

# Development of a multilocus sequence typing scheme for intestinal spirochaetes within the genus *Brachyspira*

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The purpose of this study was to evaluate a multilocus sequence typing (MLST) scheme for intestinal spirochaetes of the genus *Brachyspira*. Eight loci mainly coding for enzymes previously used in multilocus enzyme electrophoresis analysis of *Brachyspira* species were examined in 66 *Brachyspira* field isolates and type/reference strains. The isolates and strains were recovered from pigs, birds, dogs and a mouse and originated from seven European countries, the USA and Canada. Forty-six isolates represented recognized *Brachyspira* species and 20 represented provisionally designated species or isolates that have not been classified. Only two loci gave PCR products for all 66 strains and isolates, but amplicons for seven loci were obtained for 44 of the isolates. Sequences for each locus had a DNA allelic variation of 30–47 and an amino acid allelic variation of 14–47 that gave rise to the same number of sequence and amino acid types (58) for the strains and isolates studied. A population snapshot based on sequence and amino acid types showed a close phylogenetic relationship amongst the porcine isolates from the same geographical regions, and indicated a close evolutionary relationship between isolates recovered from pigs and mallards. A general concordance was obtained between the MLST groupings and classifications based on culture and biochemical tests, 16S rDNA sequence analysis and random amplified polymorphic DNA analysis. This is a first step towards establishing an MLST system for use in identifying *Brachyspira* species and determining relationships between individual strains and species in the genus.

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## INTRODUCTION

The fastidious anaerobic intestinal spirochaetes of the genus *Brachyspira* (formerly *Serpulina*) are commonly

**Abbreviations:** AAT, amino acid type; MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing; RAPD, random amplified polymorphic DNA; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the core sequences for the MLST loci used for *B. hyodysenteriae* strain WA1 and *B. pilosicoli* strain 95/1000 are EF488202–EF488215.

found colonizing the large intestines of a wide range of animals, including humans (Hampson & Stanton, 1997; Duhamel, 2001). Currently there are seven officially named species in the genus, and several provisionally named species. Some species are important causes of enteric disease, particularly in pigs and poultry. *Brachyspira hyodysenteriae* is the cause of swine dysentery (Taylor & Alexander, 1971) and *Brachyspira pilosicoli* causes intestinal spirochaetosis in pigs and other species (Taylor *et al.*, 1980; Duhamel, 2001). In poultry, *B. pilosicoli*, *Brachyspira intermedia* and *Brachyspira alvinipulli* cause reduced egg

production, wet faeces and dirty eggshells (Stephens & Hampson, 2001). *Brachyspira innocens* and *Brachyspira murdochii* have been found in various species of animals and birds, and are generally considered to be apathogenic. *Brachyspira aalborgi* appears to be largely confined to humans, and its potential role in enteric disease is controversial (Hovind-Hougen *et al.*, 1982). '*Brachyspira canis*' (Duhamel *et al.*, 1998), '*Brachyspira pulli*' (Stephens & Hampson, 2001) and '*Brachyspira suanatina*' (Råsbäck *et al.*, 2007) are provisionally designated species; the last causes a disease in pigs similar to swine dysentery. Additionally, isolates exist that cannot be classified into presently recognized *Brachyspira* species because of atypical phenotypic or genotypic features or both (Ateyo *et al.*, 1999b; Thomson *et al.*, 2001; Jansson *et al.*, 2004).

The routine identification and classification of *Brachyspira* species is normally based on culture and biochemical tests, often supported by species-specific PCR assays, which together make a very reliable combination for the clinically important porcine *Brachyspira* species. However, identification and classification of some *Brachyspira* isolates can be challenging, particularly when they have atypical features (Råsbäck *et al.*, 2005, 2006). For many bacterial genera, species identification of isolates can be achieved by 16S rDNA sequence analysis (Woese, 1987), but for the *Brachyspira* genus, phylogenetic division based on 16S rDNA sequences is only reliable for the species that are not too closely related. For example, in a phylogenetic tree based on 16S rDNA sequences, *B. hyodysenteriae* and certain *B. intermedia* isolates clustered together, as did *B. innocens* and *B. murdochii* isolates (Pettersson *et al.*, 1996). This is particularly bothersome because the two closely related species *B. hyodysenteriae* and *B. intermedia* have different veterinary medical significances in pigs. The latter is generally regarded as non-pathogenic, whereas *B. hyodysenteriae* can cause severe mucohaemorrhagic diarrhoea.

Multilocus enzyme electrophoresis (MLEE) has been an influential technique for identifying potentially new *Brachyspira* species (Lee *et al.*, 1993; McLaren *et al.*, 1997; Duhamel *et al.*, 1998). Unfortunately, MLEE is a time-consuming and tedious technique that is not available in most diagnostic microbiology laboratories. Furthermore, MLEE is often not sufficiently discriminatory at the strain level for use in detailed molecular epidemiological studies on *Brachyspira* species. More discriminatory techniques that have been used include pulsed-field gel electrophoresis (PFGE) (Ateyo *et al.*, 1996, 1999a) and random amplified polymorphic DNA (RAPD) analysis (Dugourd *et al.*, 1996; Jansson *et al.*, 2004). It should be acknowledged, however, that sometimes the results obtained by these techniques can be difficult to interpret or reproduce or both (Maiden *et al.*, 1998), and improved methods are needed. In the case of many *Brachyspira* species, the molecular epidemiology may be much more complex than is currently appreciated. For example, in swine dysentery, carrier pigs are a major cause of transmission, but dogs, birds, rats and mice are also potential vectors (Songer *et al.*, 1978; Joens, 1980;

Hampson *et al.*, 1991; Trott *et al.*, 1996a; Duhamel, 2001; Fellström & Holmgren, 2005; Råsbäck *et al.*, 2007). Such epidemiological connections could be more easily established if simple and improved strain-typing techniques were available for all *Brachyspira* species.

Multilocus sequence typing (MLST) is a robust, consistent and portable technique that now has largely replaced MLEE for analysis of bacterial population structure, determining relatedness of species, and as a molecular epidemiological tool (Maiden *et al.*, 1998; Urwin & Maiden, 2003). The present study had two main objectives: first, to determine whether MLST could be used to help delineate and define species more widely across the genus, including examination of isolates with atypical characteristics for which the species affiliation is currently uncertain; and second, to investigate whether the system was sufficiently discriminatory for use as a tool for molecular epidemiological studies. MLST was applied to 66 carefully selected *Brachyspira* isolates and strains, and the results were compared with current classifications based on culture and biochemical tests, 16S rDNA sequences, and strain-typing results from RAPD analysis.

