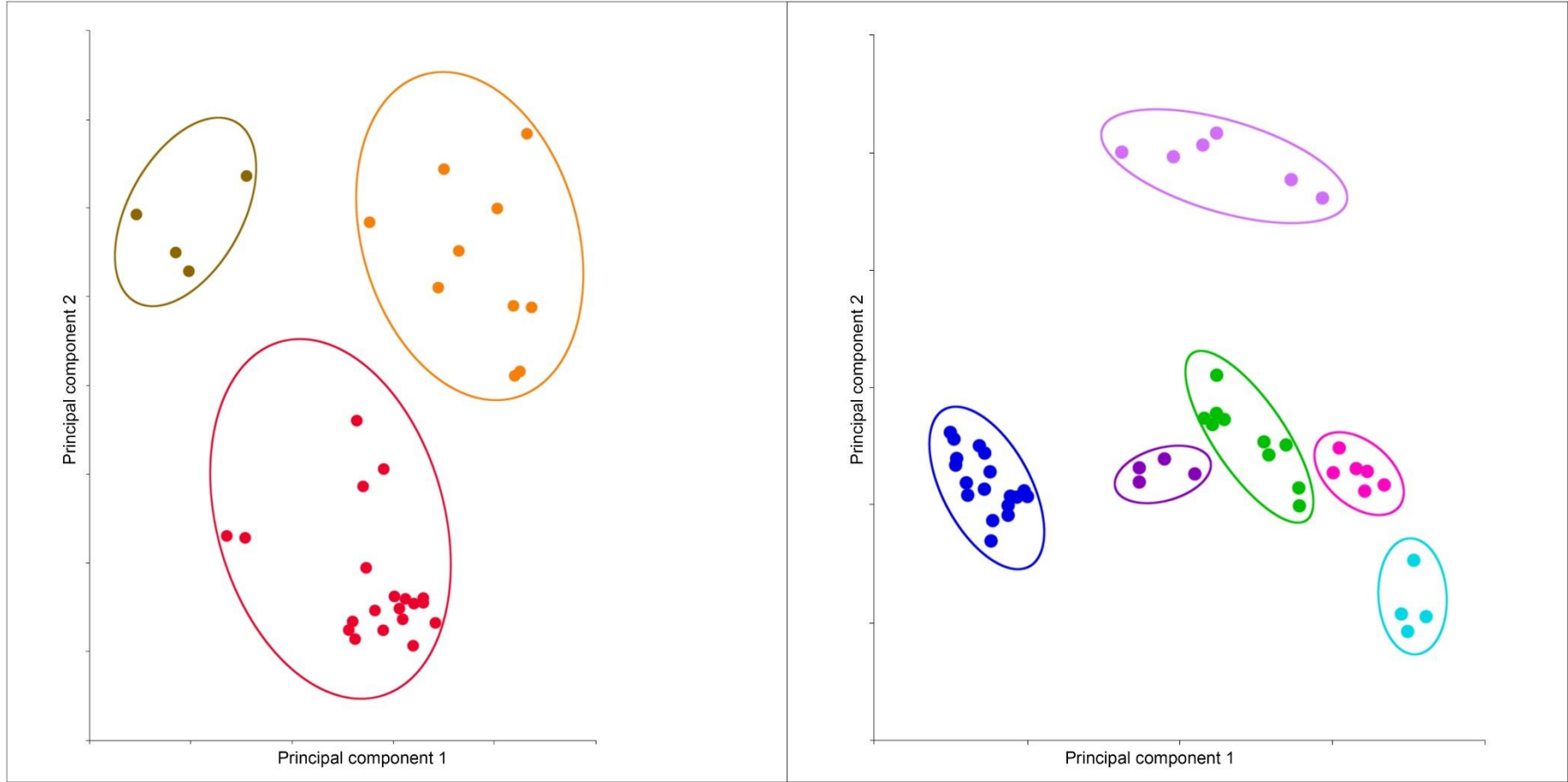
**Supporting information:**

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

```
                  10        20        30        40        50        60        70        80        90        100       110       120
FRB97T|Gq      GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
BER12|Gq       GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCACAGGAACTGCTGGAAACCGGTATCAAG
BW2/28|Gq      GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
FOD9/25|Gq     GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
BH1/65b|Gq     GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
BH1/44b|Gq     GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
CH214|Gq       GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
AT18b|Gq       GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
BH1/86|Gq      GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
Gq2134|Gq      GGCAACCCATCGACATGAAGGGTGACATCGGCGAAGAAGATCGCTGGGCGATTCACCGCGCGGCGCCAAGCTACGAAGAGTTGTCCAACTCACAGGAACTGCTGGAAACCGGTATCAAG
DSM23818T|Gd   GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCAGCGCAACTTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
JCM18389T|Gd   GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCAGCGCAACTTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
FRB224T|Gd     GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCAGCGCAACCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
USA42|Gg       GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCGGCGCCAACCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
USA15|Gg       GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCGGCGCCAACCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
USA25|Gg       GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCGGCGCCAACCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
USA56|Gg       GGCAACCCATCGACATGAAGGGCGACATCGGCGAAGAAGAGAGCTGGGCGATTCACCGCGCGGCGCCAACCTACGAAGAGTTGTCCAACTCCCAGGAACTGCTGGAAACCGGTATCAAG
FRB141T|Bg     GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAATTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
BH1/28a|Bg     GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
BH1/28b|Bg     GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
J10.1|Bg       GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAATTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
J11.1|Bg       GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
FRB171|Bg      GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
FRB186|Bg      GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
FRB193|Bg      GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAACTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
