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Title: Multilocus sequence typing as a tool for studying the molecular epidemiology and population structure of *Brachyspira hyodysenteriae*

Authors: Tom La, Nyree D. Phillips, Belinda L. Harland, Phatthanaphong Wanchanthuek, Matthew I. Bellgard, David J. Hampson

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1 **REVISED**

2 **Multilocus sequence typing as a tool for studying the molecular epidemiology**
3 **and population structure of *Brachyspira hyodysenteriae***

4

5 Tom La[1], Nyree D. Phillips[1], Belinda L. Harland[1], Phatthanaphong Wanchanthuek[2],
6 Matthew I. Bellgard[2], David J. Hampson[1]*

7

8

9 *Animal Research Institute, School of Veterinary and Biomedical Science, Murdoch*
10 *University, Murdoch, Western Australia 6150, Australia*[1]*, and Centre for*
11 *Comparative Genomics, Murdoch University, Murdoch, Western Australia 6150,*
12 *Australia*[2]

13

14 Short title: *Brachyspira hyodysenteriae* MLST

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17 *Corresponding author: Tel: +62 89360 2287; fax: +61 89310 41344.
18 *E-mail address:* d.hampson@murdoch.edu.au

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26 ABSTRACT

27 The purpose of this study was to develop and apply a multilocus sequence typing
28 (MLST) scheme to study the molecular epidemiology of *Brachyspira hyodysenteriae*,
29 the aetiological agent of swine dysentery. Sequences of seven conserved genomic loci
30 were examined in 111 *B. hyodysenteriae* strains. Fifty-eight of these previously had
31 been analysed by multilocus enzyme electrophoresis (MLEE), and for some the
32 results of pulsed field gel electrophoresis (PFGE), restriction endonuclease analysis
33 (REA) and/or serotyping also were available. The discriminatory power of these
34 methods was compared. The strains were divided into 67 sequence types (STs) and 46
35 amino acid types (AATs) by MLST. The Index of Association value was significantly
36 different from zero, indication that the population was clonal. Eleven clonal
37 complexes (Cc) comprising between 2 and 10 STs were recognised. A population
38 snapshot based on AATs placed 77.5% of the isolates from 30 of the AATs into one
39 major cluster. The founder type AAT9 included 13 strains from nine STs that were
40 isolated in Australia, Sweden, Germany and Belgium, including one from a mallard.
41 The MLST results were generally comparable to those produced by MLEE. The
42 MLST system had a similar discriminatory power to PFGE, but was more
43 discriminatory than REA, MLEE or serotyping. MLST data provided evidence for
44 likely transmission of strains between farms, but also for the occurrence of temporal
45 "micro-evolution" of strains on individual farms. Overall, the MLST system proved to
46 be a useful new tool for investigating the molecular epidemiology and diversity of *B.*
47 *hyodysenteriae*.

48

49 *Keywords: Brachyspira hyodysenteriae*; MLST; swine dysentery; molecular
50 epidemiology; spirochaete

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51 **1. Introduction**

52 Swine dysentery (SD) is a mucohaemorrhagic colitis resulting from infection of the
53 porcine caecum and colon with the anaerobic intestinal spirochaete *Brachyspira*
54 *hyodysenteriae* (Hampson et al., 2006). SD is widespread and problematic in many
55 swine-rearing countries, and the emergence of *B. hyodysenteriae* strains with reduced
56 susceptibilities to various antimicrobials has led to an increased need to understand
57 the molecular epidemiology of the disease (Karlsson et al., 2004; Duinof et al., 2008).
58 To do this, reliable strain typing methods are needed to help trace routes of
59 transmission and monitor reservoirs of *B. hyodysenteriae* infection.

60 *B. hyodysenteriae* isolates have been typed using various methods, including
61 serotyping based on reacting extracted lipooligosaccharide with hyperimmune rabbit
62 sera (Baum and Joens, 1979), DNA restriction endonuclease analysis (REA) (Combs
63 et al., 1992; Harel et al., 1994), random amplification of polymorphic DNA (RAPD)
64 (Dougard et al., 1996), DNA restriction fragment polymorphism analysis (RFLP)
65 (Fisher et al., 1997), and pulsed field gel electrophoresis (PFGE) (Atyeo et al., 1999;
66 Fellström et al., 1999). In addition, multilocus enzyme electrophoresis (MLEE) has
67 been used to examine the population structure and diversity of *B. hyodysenteriae* (Lee
68 et al., 1993; Trott et al., 1997). Although MLEE has been useful for differentiating
69 and analysing the relatedness of *B. hyodysenteriae* strains, the technique is slow and
70 cumbersome to perform, and hence it is not suitable for routine use.

71 Recently, multilocus sequence typing (MLST) has been developed as an alternative
72 method for analysis of bacterial population structure and for discriminating between
73 strains (Maiden et al., 1998; Urwin and Maiden, 2003). The purpose of the present
74 study was to develop a specific MLST system for *B. hyodysenteriae*, based on
75 modifications to the preliminary scheme described for the whole *Brachyspira* genus

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76 (Råsbäck et al., 2007). The method was assessed for its usefulness by applying it to a
77 collection of *B. hyodysenteriae* strains, some of which previously had been analysed
78 by MLEE, PFGE, REA and/or serotyping.
79
80 **2. Materials and methods**
81
82 *2.1. Brachyspira hyodysenteriae strains*
83 Ninty-five strains of *B. hyodysenteriae* were obtained as frozen stock from the
84 culture collection at the Reference Centre for Intestinal Spirochaetes at Murdoch
85 University. These included 58 representatives of a range of MLEE electrophoretic
86 types previously established in our laboratory (Lee et al., 1993; Trott et al., 1997), as
87 well as 37 other Australian field isolates. The latter included six from feral pigs in
88 Western Australia (WA) (Phillips et al., 2009), and multiple (2-13) recent isolates
89 from each of four large farms, some of which had been tested for their antimicrobial
90 susceptibilities. Many of the older strains that had been analysed by MLEE also had
91 been analysed in our laboratory by PFGE (Atyeo et al., 1999), REA (Combs et al.,
92 1992) and serotyping (Hampson et al., 1989; Combs et al., 1992; Hampson et al.,
93 1997). The 95 strains originated from different States of Australia (n=82), the USA
94 (n=7), Canada (n=3) and the UK (n=3). The names of the strains, their origins and
95 previous results of MLEE, PFGE, REA and serotyping are presented in Table 1.
96 Sequence data for 16 Scandinavian and European strains (AN174/92, AN1082/90,
97 AN3379/98, AN383:2/00, T20, T4, A5677/96, Be45, AN3730/96, AN613/98,
98 AN2420/97, AN360/03, AN551/03, P134/99, E2 and AN1409:2/01) that previously
99 were used in the *Brachyspira* MLST scheme (Råsbäck et al., 2007) were obtained
100 from PubMLST (http://pubmlst.org/) and included in the study. The full collection of