## METHODS

**Bacterial isolates and strains.** Forty-six well-described *Brachyspira* isolates and strains and 20 isolates that were either provisionally named, had atypical phenotypic characteristics, or could not be designated into any described *Brachyspira* species were included in the study (Table 1). The majority were recovered from pigs, and the selection was based on results from culture and biochemical tests (Fellström & Gunnarsson, 1995; Fellström *et al.*, 1999), in some cases supported by the results of PCR or 16S rDNA sequence analysis (Johansson *et al.*, 2004), or both. The selection of isolates for the study included *B. hyodysenteriae* and *B. pilosicoli* isolates with low or high MICs for tiamulin and tylosin. Isolates and strains were obtained from the National Veterinary Institute's strain collection (Uppsala, Sweden), except for the type strains of *B. pilosicoli* (P43/6/78<sup>T</sup>), *B. intermedia* (PWS/A<sup>T</sup>), *B. innocens* (B256<sup>T</sup>) and *B. alvinipulli* (C1<sup>T</sup>), which came from the collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. All the type and reference strains used were originally obtained from the ATCC Bacteriology Collection. Supplementary information for some of the isolates is outlined below.

The *B. hyodysenteriae* isolates T20 (A91507-6x/01), T4 [A84193-2x/99 (CCUG 47386)] and A5677/96 were indole-negative (Fellström *et al.*, 1999; Karlsson *et al.*, 2004; Pringle *et al.*, 2004; Råsbäck *et al.*, 2005), P134/99 had an atypical 23S rDNA sequence (Thomson *et al.*, 2001) and E2 was included because it was tiamulin resistant (Karlsson *et al.*, 2004). Eight isolates (A5677/96 and Be45; AN1082/90 and AN3379/98; AN3730/96 and AN613/98; AN360/03 and AN551/03) consisted pairwise of four identical PFGE types (Fellström *et al.*, 1999; Fellström & Holmgren, 2005). Five of the *B. pilosicoli* isolates (AN738/02, AN953/02, AN991/02, AN1085/02 and AN984/03) were tiamulin resistant and some were also tylosin resistant, of which two (AN1085/02 and AN984/03) originated from the same farm. The six *B. intermedia* field isolates were all weakly haemolytic, spot indole-positive, hippurate and  $\alpha$ -galactosidase-negative, and  $\beta$ -glucosidase-positive. Two of the '*B. suanatina*' isolates (AN4859/03 and AN2384/04) were shown to represent one RAPD type (Råsbäck *et al.*, 2007). Biochemical characteristics for the avian isolates described in this

**Table 1.** Strain and isolate designation, species name at selection and origin of the 66 *Brachyspira* species used in this study, and their allelic assignment (ST/AAT), sequence type (ST) and amino acid type (AAT) for each locus

Strain/isolate	Species name	Species of origin	Country of origin	ST and AAT	MLST genes							GenBank accession no.	References
					<i>adh</i>	<i>pgm</i>	<i>est</i>	<i>glp</i>	<i>gdh</i>	<i>thi</i>	<i>alp</i>		
AN174/92	<i>B. hyodysenteriae</i>	Pig	Sweden	1	1/14	1/32	1/30	6/42	2/40	3/31	1/30	U14931	Fellström <i>et al.</i> (1995)
AN1082/90	<i>B. hyodysenteriae</i>	Pig	Sweden	1	1/14	1/32	1/30	6/42	2/40	3/31	1/30	EF517531	Fellström <i>et al.</i> (1999); this study
AN3379/98	<i>B. hyodysenteriae</i>	Pig	Sweden	1	1/14	1/32	1/30	6/42	2/40	3/31	1/30	ID to EF517531	Fellström <i>et al.</i> (1999); this study
AN383 : 2/00	<i>B. hyodysenteriae</i>	Mallard	Sweden	2	1/14	1/32	2/33	1/46	1/41	1/31	5/24	AY352291	Jansson <i>et al.</i> (2004)
T20	<i>B. hyodysenteriae</i>	Pig	Germany	3	1/14	1/32	2/33	2/47	1/41	4/31	2/30	EF51730	Pringle <i>et al.</i> (2004); this study
T4	<i>B. hyodysenteriae</i>	Pig	Germany	4	1/14	1/32	3/24	2/47	1/41	4/31	2/30	DQ922564	Karlsson <i>et al.</i> (2004)
A5677/96	<i>B. hyodysenteriae</i>	Pig	Germany	3	1/14	1/32	2/33	2/47	1/41	4/31	2/30	DQ974209	Fellström <i>et al.</i> (1999)
Be45	<i>B. hyodysenteriae</i>	Pig	Belgium	3	1/14	1/32	2/33	2/47	1/41	4/31	2/30	ID to AF172986	Fellström <i>et al.</i> (1999)
B78 <sup>T</sup>	<i>B. hyodysenteriae</i>	Pig	USA	5	1/14	2/32	4/22	7/41	5/37	3/31	4/23	U14930	Harris <i>et al.</i> (1972)
AN3730/96	<i>B. hyodysenteriae</i>	Pig	Sweden	6	1/14	3/32	2/33	1/46	7/35	1/31	5/24	ID to DQ473574	Fellström <i>et al.</i> (1999); this study
AN613/98	<i>B. hyodysenteriae</i>	Pig	Sweden	6	1/14	3/32	2/33	1/46	7/35	1/31	5/24	ID to DQ473574	Fellström <i>et al.</i> (1999); this study
AN2420/97	<i>B. hyodysenteriae</i>	Pig	Sweden	7	1/14	4/31	2/33	1/46	7/35	1/31	5/24	DQ473574	Råsbäck <i>et al.</i> (2007)
AN360/03	<i>B. hyodysenteriae</i>	Pig	Sweden	8	1/14	3/32	2/33	4/44	3/39	1/31	1/30	EF517534	Fellström & Holmgren (2005); this study
AN551/03	<i>B. hyodysenteriae</i>	Mouse	Sweden	8	1/14	3/32	2/33	4/44	3/39	1/31	1/30	ID to EF517534	Fellström & Holmgren (2005); this study
P134/99	<i>B. hyodysenteriae</i>	Pig	UK	9	1/14	3/32	2/33	9/39	6/38	1/31	4/23	EF517541	Thomson <i>et al.</i> (2001); this study
E2	<i>B. hyodysenteriae</i>	Pig	UK	10	1/14	3/32	2/33	3/45	6/38	1/31	4/23	EF517540	Karlsson <i>et al.</i> (2004); this study
AN1409 : 2/01	<i>B. hyodysenteriae</i>	Mallard	Sweden	11	2/14	1/32	2/33	5/43	5/37	2/31	1/30	AY352281	Jansson <i>et al.</i> (2004)
B204 <sup>R</sup>	<i>B. hyodysenteriae</i>	Pig	USA	12	2/14	5/32	2/33	8/40	4/36	2/31	3/30	U14932	Kinyon <i>et al.</i> (1977)
PWS/A <sup>T</sup>	<i>B. intermedia</i>	Pig	UK	13	4/14	6/27	5/25	0/0	10/34	10/27	11/22	U23033	Stanton <i>et al.</i> (1997)
C1 <sup>T</sup>	<i>B. alvinipulli</i>	Chicken	USA	14	1/14	17/29	18/18	19/29	27/15	25/23	10/28	U23030	Stanton <i>et al.</i> (1998)
AN6050 : 2/1/00	<i>Brachyspira</i> sp.	Mallard	Sweden	15	3/13	18/8	20/17	20/28	17/25	26/22	0/0	EF371459	This study

Table 1. cont.