BH4/25a|Bg     GTGACCCGGTCGACATGAAAGGCGACATCGGTGAAGAAGAGCGTTGGGCTATTCACCGCGCTGCGCCAAGCTACGAAGAATTGTCCAGCTCGCAAGACCTGCTGGAAACCGGCATCAAG
FRB222T|Brr    GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGTCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
BH1/58|Brr     GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGTCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
BH1/82b|Brr    GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGTCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
BH1/40b|Brr    GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCAATTCACCGTCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
BH1/43f|Brr    GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGTCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
FRB223T|Bra    GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGCCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
USA52b|Bra     GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGCCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
USA32|Bra      GCGAACCAGTCGACATGAAAGGTCCGATCGGCGAAGAAGACCGCTGGGCGATTCACCGCCCGGCGCCAACCTATGAAGAACTGTCAAACTCACAGGAACTGCTGGAAACCGGCATCAAG
LMG2724T|Lqq   GCGAGCCAGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGTGAAGCGCCGGTTTACGAAGATCTGTCCAACTCACAGGAACTGCTGGAAACGGGTATCAAG
USA44|Lqq      GCGAGCCAGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGTTTACGAAGATCTGTCCAACTCACAGGAACTGCTGGAAACGGGTATCAAG
USA1b|Lqq      GCGAGCCAGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGTTTACGAAGATCTGTCCAACTCACAGGAACTGCTGGAAACGGGTATCAAG
FRB18T|Lqb     GCGAGCCGGTCGACATGAAAGGCGATATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGCGTACGAAGAACTGTCCAACTCCCAGGAACTGCTGGAAACGGGTATCAAG
J2.1|Lqb       GCGAGCCGGTCGACATGAAAGGCGATATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGCGTACGAAGAACTGTCCAACTCCCAGGAACTGCTGGAAACGGGTATCAAG
1915/14T|Lqi   GCGAGCCGGTCGACATGAAGGGCGACATCGGCGAAGAAGAGCGTTGGGCTATCCACCGCGAAGCGCCGGACTATGAAGACCTGTCCAACTCTCAGGAACTGCTGGAAACGGGTATCAAG
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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.

CH214
AT18b
BH1/44b
BH1/65b
85 — FOD9/25
BW2/28
FRB 97[T]
BH1/86
BER12
64 — 2134

90

88 — DSM 23818[T]
JCM 18389[T]

FRB 224[T]
USA42
85 — USA15
USA25
USA56

USA52b
USA32
FRB 223[T]

100 — BH1/40b
FRB 222[T]
BH1/58
64 — BH1/82b
BH1/43f

USA44
99 — USA1b
62 — LMG 2724[T]
1915/14[T]

97

FRB 18[T]
98 — J2.1

J10.1
65 — BH4/25a
FRB 141[T]
BH1/28a
BH1/28b
99 — J11.1
FRB 171
FRB 186
FRB 193

*Cronobacter sakazakii* ATCCBAA 894

0.05

*Gibbsiella quercinecans*

*Gibbsiella dentisursi*

*Gibbsiella greigii*

*Brenneria roseae* ssp. *americana*

*Brenneria roseae* ssp. *roseae*

*Lonsdalea quercina* ssp. *quercina*

*Lonsdalea quercina* ssp. *iberica*

*Lonsdalea quercina* ssp. *britannica*

*Brenneria goodwinii*