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101 111 strains that was analysed included 101 from commercial pigs, six from feral pigs,
102 two from mallards, one from a rhea and one from a mouse (Table 1).
103
104 *2.2. Bacterial culture*
105 Spirochaete strains were propagated at 37°C in Kunkle's pre-reduced anaerobic
106 broth containing 2 % (vol/vol) foetal bovine serum and a 1 % (vol/vol) ethanolic
107 cholesterol solution (Kunkle et al., 1986). Cells were harvested from mid-log phase
108 culture by centrifuging at 10,000 x *g*, and counted in a haemocytometer chamber
109 under a phase contrast microscope.
110
111 *2.3. DNA extraction*
112 The DNeasy kit (QIAGEN Pty Ltd, Doncaster, Australia) was used to extract
113 chromosomal DNA, using the Gram-negative bacterial protocol. Ten ml of a 10^8
114 cells/ml culture of *B. hyodysenteriae* was centrifuged at 5,000 x *g*. The cell pellet was
115 resuspended in 180 µl of lysis buffer containing 20 µl of proteinase K (10 mg/ml) and
116 incubation at 55 °C for 30 min. After lysis, 180 µl of AL Buffer was added and the
117 sample incubated at 70 °C for 10 min. Two hundred µl of absolute ethanol was added
118 to the sample and this was transferred to a DNeasy spin-column. Ethanol (70%,
119 vol/vol)-based buffers AW1 and AW2 were added sequentially to the columns and
120 centrifuged at 6,000 x *g*. The supernatants were discarded, and the DNA resuspended
121 in sterile water and stored at -20 °C.
122
123 *2.4. Multilocus sequence typing*
124 Seven of the eight MLST loci previously described for use with the genus
125 *Brachyspira* (Råsbäck et al., 2007) were selected for this study. These were the genes

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126 encoding alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*),
127 glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*)
128 and acetyl-CoA acetyltransferase (*thi*). The DNA mismatch repair protein gene (*mutS*)
129 was not discriminatory in the previous study, and so it was not used. The primers and
130 PCR conditions were as previously described (Råsbäck et al., 2007), except that only
131 primer pairs ADH-F206 and ADH-R757 were used for *adh*, and ALP-F354 and ALP-
132 R1262 for *alp*.

133 PCRs were performed in 50 μl reaction mixtures with *Taq* DNA polymerase
134 (Invitrogen, Carlsbad, USA). Each PCR reaction set included *B. hyodysenteriae* strain
135 WA1 as a positive control and double distilled water as a negative control. The PCR
136 conditions were 95 ^{o}C for 2 min, followed by 33 cycles at 95 ^{o}C for 30 sec, 50 ^{o}C for
137 15 sec, 72 ^{o}C for 1 min, an extension period of 5 min at 72 ^{o}C then cooling to 10 ^{o}C.
138 The PCR products were purified with the AxyPrep^{TM} PCR Clean-up Kit according to
139 the manufacturer's instructions (Axygen Scientific, Inc., Union City, USA).

140 The purified PCR products were sequenced using the BigDye Terminator v3.1
141 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to the
142 manufacturer's instructions, using the same primers. Sequencing was performed using
143 the 373A sequencing system (Applied Biosystems). Sequence results were analysed
144 and assembled using the ContigExpress component of VectorNTI Advanced 10
145 (Invitrogen).

146 To ensure that the sense strand was being used for the analysis, the sequences for
147 each locus were aligned with the original *B. hyodysenteriae* strain WA1 sequence
148 (Råsbäck et al., 2007), using ClustalW (from EMBL-EBI, European Bioinformatics
149 Institute [http://www.ebi.ac.uk/clustalw/]). The aligned loci sequences were trimmed
150 using GeneDoc (Nicholas et al., 1997) such that a 345 bp *adh* allele (nucleotide 303 to

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151 647), 648 bp *alp* allele (nucleotide 460 to 1107), 503 bp *est* allele (nucleotide 288 to
152 790), 415 bp *gdh* allele (nucleotide 634 to 1049), 687 bp *glpK* allele (nucleotide 320
153 to 1006), 744 bp *pgm* allele (nucleotide 297 to 1040), and a 745 bp *thi* allele
154 (nucleotide 224 to 968) would be used for the subsequent MLST analysis. The DNA
155 sequences also were translated to predicted amino acid sequences using BioEdit
156 version 7.0.9.0 (Hall, 1999).
157
158 *2.5. Data analysis*
159 The aligned sequences for each of the seven loci were analysed using the non-
160 redundant databases (NRDB) program (http://pubmlst.org/analysis/) to identify strain
161 sequences that were identical. Each unique nucleotide sequence was assigned a
162 unique allele number. The allelic profile for each isolate was determined and
163 consisted of a line listing the allele number for each locus in turn. Isolates were
164 assigned a sequence type (ST) according to their allelic profiles. Isolates were
165 considered genetically identical and hence of the same ST if they were identical at all
166 seven loci. An analysis was also undertaken with five loci, omitting *adh* and *pgm.* The
167 sequences of the *B. hyodysenteriae* MLST alleles for each locus were deposited at the
168 PubMLST site at Oxford University (http://pubmlst.org/). Unique amino acid types
169 (AATs) predicted from the sequences also were recorded. An MLST dendrogram was
170 constructed from the data matrix of allelic mismatches using the unweighted pair
171 group method with averages method with 1000 bootstrap replicates in START2
172 (Jolley et al., 2001). Isolates were grouped into clonal complexes (Cc) by the BURST
173 algorithm using the eBURST v3 program (Feil et al., 2004). Within the program a
174 population snapshot was obtained by setting the group definition to 0/7, and assigning
175 a zero for loci without sequence data. The same analysis was used for the AATs. The

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176 degree of linkage disequilibrium in the population was estimated by calculating the
177 Index of Association (I_A) for the 111 strains and the 67 STs (Smith et al., 1993), using
178 the START2 program. A Diversity Index (DI) based on Simpson's index of diversity
179 was calculated for the results of MLST, MLEE, PFGE and REA, as previously
180 described (Hunter and Gaston, 1988).
181

182 **3. Results**

183 *3.1. Sequence types and population structure analysis*

184 The 111 strains analysed were divided into 67 STs and 46 AATs (Table 1). A total
185 of 64 STs and 41 AATs were recognised when *adh* and *pgm* were excluded from the
186 analysis. The corresponding allele numbers assigned for all the STs are shown in the
187 supplementary table, and these raw sequences are recorded in the PubMLST site.
188 Allelic frequency over the seven loci ranged from 9 (for *adh*) to 20 (for *thi*), with a
189 mean of 15.4. Based on the number of strains tested the population had an I_A value of
190 1.05, whilst based on the number of STs the I_A was 0.175. Significant linkage
191 disequilibrium (P = 0.000) was found in both analyses.