Strain/isolate	Species name	Species of origin	Country of origin	ST and AAT	MLST genes							GenBank accession no.	References
					<i>adh</i>	<i>pgm</i>	<i>est</i>	<i>glp</i>	<i>gdh</i>	<i>thi</i>	<i>alp</i>		
AN4578/01	<i>Brachyspira</i> sp.	Dog	Sweden	16	1/14	19/8	21/16	0/0	28/14	27/21	0/0	AY349933	Johansson <i>et al.</i> (2004)
AN3949:2/02	' <i>B. suanatina</i> '	Mallard	Sweden	17	6/14	7/28	10/20	10/38	8/26	13/25	17/16	AY352290	Jansson <i>et al.</i> (2004); Råsbäck <i>et al.</i> (2007)
AN1418:2/01	' <i>B. suanatina</i> '	Mallard	Sweden	18	6/14	7/28	10/20	10/38	8/26	12/24	20/13	AY352282	Jansson <i>et al.</i> (2004); Råsbäck <i>et al.</i> (2007)
AN2384/04	' <i>B. suanatina</i> '	Pig	Sweden	19	7/14	7/28	15/27	10/38	9/27	13/25	18/15	DQ473577	Råsbäck <i>et al.</i> (2007)
Dk12570-2	' <i>B. suanatina</i> '	Pig	Denmark	20	7/14	7/28	10/20	10/38	8/26	12/24	17/16	DQ473578	Råsbäck <i>et al.</i> (2007)
AN4859/03	' <i>B. suanatina</i> '	Pig	Sweden	19	7/14	7/28	15/27	10/38	9/27	13/25	18/15	DQ473575	Råsbäck <i>et al.</i> (2007)
AN983/90	<i>B. intermedia</i>	Pig	Sweden	21	5/14	11/25	14/28	14/37	11/33	11/26	15/18	U14933	Fellström <i>et al.</i> (1995)
AN517/97	<i>B. intermedia</i>	Pig	Finland	22	8/14	8/30	6/23	17/34	13/31	6/31	12/19	EF517535	This study
AN519/97	<i>B. intermedia</i>	Pig	Finland	23	8/14	8/30	6/23	16/35	13/31	7/31	12/19	EF517536	This study
AN1707/96	<i>B. intermedia</i>	Pig	Sweden	24	9/14	9/30	7/26	13/31	15/28	8/30	13/21	EF517532	This study
AN621/97	<i>B. intermedia</i>	Pig	Sweden	25	10/14	10/30	9/29	18/36	16/29	5/29	14/20	EF517537	This study
AN885/94	<i>B. intermedia</i>	Pig	Sweden	26	10/14	10/30	9/29	18/36	14/30	5/29	14/20	ID to EF517537	This study
AN3930:2/02	<i>Brachyspira</i> sp.	Mallard	Sweden	27	11/12	16/21	8/32	12/32	12/32	9/28	16/17	AY352288	Jansson <i>et al.</i> (2004)
AN1268/7/04	<i>Brachyspira</i> sp.	Chicken	Sweden	28	14/3	12/26	16/19	11/30	0/0	0/0	19/14	EF164991	This study
AN2929/1/03	<i>Brachyspira</i> sp.	Chicken	Sweden	29	15/3	13/23	11/21	15/33	18/24	30/14	6/26	EF164984	This study
AN1788/01	<i>Brachyspira</i> sp.	Dog	Sweden	30	17/4	15/22	13/31	23/24	20/22	29/12	0/0	AY349935	Johansson <i>et al.</i> (2004)
CN2	<i>Brachyspira</i> sp.	Dog	Norway	31	16/1	14/24	12/31	21/26	19/23	28/13	0/0	AY349934	Johansson <i>et al.</i> (2004)
AN3927:3/2/02	<i>Brachyspira</i> sp.	Mallard	Sweden	32	18/5	30/7	0/0	45/16	31/11	0/0	21/12	EF371460	This study
AN497/93	<i>B. pilosicoli</i>	Pig	Sweden	33	19/1	36/3	36/3	34/8	41/2	15/7	32/9	ID to U14927	Fellström <i>et al.</i> (1995)
AN652/02	<i>B. pilosicoli</i>	Pig	Sweden	34	24/1	31/1	29/2	33/13	37/5	14/2	27/4	EF517538	Råsbäck <i>et al.</i> (2005); this study
C62	<i>B. pilosicoli</i>	Pig	Sweden	35	21/1	37/6	37/3	43/5	32/10	21/3	25/7	ID to U14927	Fellström <i>et al.</i> (1995)
AN1085/02	<i>B. pilosicoli</i>	Pig	Sweden	36	24/1	32/1	35/3	32/12	34/7	23/1	23/2	ID to U14927	Pringle <i>et al.</i> (2006); this study
AN984/03	<i>B. pilosicoli</i>	Pig	Sweden	36	24/1	32/1	35/3	32/12	34/7	23/1	23/2	ID to U14927	Pringle <i>et al.</i> (2006); this study
P43/6/78 <sup>T</sup>	<i>B. pilosicoli</i>	Pig	UK	37	22/1	33/1	31/1	40/1	40/1	22/1	22/1	U14927	Trott <i>et al.</i> (1996b)
AN390/00	<i>Brachyspira</i> sp.	Mallard	Sweden	38	19/1	41/5	32/4	42/3	36/6	19/4	28/5	EF371457	This study
AN2448/02	<i>B. pilosicoli</i>	Pig	Sweden	39	24/1	40/2	22/8	41/2	39/3	18/5	24/3	EF517533	Råsbäck <i>et al.</i> (2005); this study

Table 1. cont.

Strain/isolate	Species name	Species of origin	Country of origin	ST and AAT	MLST genes							GenBank accession no.	References
					<i>adh</i>	<i>pgm</i>	<i>est</i>	<i>glp</i>	<i>gdh</i>	<i>thi</i>	<i>alp</i>		
C162	<i>B. pilosicoli</i>	Pig	Sweden	40	24/1	42/1	39/3	38/7	38/4	16/8	29/11	U14928	Fellström <i>et al.</i> (1995)
AN953/02	<i>B. pilosicoli</i>	Pig	Sweden	41	23/2	42/1	39/3	39/6	38/4	0/0	29/11	EF517539	Pringle <i>et al.</i> (2006); this study
AN4170/01	<i>B. pilosicoli</i>	Pig	Sweden	42	20/1	38/1	38/3	44/4	36/6	20/1	26/6	ID to U14927	Råsbäck <i>et al.</i> (2005); this study
AN76/92	<i>B. pilosicoli</i>	Pig	Sweden	43	20/1	39/1	30/1	37/11	41/2	17/6	31/10	ID to U14927	Fellström <i>et al.</i> (1995)
AN738/02	<i>B. pilosicoli</i>	Pig	Sweden	44	24/1	34/4	33/5	36/10	33/9	0/0	30/8	ID to U14927	Pringle <i>et al.</i> (2006); this study
AN991/02	<i>B. pilosicoli</i>	Pig	Sweden	45	20/1	35/4	34/3	35/9	35/8	0/0	33/9	ID to U14927	Pringle <i>et al.</i> (2006); this study
AN1263/2/04	<i>Brachyspira</i> sp.	Chicken	Sweden	46	25/11	20/20	19/15	0/0	0/0	24/9	0/0	EF164988	This study
AN3491: 1/1/02	<i>Brachyspira</i> sp.	Eider	Sweden	47	12/9	21/15	17/6	24/27	29/13	31/10	0/0	EF371458	This study
AN4771/01	' <i>B. canis</i> '	Dog	Sweden	48	13/10	22/19	28/14	22/25	30/12	32/44	0/0	AY349937	Johansson <i>et al.</i> (2004)
B256 <sup>T</sup>	<i>B. innocens</i>	Pig	USA	49	1/14	29/16	26/10	28/20	26/18	35/18	7/27	U14920	Kinyon & Harris (1979)
56-150 <sup>T</sup>	<i>B. murdochii</i>	Pig	Canada	50	27/7	24/9	22/7	25/17	21/16	38/15	0/0	AY312492	Stanton <i>et al.</i> (1997)
C378	<i>B. murdochii</i>	Pig	Sweden	51	27/7	23/14	23/9	27/19	22/17	37/16	0/0	U14918	Fellström <i>et al.</i> (1995)
C301	<i>B. murdochii</i>	Pig	Sweden	52	27/7	25/10	22/8	26/18	21/16	38/15	0/0	U14917	Fellström <i>et al.</i> (1995)
C173	<i>B. innocens</i>	Pig	Sweden	53	29/6	26/18	25/11	30/21	23/21	33/19	0/0	U14921	Fellström <i>et al.</i> (1995)
C336	<i>B. innocens</i>	Pig	Sweden	54	28/7	27/17	24/12	31/22	24/20	34/20	0/0	ID to U14919	Fellström <i>et al.</i> (1995)
C555	<i>B. innocens</i>	Pig	Sweden	55	30/7	28/17	27/13	29/23	25/19	36/17	0/0	U14926	Pettersson <i>et al.</i> (1996)
AN968/2/04	<i>Brachyspira</i> sp.	Corvids	Sweden	56	26/8	45/13	0/0	46/15	0/0	0/0	9/29	EF371462	This study
AN2181/1/00	<i>Brachyspira</i> sp.	Corvids	Sweden	57	1/14	44/12	0/0	0/0	0/0	0/0	8/25	EF371461	This study
AN1865/1/04	<i>Brachyspira</i> sp.	Corvids	Sweden	58	1/14	43/11	0/0	47/14	0/0	0/0	0/0	EF371463	This study
Total no. ST/ATT				58	30/14	45/32	39/33	47/47	41/41	38/31	33/30		

study are listed in Table 2. Characterization of these isolates will be further described elsewhere (D. S. Jansson, unpublished data).

**16S rDNA sequence analysis.** Twenty-two porcine isolates and 10 avian isolates were subjected to 16S rRNA gene sequencing (Table 1; this study), as previously described (Pettersson *et al.*, 1996; Johansson *et al.*, 2004). The corresponding sequences of the type strains of *Borrelia burgdorferi* (GenBank accession number X98228), and *Treponema denticola* (GenBank accession number AF139203), were used as out-group when constructing a phylogenetic tree for all 66 isolates and strains used in this study.

**RAPD analysis.** To identify possible clones, two isolates of tiamulin-resistant *B. pilosicoli* recovered from a single farm, and the eight *B. hyodysenteriae* isolates previously recognized as four PFGE types were analysed by RAPD. The 26 isolates that showed identical sequence type (ST) or closely related STs or amino acid types (AATs) in MLST analysis (see Figs 2 and 3; isolates with coloured designations) were also analysed by RAPD for comparison of data. Two primers, 5'-ACG CGC CCT-3' (P73) (Quednau *et al.*, 1998) and 5'-CCG CAG CCA A-3' (P1254) (Torriani *et al.*, 1999), were used separately for RAPD. The PCR was performed under standard conditions in a 50 µl reaction mixture with the *Taq* DNA polymerase (Biotech

**Table 2.** Characteristics of 10 atypical avian *Brachyspira* isolates with rare phenotypes used in MLST

A comprehensive description of these isolates will be published elsewhere (D. S. Jansson).

Strain designation	Bird of origin	Biochemical tests				
		Haemolysis	Spot indole	Hippurate	$\alpha$ -Galactosidase	$\beta$ -Glucosidase
AN6050:2/1/00	Mallard	+	–	+	–	+
AN3927:3/2/02	Mallard	+++	–	+	+	+
AN390/00	Mallard	+++	–	+	+	–
AN3491:1/1/02	Common eider	+++	–	–	–	+
AN1263/2/04	Chicken	+	–	+	–	+
AN2929/1/03	Chicken	+	–	–	+	+
AN1268/7/04	Chicken	+	+	–	+	+
AN2181/1/00	Eurasian jackdaw*	+	–	–	+	–
AN968/2/04	Hooded crow*	+	–	–	+	–
AN1865/1/04	Rook*	+	–	–	+	(+)

\*Corvids.

International). The PCR programme consisted of four cycles of 94 °C for 45 s, 30 °C for 2 min, and 72 °C for 1 min, followed by 26 cycles of 94 °C for 5 s, 36 °C for 30 s and 72 °C for 30 s. A final extension period of 10 min at 72 °C was included before cooling to 14 °C.

**Multilocus sequence typing.** Chromosomal DNA was prepared by boiling the bacterial cells, as previously described (Råsback *et al.*, 2006). In addition, DNA was prepared and purified by conventional protein K lysis and phenol/chloroform extraction as well as by robot extraction (BioRobot EZ1, EZ1 DNA Tissue kit; Qiagen) for a subset of 10 of the isolates for which all loci could not be amplified by PCR from DNA extracted by boiling. Five of the eight genes tested in the MLST scheme represented genes coding for enzymes of *Brachyspira* species previously used in MLEE analysis: alcohol dehydrogenase (ADH), alkaline phosphatase (ALP), esterase (EST), glutamate dehydrogenase (GDH) and phosphoglucomutase (PGM) (Lee *et al.*, 1993). MLEE enzymes have previously been shown to be present in all named *Brachyspira* species as well as in the proposed species '*B. pulli*' and '*B. canis*'. Further included were three genes: *glp* [glucose kinase (*glpK*)], *thi* [acetyl-CoA acetyltransferase (*yqi*, also known as thiolase)] and *mut* [DNA mismatch repair protein (*mutS*)], used for the MLST systems of *Staphylococcus aureus* (*glpK* and *yqi*) and *Streptococcus pyogenes* (*mutS*) (<http://pubmlst.org/>). The likely existence of single copies for all genes was confirmed by examining the near-complete (~90%) genome sequences of the *B. hyodysenteriae* strain WA1 and *B. pilosicoli* strain 95/1000 genomes, obtained in a sequencing project conducted at Murdoch University (unpublished data). Primers for MLST were designed by using the Primer3 program (Rozen & Skaletsky, 2000). Previously a small set of Australian isolates had been analysed primarily with primers amplifying the complete genes at the five MLEE loci (unpublished data). These isolates were not used in the current study, but the available sequences for each locus were aligned and primers were designed targeting conserved regions. The annealing temperature of the primers was 45 °C for whole genes, and 50 °C for shorter fragments. Primers used for all isolates are listed in Table 3.