192 **[Table 1 about here]**

193 The dendrogram showing the relative relationships of the 67 STs is presented as
194 Figure 1. The basic structure of the tree was unchanged when only five loci were
195 used. STs 1-27, 28-48 and 49-54 each formed a large cluster, with the other groups of
196 STs generally comprising single strains and being arranged in a stepwise fashion with
197 increasing genetic distances between the STs. The two most genetically distinct STs
198 (ST66 and ST67) each contained single strains from Sweden, including one from a
199 mallard duck (Jansson et al., 2004). The small cluster comprising ST61-ST65
200 contained recent isolates from Western Australia. Overall, the number of isolates in an

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201 ST varied from 1 to 8 (ST19), and the number in an AAT varied from 1 to 14
202 (AAT17). The 82 strains from Australia were divided into 46 STs and the 29 non-
203 Australian strains were in the other 21 STs. Only in the case of ST52 did strains from
204 different countries share an ST (Germany and Belgium).

205 **[Fig 1 about here]**

206 Eleven clonal complexes (Cc) of STs were identified by e-Burst analysis (Figure
207 2), and these are marked on Figure 1 and highlighted in Table 1. The Ccs contained
208 between 2 and 10 STs, and between 2 and 26 strains. In seven cases the Ccs were
209 made up of strains just from Australia (Cc1, Cc31, Cc33, Cc36, Cc39, Cc46, Cc64),
210 and these varied in composition from all the strains being from the same farm in the
211 same year (Cc1), and hence likely to be epidemiologically linked, to the strains
212 coming from farms in different States and spanning three decades (Cc46). Cc15
213 contained 25 Australian strains isolated in different States and decades, and one strain
214 isolated from the USA in the 1980s. Cc51 contained three strains from Germany and
215 one from Belgium, all of which had the unusual phenotype of being indole negative,
216 and which also were considered to be related based on their PFGE patterns (Fellström
217 et al., 1999).

218 **[Fig 2 about here]**

219 For each of the four Australian farms where recent multiple *B. hyodysenteriae*
220 isolates were available, some of the isolates belonged to different STs (Table 1). Farm
221 A had isolates in STs 1, 2, 64 and 65; farm B had isolates in STs 61, 62, 63 and 64;
222 farm C had isolates in STs 3, 49 and 64; farm D had isolates in STs 14, 19 and 21,
223 with all the isolates in the later two STs being resistant to tiamulin.

224 The six recent isolates from feral pigs in WA all had unique STs. FP1 and FP5
225 belonged to the same clonal complex (Cc39), together with a WA isolate from a

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226 farmed pig from the 1980s, whilst FP3 was part of a clonal complex (Cc46) with a
227 WA farmed pig isolate from the 1980s, and a Queensland isolate from the 1990s.
228 A population snapshot obtained by using AATs rather than STs is shown in Figure
229 3. Thirty (65%) of the AATs were contained in one major cluster, which was made up
230 of two linked sub-clusters. The larger sub-cluster had AAT9 as the founder member,
231 and the other sub-cluster had AAT17 at its centre. This major cluster contained 86 of
232 the strains that were analysed (77.5%). There were three other clusters containing two
233 AATs, one containing four AATs, and each of the other 6 AATs were separate. The
234 founder AAT9 profile was shared by 13 strains from nine STs, comprising seven
235 strains from Australia, three from Sweden (including a strain from a mallard), two
236 from Germany and one from Belgium.

237 **[Fig 3 about here]**

238 *3.2. Strain discrimination*

239 Results of the diversity index (DI) using MLST, and a comparison of results for 36
240 isolates using the different typing methods are summarised in Table 2. MLST was the
241 most discriminatory method of strain typing, with PFGE being the next most
242 discriminatory method, then REA, then MLEE, and then serotyping. The MLST
243 results appeared to be consistent, since in most cases multiple isolates from the same
244 farm isolated in the same year had the same ST. When only five loci were included in
245 the MLST analysis the DI was 0.972.

246 **[Table 2 about here]**

247 **4. Discussion**

248 This is the first study in which an MLST scheme has been used to analyse the
249 molecular epidemiology and population structure of *B. hyodysenteriae.* Eighteen *B.*
250 *hyodysenteriae* strains were included in our previous MLST analysis of species in the

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251 genus *Brachyspira*, but as that study investigated relationships between species, the
252 sequence data were concatenated, and within-species analysis received little attention.
253 With the current larger data set of 111 strains, and by undertaking a comparison of
254 allelic differences at each locus, a more detailed analysis of the population was
255 possible.

256 Based on calculated I_A values obtained in a previous study using MLEE, it was
257 deduced that *B. hyodysenteriae* is a recombinant species, with an epidemic population
258 structure (Trott et al., 1997). Such populations are basically recombinant, with
259 frequent genetic exchange, but also have clonal groups that have emerged due to them
260 possessing some selective advantage. In the current work, the I_A value obtained was
261 significantly different from zero whether or not it was calculated on the number of
262 strains or number of STs, and this result was consistent with the *B. hyodysenteriae*
263 population being clonal (Smith et al., 1993). The reasons for these different findings
264 with MLEE and MLST are uncertain, but the earlier MLEE study analysed more
265 strains (231) than this MLST study. The current study also was somewhat biased by
266 the fact that 82 (74%) of the strains examined were from Australia, and these included
267 a number of sets of isolates from the same farms. On the other hand, the isolates
268 originated from the 1970s to the 2000s, so a broad timeframe was examined. In the
269 future a more definitive conclusion about the population structure of *B.*
270 *hyodysenteriae* should become available once MLST results for more strains from
271 around the world are added to the PubMLST database, and the data are reanalysed.