PCR was performed under standard conditions in a 25  $\mu$ l reaction mixture generally with *Taq* DNA polymerase (Biotech International). Each PCR reaction set included a positive control represented by either *B. hyodysenteriae* B78<sup>T</sup> or WA1, or *B. pilosicoli* P43/6/76<sup>T</sup>, and a negative control (double-distilled water). The conditions of the PCR programme were 95 °C for 3 min, followed by 33 cycles at 95 °C for

30 s, 50 °C for 30 s and 72 °C for 1 min if the fragment was shorter than 600 bp, and 1.5 min for longer fragments. A final extension period of 7 min at 72 °C was included before cooling to 14 °C. For those isolates and strains that were not amplified under the above-described conditions, the annealing temperature was lowered gradually by 2–3 °C to an annealing temperature of 43 °C. *Pfu* DNA polymerase (Promega) was used for isolates that could not be amplified with *Taq* DNA polymerase. Amplification conditions with *Pfu* were 96 °C for 2 min and 20 s, followed by 33 cycles of 96 °C for

**Table 3.** Primers used for MLST of *Brachyspira* species

Gene	Primer designation*	PCR and sequencing primers (5'→3')
<i>adh</i>	ADH-F206	GAAGTTTAGTAAAAGACTTTAAACC
	ADH-R757	CTGCTTCAGCAAAAAGTTTCAAC
	Bh-ADH-F1	ATGAAAGGATATGCTATGTTA
	Bh-ADH-R1059	TTTCTCAATTCTTACTACAGG
	Bp-ADH-F22	AAAATAGGTCAATCAGGTTG
	Bp-ADH-R1057	TTTCAATTCTTACTACAGGTTA
<i>alp</i>	ALP-F354	TCCAGATGAGGCTATACTTC
	ALP-R1262	TATGCTCTTTTGTCTAATATTG
	Bh-ALP-F24	TATAAAGATTGGAGAAAAATC
	Bh-ALP-R1617	TACCTCAGCAGGGAAATAC
	Bp-ALP-F26	TTTTATCCGCATTTTTTATC
	Bp-ALP-R1573	AAACCTGAGAAGGGAAATAC
<i>est</i>	EST-F229	GATGCTTCAGGCGGAGTTATG
	EST-R847	CCACACTCATAGCATAAATACTG
<i>gdh</i>	GDH-F514	GGAGTTGGTGCTAGAGAGAT
	GDH-R1157	ATCTCTAAAGCAGAAGTAGCA
<i>glp</i>	GLP-F123	AGGCTGGGTAGAACATAATGC
	GLP-R1158	TCTTTACTTTGATAAGCAATAGC
<i>pgm</i>	PGM-F172	GTTGGTACTAACAGAATGAATA
	PGM-R1220	CCGTCTTTATCGCGTACATT
<i>thi</i>	THI-F163	TGTGTTATAACAATCAGCACTTC
	THI-R1079	GTAGTAAGTATTCTAGCTCCAG

\*F, Forward; R, reverse.

30 s, 50 °C for 30 s and 72 °C for 3 min. The amplification was ended with an extension step of 72 °C for 5 min before cooling to 14 °C. The annealing temperature was regulated as described above for isolates that could not be amplified. For isolates for which only weak bands were obtained, the number of cycles was increased to 40. The PCR products were purified with the UltraClean PCR Clean-up kit according to the manufacturer's instructions (Mo Bio Laboratories). For weak bands, a QIAquick PCR Purification kit (Qiagen) was used to increase the amount of DNA in the eluate. For cycle sequencing, an annealing temperature of 43–45 °C was used with one-eighth of the amount of Big dye. Sequencing was performed with a 3730 DNA analyser (Applied Biosystems and Hitachi).

The sequences were edited and analysed manually by using NTI Vector 9.0 (<http://www.invitrogen.com>). The highest peak was consistently selected for further analysis. Allelic numbers were assigned manually and a different allelic number was given if any nucleotide or amino acid differences were registered. Isolates with the same allelic numbers were assigned to the same ST or AAT. The core sequences for the MLST loci used for the *B. hyodysenteriae* strain WA1 and *B. pilosicoli* strain 95/1000 were deposited with GenBank under accession numbers EF488202–EF488215. Sequences of the MLST alleles for each locus were deposited at the PubMLST site at Oxford University (<http://pubmlst.org/>). Each DNA sequence was translated into amino acid sequences by using Vector NTI 10.0 (<http://www.invitrogen.com>). The nucleotide sequences for the seven genes of each isolate were concatenated in the order *adh*, *pgm*, *est*, *glp*, *gdh*, *thi* and *alp*. All sequences (66 isolates) were placed into a single FASTA formatted file and aligned by using CLUSTAL W [from EMBL-EBI, European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/>)]. The file with the aligned sequences was converted to the MEGA format (<http://ccg.murdoch.edu.au/tools/clustalw2mega/>). A phylogenetic tree for the aligned DNA sequences was constructed by using the 'number of difference' model and the neighbour-joining tree in MEGA version 2.0 (Kumar *et al.*, 2001). For the amino acid trees, the translated DNA sequences were aligned (CLUSTAL W) and a Poisson correction model was used to construct a neighbour-joining tree in MEGA. A population snapshot was obtained by using the program eBURST (<http://eburst.mlst.net>) by setting the group definition to 0/7 (Feil *et al.*, 2004), assigning a zero for loci without sequence data.

## RESULTS

### 16S rDNA sequence analysis

A phylogenetic tree based on almost complete 16S rDNA sequences of the 66 strains and isolates is shown in Fig. 1. Despite having been repeatedly subcultured, isolate AN652/02 was shown to be mixed as the 16S rDNA sequence contained ten ambiguities. However, none of the nucleotides of the ambiguities could be identified as representing any recognized *Brachyspira* species other than *B. pilosicoli* (data not shown).

### MLST analysis

In MLST, the *mut* gene was excluded from the analysis due to poor sequence quality and the presence of too many ambiguous positions. All the isolates were successfully sequenced at two loci (*adh* and *pgm*), and 44 isolates were successfully sequenced at seven loci, however with a few ambiguities (superimposed peaks in the raw data) in the

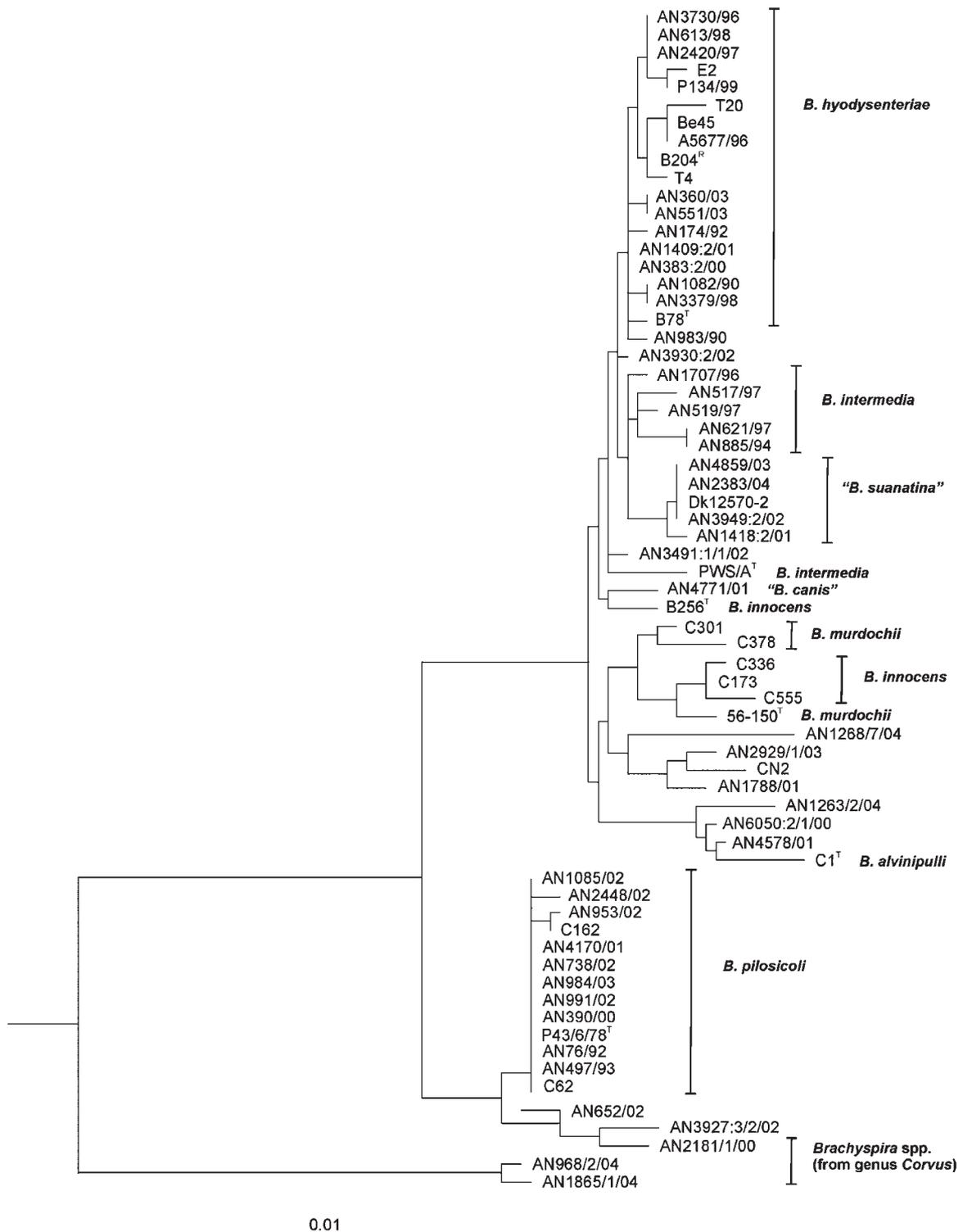
sequences of the type strains of *B. intermedia* (PWS/A<sup>T</sup>), *B. innocens* (B256<sup>T</sup>) and *B. alvinipulli* (C1<sup>T</sup>), and a field isolate of *B. pilosicoli* (AN652/02). The different combinations of the seven genes that were successfully sequenced for the remaining isolates are shown in Table 4. It was still not possible to amplify the loci in the 10 isolates examined after extraction and purification of DNA by other methods. The gene-sequence lengths after editing were 492, 783–788, 569–587, 585–588, 793–797, 909–913 and 810–822, for the *adh*, *alp*, *est*, *gdh*, *glp*, *pgm* and *thi* genes, respectively. For the *adh* gene, primers for the whole gene were used to obtain sequences for the type strains of *B. alvinipulli* (C1<sup>T</sup>) and *B. innocens* (B256<sup>T</sup>).

Deletions/insertions of segments of 2–92 nt were found in the sequenced fragment of the *est* gene. The deletion/insertion patterns showed intra-species similarities. The *alp* and *glp* genes had 7–10 point mutations/insertions/deletions, respectively. The sequences for the *glp* genes were sometimes of poor quality, mainly at the 3'-end of the fragment. In the translation to amino acids, the field isolate C378 showed a diverging 3'-end of 102 amino acids in the sequence when compared to other *B. murdochii* isolates. The sequences from the three isolates recovered from corvids (birds of the genus *Corvus*) (Table 2) showed a 3 nt insertion in the *pgm* gene. The *B. pilosicoli* isolates and the '*B. canis*' isolate had a 3 nt deletion in the *gdh* gene. One tiamulin-resistant *B. pilosicoli* (AN984/03) could not be sequenced with the reverse primer for the *adh* and *est* genes, respectively, which resulted in sequenced fragments that were 21 and 20 nt shorter, respectively.

A dendrogram based on concatenated DNA sequences of *adh* and *pgm* (approx. 1400 nt) for all 66 isolates is shown in Fig. 2(a), and the equivalent dendrogram based on amino acid sequences (approx. 460 positions) is shown in Fig. 2(b). A dendrogram based on concatenated DNA sequences of the 44 isolates for which all seven MLST loci (up to 4953 nt) were successfully sequenced is shown in Fig. 3. A large distance was observed between the cluster embracing the field isolates of *B. murdochii*/*B. innocens* and the *B. hyodysenteriae* cluster, particularly relative to the differences in their 16S rRNA gene sequences (Fig. 1). Also, the type strains of *B. innocens* (B256<sup>T</sup>) and *B. intermedia* (PWS/A<sup>T</sup>) did not cluster with other members of their respective species in the MLST dendrograms or the 16S rDNA-based phylogenetic tree. For some of the atypical isolates there were discrepancies between their positions in the dendrograms based on 16S rRNA gene sequences and those based on MLST data.

### eBURST and RAPD analysis

An allelic assignment for each isolate is presented in Table 1. Allelic frequency ranged from 30 to 46 alleles per locus, with a mean of 38.9. In total, 58 STs were identified. For the amino acid sequences, an allelic range of between 14 and 47 was identified, with a mean of 32.6. The number of AATs identified (58) was the same as the number of STs.