272 The dendrogram shown as Figure 1 confirmed previous observations that the
273 species is diverse (Lee at al., 1993; Trott et al., 1997), and it helped to depict the
274 relationships between the identified STs. The three large groups of STs (ST1-ST27;
275 ST28-ST48; ST49-ST54) each contained strains isolated in different decades, in

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276 different Australian States and in different countries. Other more distantly related
277 members of the species at the periphery of the dendrogram also were identified.
278 Despite evidence for a general persistence and stability of strains in the larger ST
279 groups, on individual farms there was evidence for the emergence of variants of
280 original strains. A similar phenomenon previously has been observed where PFGE
281 was used to analyse strains from Australian farms, and was described as "micro-
282 evolution" (Atyeo et al., 1999). These apparent minor changes were seen on several
283 occasions, where some strains from the same farm had different STs but belonged to
284 the same Cc (eg ST1 and ST2 in Cc1 on farm A; and ST15, ST19 and ST21 in Cc15
285 on farm D). In the latter case the isolate in ST15 was recovered in the 1990s and was
286 susceptible to tiamulin, whilst those in ST19 and ST21 were isolated in 2004 and
287 2005, and were resistant to tiamulin. It appeared that during this period the original
288 strain had acquired mutations in the housekeeping genes that were responsible for
289 assigning it to an ST, and during the same period it also had acquired tiamulin
290 resistance. It was known that tiamulin had been used on this farm to help control SD
291 prior to the emergence of resistance, and this use presumably applied pressure for
292 selection of resistant strains. Mutations in the genes for ribosomal protein L3 and/or
293 23S rRNA are known to confer resistance to tiamulin in *Brachyspira* species (Pringle
294 et al., 2004).

295 The MLST data also provided useful molecular epidemiological data at a broader
296 level. For example, some strains with the same ST were found on different farms –
297 and this can be used to provide evidence for the likely transmission of strains between
298 the farms. This was the case for strain WA40; it was both tiamulin resistant and
299 shared ST19 with tiamulin resistant strains from a farm in New South Wales that were
300 isolated in the same year. Based on STs, there was no direct evidence for transmission

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301 of strains between feral and farmed pigs in WA, but in some cases strains were similar
302 enough to suspect that such transmission may have occurred in the recent past. The
303 fact that the feral pig strains were different from each other suggests that these
304 animals are exposed to multiple sources of infection. In farms A, B and C where
305 multiple *B. hyodysenteriae* isolates were obtained, there was clear evidence for the
306 presence of between two and three major distinct strains in each farm, over and above
307 any minor variants of a given strain that were present. This is important information
308 as the different strains may vary in their biological properties, including antimicrobial
309 resistance, and this could influence the success of control programmes. Similarly,
310 where different strains co-exist on a farm this increases the opportunity for exchange
311 of genetic information between them, for example via the activity of the prophage-
312 like gene transfer agents (Humphrey et al., 1997; Motro et al., 2009).
313 Analysis of AATs in a population snapshot revealed that the majority (86%) of
314 strains belonged to one major cluster (Fig. 3). This greater clustering of AATs
315 compared to STs suggests that, against a backdrop of ongoing mutational changes,
316 there is negative selection on changes in nucleotide sequences that result in changes to
317 amino acid sequences that are essential for protein functionality. Based on this
318 consideration, analysis of AATs appeared to be a useful means of deducing putative
319 ancestral relationships between strains. It was interesting that AAT9 at the centre of
320 the main cluster was shared by strains from different countries, and even included a
321 strain from a mallard that had seemed distantly related from most of the other *B.*
322 *hyodysenteriae* strains on the basis of its position on the ST dendrogram (Fig. 1). It
323 seems likely that AAT9 represents an ancestral type, from which other AATs have
324 evolved.

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325 As a strain typing method, the use of MLST with seven loci was highly
326 discriminatory, and it was only slightly less discriminatory when used with five loci.
327 Currently it is still recommended to use seven loci until more strains have been
328 examined, and the full extent of diversity in the species has been uncovered. When
329 compared over the same 36 strains, MLST with seven loci had a similar DI to PFGE,
330 but was more discriminatory than REA, MLEE or serotyping. The groupings of
331 strains that were obtained with MLST broadly agreed with those previously
332 established by MLEE and/or PFGE, although there were some exceptions. On the
333 other hand, in agreement with previous observations (Lee et al., 1993), there was no
334 clear association between the serotype and the genetic grouping (ST) of the strains.
335 Compared to MLEE the MLST system was quicker and easier to use, while the results
336 should be more easily compared between laboratories than those of PFGE. An MLST
337 database for *B. hyodysenteriae* has been set up at the PubMLST website, and its future
338 use will allow easy comparisons of other strains isolated in different regions in the
339 world, and from different animal species.
340
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345 *hyodysenteriae* database at PubMLST.
346
347 **Conflicts of interest**
348 There are no conflicts of interest.
349

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445 **Figure captions**

446 **Fig. 1.** Dendrogram constructed from combined individual distance matrices of
447 sequences from seven genes *adh*, *alp*, *est*, *gdh*, *glpK*, *pgm* and *thi* in 111 *B.*
448 *hyodysenteriae* isolates divided into 67 sequence types (STs). The ST numbers that
449 formed part of the previous study by Råsbäck et al. (2007) are marked in bold. The
450 branch lines of the STs in the 11 clonal complexes (Cc) that were identified in the
451 current study are similarly outlined in bold. The length of the scale bar represents 1
452 nucleotide substitution in 100 base pairs of the sequenced gene fragment.
453 **Fig. 2.** Population snapshot obtained with eBURST using nucleotide sequences at
454 seven gene loci, with 111 *B. hyodysenteriae* isolates divided in 67 sequence types
455 (ST). Clusters of related isolates and individual unlinked isolates within the
456 population are displayed as a single eBURST diagram using a group definition of 0/7
457 shared alleles. The numbers represent the ST, and the size of the dots represents the
458 relative number of isolates in the ST. The eleven sets of linked isolates correspond to
459 clonal complexes (Cc), with the predicted founders positioned centrally in the cluster
460 line. The names assigned to the Ccs are indicated on Fig. 1.
461 **Fig. 3.** Population snapshot obtained from the 46 amino acid types (AATs) of 111 *B.*
462 *hyodysenteriae* isolates using eBURST. The parameters used to generate the snapshot
463 were the same as for the data in Fig. 2, except that translated allele sequences were
464 used. There is one major cluster of AATs, made up of two sub-clusters, with the
465 predicted founder (AAT9) positioned centrally in the larger sub-cluster. There are
466 four other linked groups made up of 2 or 3 AATs, and the other six AATs are
467 unlinked. The numbers represent the AAT and the size of the dots represents the
468 relative number of isolates in the AAT.