**Fig. 1.** Phylogenetic tree based on almost complete sequences of 16S rDNA from the 66 *Brachyspira* strains/isolates used in this study. The negative similarity value for AN652/02 indicates that the sequence contains ambiguities. Isolates without species designation are either genotypically deviating species or have not been fully characterized to species level. The scale bar shows the distance equivalent to one substitution per 100 nucleotide positions, corresponding to approximately 14 substitutions in the sequenced gene fragment.

**Table 4.** PCR amplification and successful sequencing for *Brachyspira* isolates for which fewer than seven MLST loci sequences were obtained

The eighth locus, *mut*, was not used in the MLST analysis.

Species	Isolates	MLST genes						
		<i>adh</i>	<i>pgm</i>	<i>est</i>	<i>glp</i>	<i>gdh</i>	<i>thi</i>	<i>alp</i>
<i>B. pilosicoli</i>	AN738/02	+	+	+	+	+	-	+
	AN953/02	+	+	+	+	+	-	+
	AN991/02	+	+	+	+	+	-	+
<i>B. intermedia</i>	PWS/A <sup>T</sup>	+	+	+	-	+	+	+
<i>B. innocens</i>	C336	+	+	+	+	+	+	-
	C555	+	+	+	+	+	+	-
	C173	+	+	+	+	+	+	-
<i>B. murdochii</i>	C301	+	+	+	+	+	+	-
	C378	+	+	+	+	+	+	-
	56-150 <sup>T</sup>	+	+	+	+	+	+	-
' <i>B. canis</i> '	AN4771/01	+	+	+	+	+	+	-
<i>Brachyspira</i> spp.	AN4578/01	+	+	+	-	+	+	-
	AN1788/01	+	+	+	+	+	+	-
	CN2	+	+	+	+	+	+	-
	AN6050: 2/1/00	+ (449)*	+	+	+	+	+	-
	AN3927: 3/2/02	+	+	-	+	+	-	+
	AN3491: 1/1/02	+	+	+	+	+	+	-
	AN1263/2/04	+	+	+	-	-	+	-
	AN1268/7/04	+	+	+	+	-	-	+
	AN2181/1/00	+	+	-	-	-	-	+
	AN968/2/04	+ (472)*	+	-	+	-	-	+
	AN1865/1/04	+	+	-	+	-	-	-

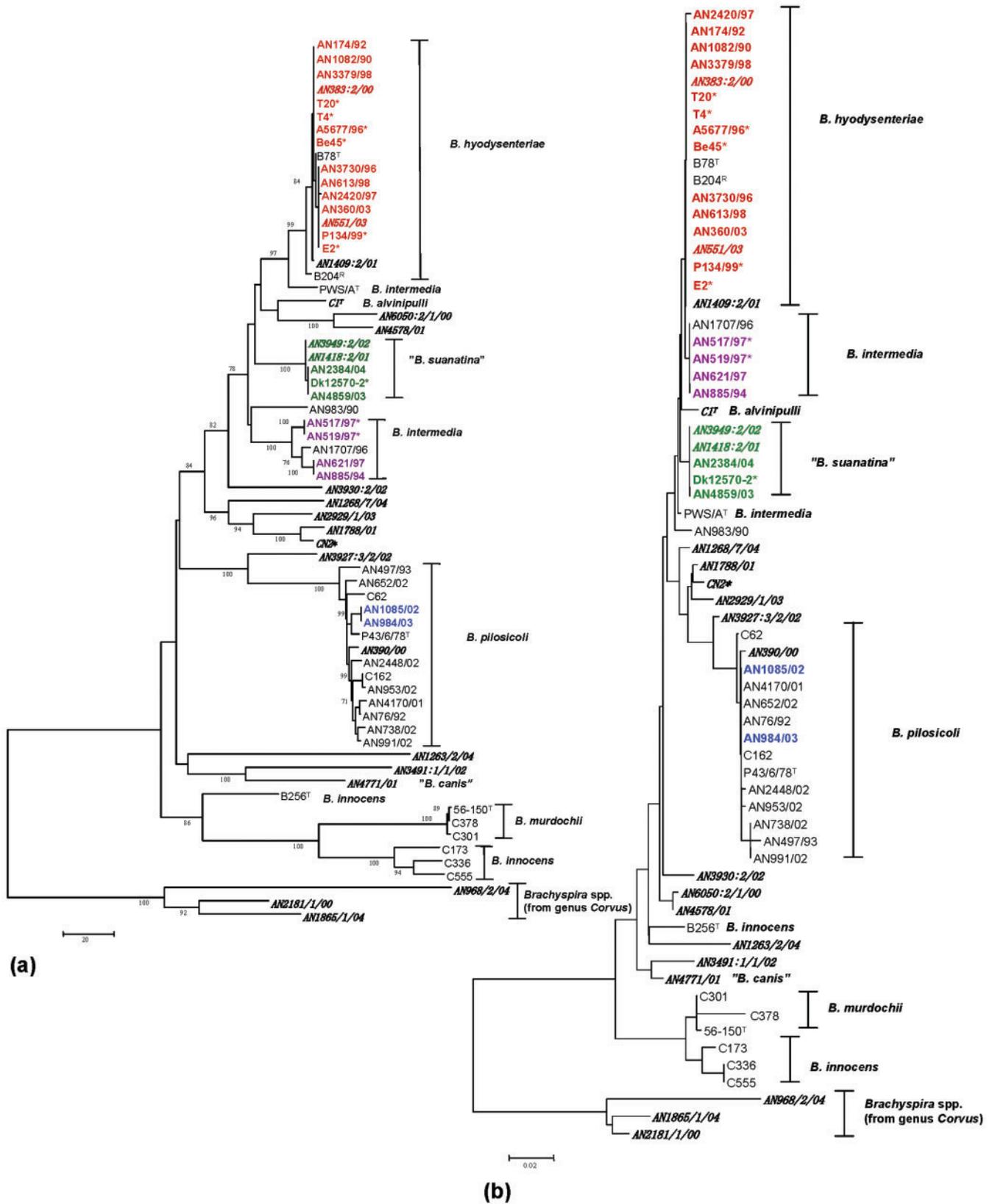
\*Length of sequence.