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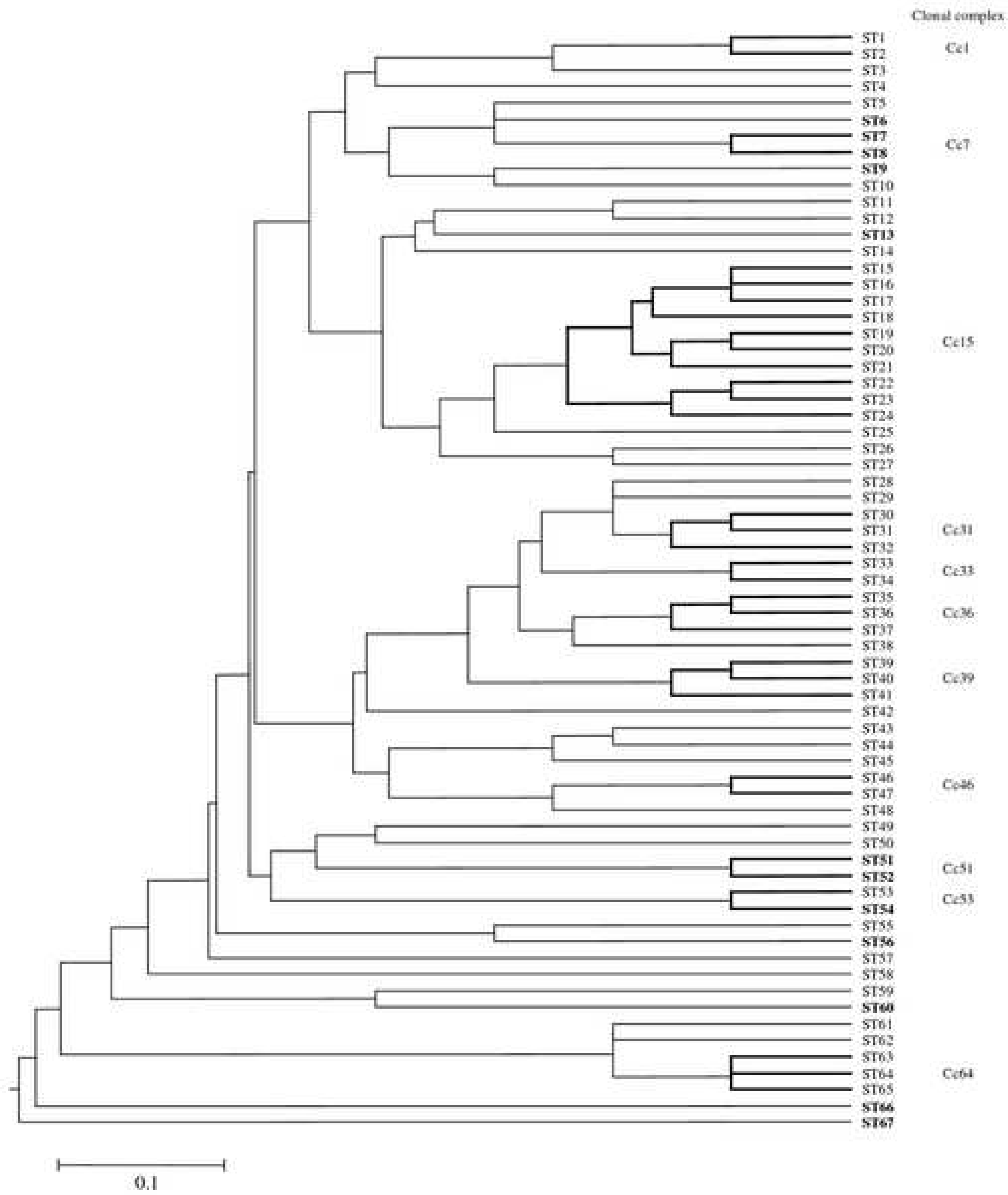

Figure 2

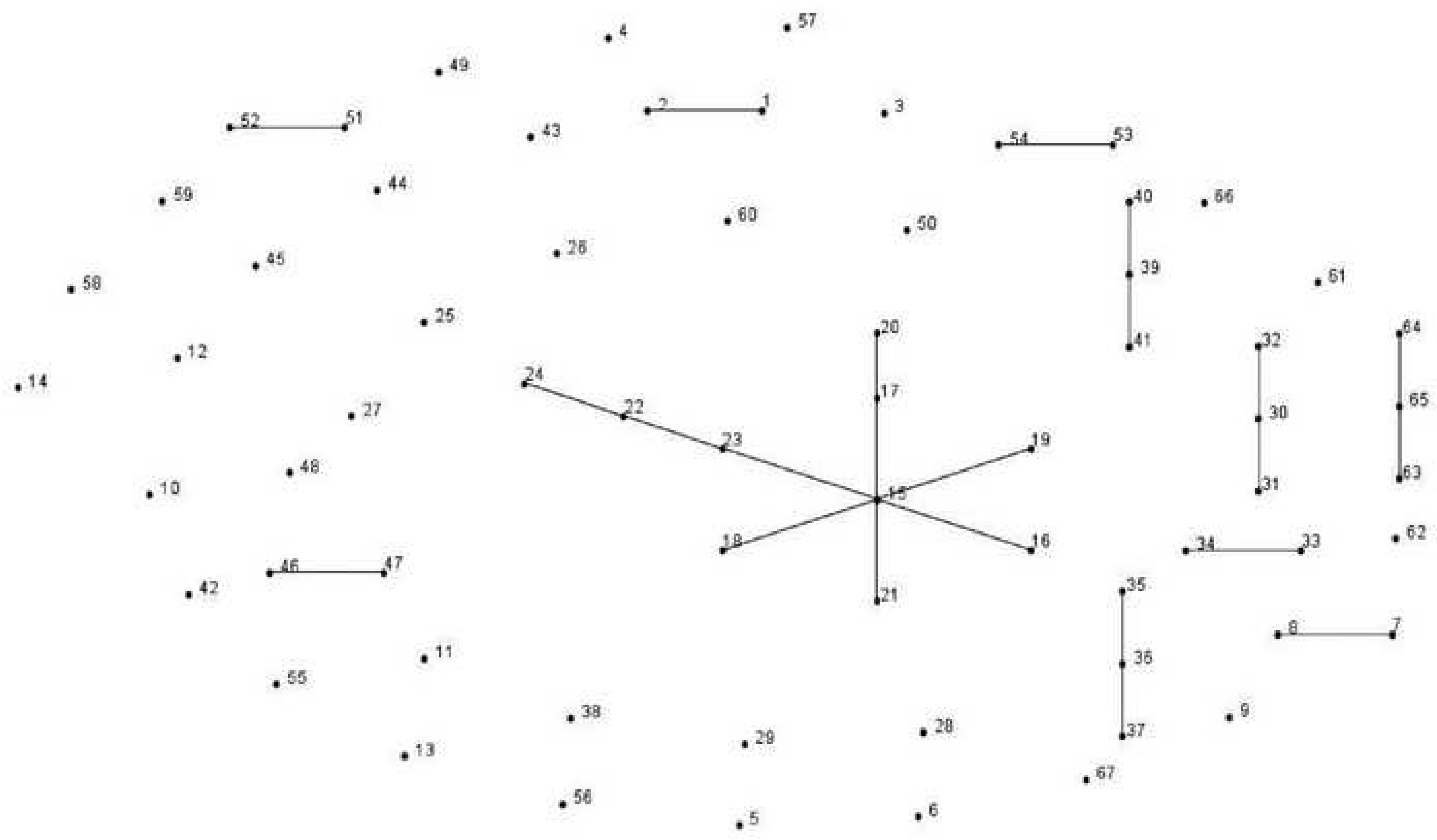

Figure 3

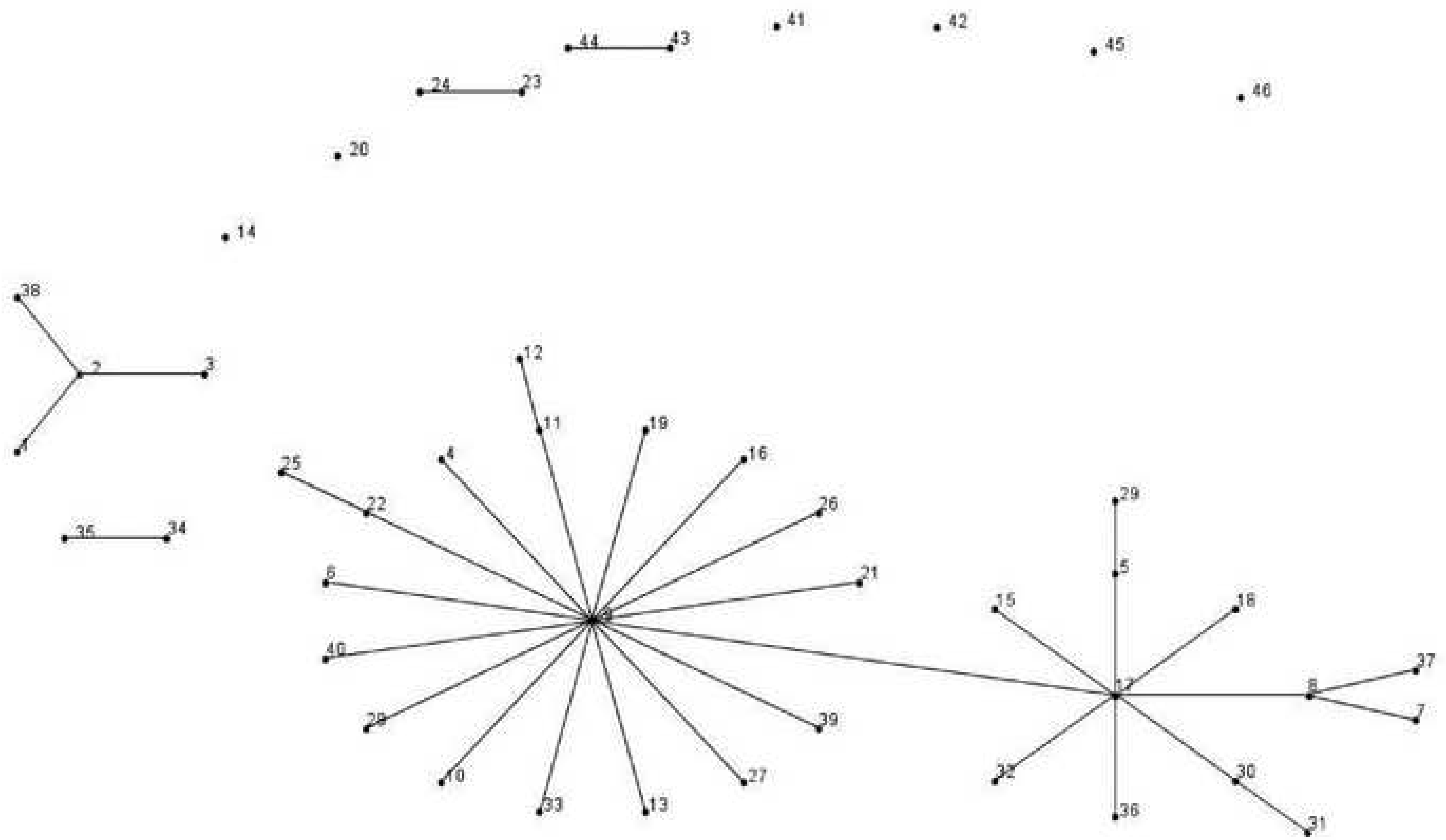

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Table 1

Brachyspira hyodysenteriae strain names and origin, sequence type and amino acid type in MLST, and comparison with previous results of multilocus enzyme electrophoresis, pulsed field gel electrophoresis, DNA restriction endonuclease analysis and serotyping, where these were available.

				Previous typing results			
Strain	Origin[a]	ST[b]	AAT[c]	ET[d]	PFGE[e]	REA[f]	Serogroup[g]
WA46	WA, 2007 (farm A)	1	1	NT	NT	NT	NT
WA41	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
WA52	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
WA53	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
WA56	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
WA57	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
WA42	WA, 2007 (farm A)	2	2	NT	NT	NT	NT
SA12	SA, 2007 (farm C)	3	3	NT	NT	NT	NT
FMV89.3323	Canada, 1989	4	4	12 (9)	NT	NT	K
ACK300/8	USA, 1970s	5	5	20 (15)	NT	NT	B
AN3730/96	Sweden, 1996	6 (6)	6 (6)	NT	NT	NT	NT
AN613/98	Sweden, 1998	6 (6)	6 (6)	NT	NT	NT	NT
P134/99	UK, 1999	7 (9)	7 (9)	NT	NT	NT	NT
E2	UK, unknown	8 (10)	8 (10)	NT	NT	NT	NT
AN360/03	Sweden, 2003	9 (8)	9 (8)	NT	NT	NT	NT
AN551/03	Sweden (mouse), 2003	9 (8)	9 (8)	NT	NT	NT	NT
KF9	UK, 1970s	10	10	22 (12)	NT	G	E
Vic36	VIC, 1991	11	11	6 (4)	B1	H5	B
Vic30	VIC, 1980s	12	12	11 (8)	D1	H8	B
Vic32	VIC, 1980s	12	12	11 (8)	D1	H8	B

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AN1082/90	Sweden, 1990	13 (1)	13 (1)	NT	NT	NT	NT
AN174/92	Sweden, 1992	13 (1)	13 (1)	NT	NT	NT	NT
AN3379/98	Sweden, 1998	13 (1)	13 (1)	NT	NT	NT	NT
Vic38	VIC, 1990	14	14	47 (26)	L1	M	B
NSW13	NSW, 1990s	15	15	35 (21)	M2	NT	D
NSW14	NSW, 1990s (farm D)	15	15	35 (21)	M2	NT	D
SA2	SA, 1980s	16	16	36 (22)	K1	L8	D
Vic4	VIC, 1980s	17	15	40 (17)	NT	NT	B
NSW2	NSW, 1990s	18	15	36 (22)	K2	L9	D
NSW42	NSW, 2005 (farm D)*	19	17	NT	NT	NT	NT
NSW45	NSW, 2005 (farm D)*	19	17	NT	NT	NT	NT
NSW46	NSW, 2005 (farm D)*	19	17	NT	NT	NT	NT
NSW49	NSW, 2005 (farm D)*	19	17	NT	NT	NT	NT
NSW27	NSW, 2004 (farm D)*	19	17	NT	NT	NT	NT
NSW28	NSW, 2004 (farm D)*	19	17	NT	NT	NT	NT
NSW32	NSW, 2005 (farm D)*	19	17	NT	NT	NT	NT
WA40	WA, 2005 (farm B)*	19	17	NT	NT	NT	NT
Vic24	VIC, 1988	20	17	43 (18)	H2	L5	D
Vic25	VIC, 1980s	20	17	43 (18)	H2	L5	D
Vic33	VIC, 1980s	20	17	43 (18)	H3	L5	NT
NSW44	NSW, 2005 (farm D)*	21	18	NT	NT	NT	NT
NSW30	NSW, 2005 (farm D)*	21	18	NT	NT	NT	NT
NSW34	NSW, 2005 (farm D)*	21	18	NT	NT	NT	NT
Q1	Q, 1980s	22	16	35 (21)	J1	L2	D
Q21	Q, 1980s	22	16	35 (21)	NT	NT	B
Q3	Q, 1980s	22	16	35 (21)	J1	L2	D
Q8	Q, 1980s	22	16	35 (21)	J2	L3	D

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Q9	Q, 1980s	22	16	35 (21)	J2	L3	D
SA1	SA, 1980s	23	16	36 (22)	K1	L8	D
B8044	USA, 1980s	24	19	42	NT	NT	B
SA3	SA, 1980s	25	9	50 (29)	NT	NT	A
B6933	USA, 1980s	26	20	35 (21)	NT	NT	A
Vic23	VIC, 1988	27	9	43 (18)	H1	L5	D
NSW9	NSW, 1991	28	17	NT	M1	NT	NT
Q33a	Q, 1991	28	17	NT	N1	NT	NT
Vic35	VIC, 1980s	29	21	4 (3)	A2	H1	B
Vic2	VIC, 1987	30	9	12 (9)	E2	H6	H
WA26	WA, 1980s	31	9	6 (4)	B2	H5	B
Q10	Q, 1980s	32	22	16 (11)	F1	H9	A
Q11	Q, 1980s	32	22	16 (11)	F1	H9	B
Q14	Q, 1988	32	22	10 (7)	C1	H9	G
Vic31	VIC, 1980s	32	22	10 (7)	C1	H9	B
NSW1	NSW, 1990s	33	23	7 (5)	NT	NT	I
NSW3	NSW, 1990s	34	24	4 (3)	A1	H2	B
Q17	Q, 1990s	35	25	10 (7)	C2	H10	B
Q22	Q, 1990s	36	22	10 (7)	C3	H11	G
WA1	WA, 1980s	36	22	4 (3)	A1	H1	B
WA2	WA, 1980s	36	22	4 (3)	A2	H1	B
WA4	WA, 1980s	36	22	4 (3)	A1	H1	B
WA5	WA, 1980s	36	22	25 (14)	G1	I1	E
WA8	WA, 1980s	36	22	4 (3)	A1	H1	B
WA9	WA, 1980s	36	22	4 (3)	A2	H1	B
WA13	WA, 1980	37	26	4 (3)	NT	NT	B
FP2	WA, 2006	38	22	NT	NT	NT	NT

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FP1	WA, 2006	39	27	NT	NT	NT	NT
WA28	WA, 1980s	40	9	16 (11)	F1	H4	A
FP5	WA, 2006	41	27	NT	NT	NT	NT
R301	USA (Rhea), 1996	42	28	20 (15)	NT	NT	A
WA27	WA, 1980s	43	29	38 (19)	I2	J	A
WA14	WA, 1980	44	5	38 (19)	I1	J	A
WA34	WA, 1990s	44	5	25 (14)	P1	NT	E
FP6	WA, 2006	45	17	NT	NT	NT	NT
FP3	WA, 2006	46	30	NT	NT	NT	NT
Q18	Q, 1990s	47	31	10 (7)	C3	H10	G
WA6	WA, 1980s	47	31	25 (14)	G1	I2	E
FP4	WA, 2006	48	32	NT	NT	NT	NT
SA11	SA, 2007 (farm C)	49	9	NT	NT	NT	NT
WA62	WA, 2007 (farm E)	49	9	NT	NT	NT	NT
Q20	Q, 1990s	50	16	49 (28)	NT	NT	A
T4	Germany, 1990s	51 (4)	33 (4)	NT	NT	NT	NT
A5677/96	Germany, 1996	52 (3)	9 (3)	NT	NT	NT	NT
Be45	Belgium, 1990s	52 (3)	9 (3)	NT	NT	NT	NT
T20	Germany, 1990s	52 (3)	9 (3)	NT	NT	NT	NT
B234	USA, 1970s	53	34	1 (1)	NT	A	A
B204	USA, 1970s	54 (12)	35 (12)	20 (15)	NT	C	B
FM88.90	Canada, 1990	55	36	28	NT	NT	J
B78[T]	USA, 1970s	56 (5)	37 (5)	12 (9)	E1	A	A
P18A	UK, 1970s	57	38	21 (13)	NT	E2	D
VS1	UK, 1970s	58	39	39	NT	NT	NT
B169	Canada, 1970s	59	40	27 (16)	NT	D	C
AN383:2/00	Sweden (mallard),	60 (2)	9 (1)	NT[h]	NT	NT	NT

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	2000						
WA73	WA, 2007 (farm B)	61	41	NT	NT	NT	NT
WA71	WA, 2007 (farm B)	62	42	NT	NT	NT	NT
WA75	WA, 2007 (farm B)	63	43	NT	NT	NT	NT
WA65	WA, 2007 (farm C)	64	44	NT	NT	NT	NT
WA69	WA, 2007 (farm C)	64	44	NT	NT	NT	NT
WA48	WA, 2007 (farm B)	64	44	NT	NT	NT	NT
WA48	WA, 2007 (farm A)	64	44	NT	NT	NT	NT
WA47	WA, 2007 (farm A)	65	43	NT	NT	NT	NT
AN2420/97	Sweden, 1997	66 (7)	45 (7)	NT	NT	NT	NT
AN1409:2/01	Sweden (mallard), 2001	67 (11)	46 (11)	NT	NT	NT	NT

Adjacent strains in the same clonal complex are highlighted with the same background shade. Unshaded strains are not included in a clonal complex.

[a]NSW, New South Wales; WA, Western Australia; VIC, Victoria; Q, Queensland; SA, South Australia. The isolates were recovered from pigs, except where noted in parenthesis. Isolates from feral pigs are marked FP. The year or approximate year of isolation is marked. The farm of origin is marked for recent Australian isolates, if known. Strains that were tested for tiamulin susceptibility and were found to be resistant (MIC $\geq$ 4 mg/l) are marked with an asterisk (*).

[b]ST, sequence type, with results for the 18 strains previously investigated (Råsbäck et al., 2007) in parenthesis.

[c]AAT, amino acid type, with results for the 18 strains previously investigated (Råsbäck et al., 2007) in parenthesis.

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[d]ET, electrophoretic type in multilocus enzyme electrophoresis for 58 strains (Trott et al., 1997), with previous results for 55 strains (Lee et al., 1993) in parenthesis.

[e]PFGE, pulse field gel electrophoresis type for 42 strains (Atyeo et al., 1999). In addition, each of the five pairs of isolates AN3730/96 and AN613/98; AN360/03 and AN551/03; AN1082/90 and AN3379/98; and A5677/96 and Be45 each had identical PFGE patterns (Råsbäck et al., 2007).

[f]REA, restriction endonuclease analysis type for 42 strains (Combs et al., 1992).

[g]Serogroup, for 56 strains (Hampson et al., 1989; Combs et al., 1992; Hampson et al., 1997).

NT, not tested.

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Table 2

Diversity Index (DI) for MLST and comparison with other previously described methods of sub-species differentiation of *B. hyodysenteriae* using members of the same subset of 36 strains

Method[a]	Number of strains analysed	Number of types	Frequency (%) of the largest type	Diversity Index (DI)
MLST	111[b]	67	7.2%	0.974
MLST	36[c]	21	19.4%	0.943
PFGE	36	21	11.1%	0.941
REA	36	19	16.7%	0.924
MLEE	36	12	19.4%	0.894
Serotyping	36	6	41.7%	0.719

[a]MLST, multilocus sequence typing (this study)

PFGE, pulse field gel electrophoresis (Atyeo et al., 1999)

REA, restriction endonuclease analysis (Combs et al., 1992).

MLEE, multilocus enzyme electrophoresis (Lee et al., 1993; Trott et al., 1997)

Serogroup, as defined (Hampson et al., 1989; Hampson et al., 1997)

[b]111 refers to the MLST results for all 111 strains in the current study

[c]36 relates to strains that were also used in earlier studies for which there is MLEE, PFGE, REA and serotyping data for all of these strains, as shown in the subsequent rows.

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Optional e-only supplementary files ACCEPTED MANUSCRIPT

Supplementary table. Sequence types and the corresponding allelic numbers for the seven loci

Sequence type	Loci[a]						
	adh	*alp*	*est*	*gdh*	*glpK*	*pgm*	*thi*
1	2	6	3	1	18	4	3
2	2	6	3	1	17	4	3
3	2	6	3	1	17	10	20
4	2	3	3	1	4	3	16
5	2	10	3	10	8	1	3
6	2	7	3	1	12	1	3
7	2	2	3	12	14	1	3
8	2	2	3	12	11	1	3
9	2	11	3	6	9	1	3
10	2	11	3	1	9	8	13
11	2	11	10	14	3	2	3
12	2	1	10	14	6	2	3
13	2	11	6	6	12	2	3
14	2	14	11	8	3	2	3
15	2	8	3	11	6	2	3
16	2	8	3	5	6	2	3
17	2	8	3	10	6	2	3
18	2	8	3	11	6	2	1
19	2	11	3	11	6	2	3
20	2	11	3	10	6	2	3
21	2	12	3	11	6	2	3
22	2	8	3	4	8	2	3
23	2	8	3	4	6	2	3
24	2	12	3	4	8	2	3
25	2	11	3	9	6	2	17
26	2	16	5	4	6	3	3
27	2	11	3	4	6	3	3
28	2	11	10	10	1	2	1
29	2	11	10	16	1	2	4
30	2	11	10	15	1	2	3
31	2	11	10	7	1	2	3
32	2	11	10	15	1	2	8
33	2	11	10	15	1	9	12
34	2	11	10	15	1	5	12
35	2	11	10	15	13	2	15
36	2	11	10	15	13	2	1
37	2	11	10	18	13	2	1
38	2	11	10	15	7	2	6

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39	2	11	9	4	1	2	14
40	2	11	10	4	1	2	14
41	2	11	9	5	1	2	14
42	2	11	8	5	9	2	10
43	2	6	10	10	8	2	5
44	2	6	2	10	8	2	3
45	2	11	2	10	8	2	6
46	2	11	9	10	7	2	6
47	2	11	9	10	7	4	6
48	2	11	10	10	1	4	6
49	2	13	14	17	16	2	6
50	2	8	14	1	13	2	2
51	2	13	4	6	8	2	17
52	2	13	3	6	8	2	17
53	2	6	7	2	4	2	7
54	2	6	7	1	4	2	7
55	2	11	1	10	6	1	9
56	2	2	1	10	5	1	11
57	2	9	5	3	4	4	3
58	4	4	10	4	10	2	2
59	1	16	3	4	2	3	6
60	1	13	3	5	8	1	6
61	8	17	16	8	18	3	14
62	7	17	15	8	18	3	14
63	6	17	11	8	18	3	14
64	2	17	11	8	18	3	14
65	9	17	11	8	18	3	14
66	3	15	12	1	5	6	18
67	5	5	13	13	15	7	19

[a] *adh*, alcohol dehydrogenase; *alp*, alkaline phosphatase; *est*, esterase: *gdh,* glutamate dehydrogenase; *glpK*, glucose kinase; *pgm*, phosphoglucomutasee; *thi*, acetyl-CoA acetyltransferase.

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