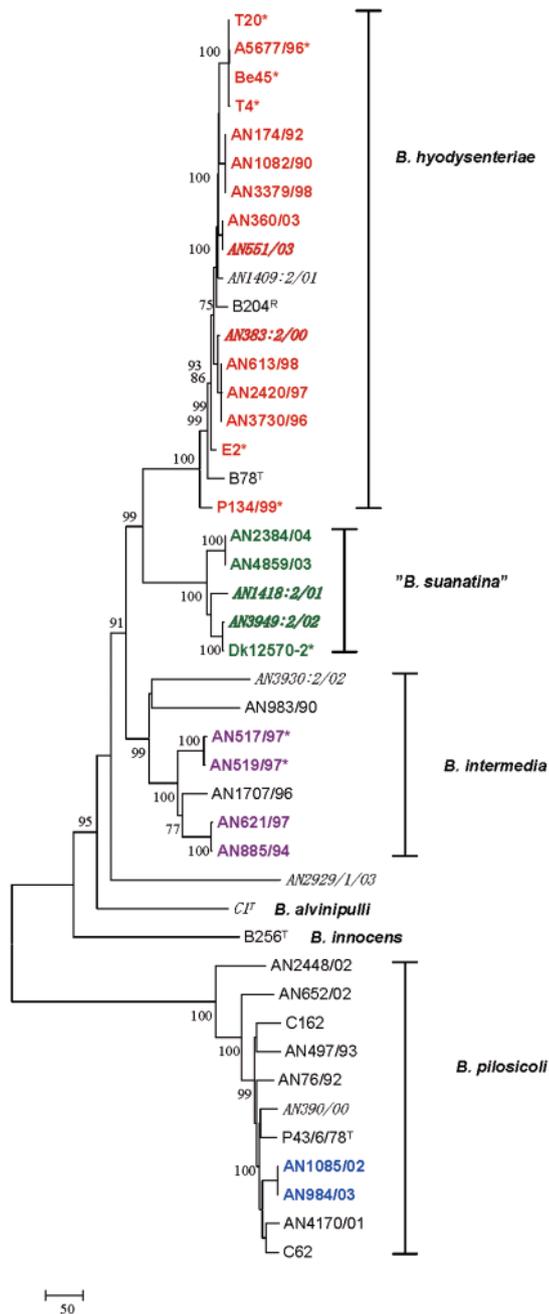
Population snapshots obtained by using STs and AATs are shown in Fig. 4. Isolates in the population snapshot that had a close evolutionary relationship originated from the same geographical areas. The results of RAPD analysis were concordant with those of MLST for isolates in the same or in closely related STs or AATs in the eBURST population snapshot, with one exception. Although the four indole-negative isolates of *B. hyodysenteriae* (T4, T20, Be45 and A5677/96) showed very similar banding patterns, two different patterns could be distinguished. The two German tiamulin-resistant isolates (T4 and T20) could be distinguished from the two tiamulin-susceptible isolates from Belgium and Germany (Be45 and A5677/96). For all other clones identified by eBURST and/or PFGE, the results agreed with those of RAPD analysis.

## DISCUSSION

Presently, phylogeny based on 16S rDNA is considered to be the gold standard for the classification of bacteria (Woese, 1987; Ludwig & Klenk, 2001), but this technique is not completely satisfactory for all species in the genus *Brachyspira* (Pettersson *et al.*, 1996; Stanton *et al.*, 1996). Here we describe an MLST system designed for the entire *Brachyspira* genus that was developed to help understand relationships between *Brachyspira* species and strains, as well as to provide an easy and reliable method for identification and classification. Most other published MLST schemes only cover a single species, and this emphasizes that it is difficult to cover an entire genus with the same sets of primers. Similar difficulties have been

**Fig. 2.** Dendrograms of the 66 *Brachyspira* isolates used in this study based on concatenated (a) DNA sequences, and (b) amino acid sequences obtained from two MLST loci (*adh* and *pgm*). Isolates without species designation are either genotypically deviating species or have not been fully characterized to species level. Isolates that were identified as being related by eBURST (Fig. 4) are indicated in red for *B. hyodysenteriae*, green for '*B. suanatina*', lilac for *B. intermedia* and blue for *B. pilosicoli*. Isolates recovered from sources other than pigs are indicated in italics, and field isolates originating from outside Sweden are marked with an asterisk. Bootstrap values are shown for stable nodes in Fig. 2(a). The scale bars show the distance of (a) 20 substitutions and (b) the distance equivalent to two substitutions per 100 amino acid positions, corresponding to approximately 9 substitutions in the sequenced gene fragment.





**Fig. 3.** Dendrogram based on concatenated DNA sequences from the 44 *Brachyspira* isolates with all seven MLST loci successfully sequenced. Isolates indicated in colours are identified as being related by eBURST (Fig. 4), italicized designations indicate isolates from sources other than pigs, and designations marked with an asterisk represents field isolates originating from outside Sweden. Bootstrap values are shown for stable nodes. The length of the scale bar is equivalent to 50 substitutions in the sequenced gene fragment.

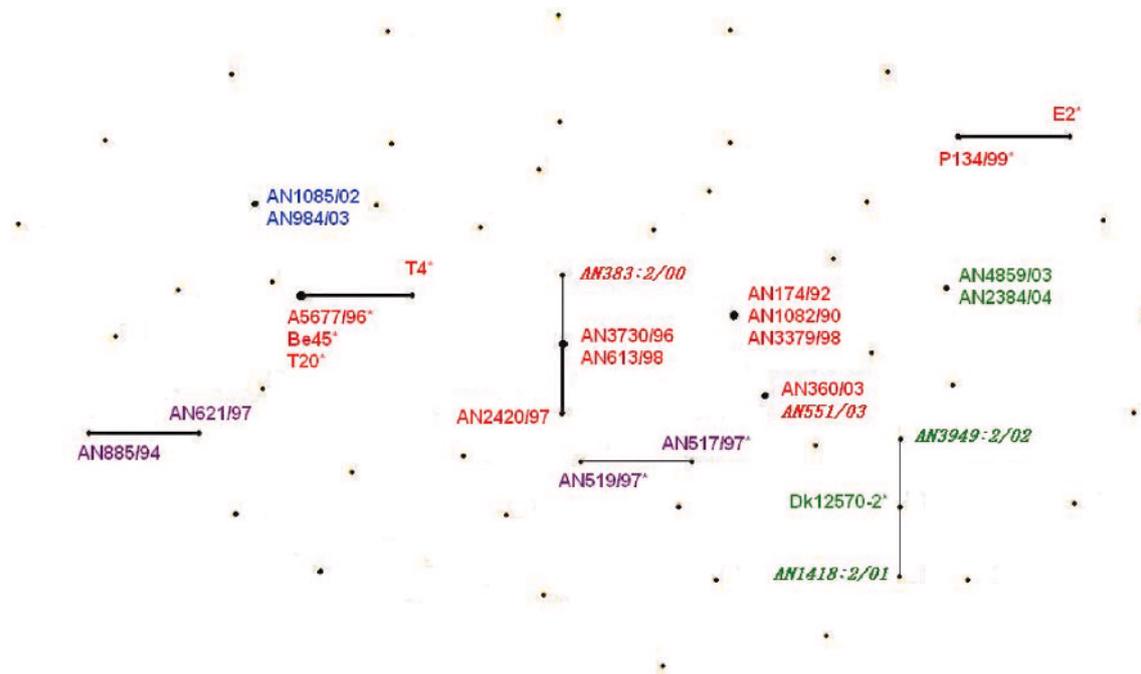
Two isolates of *B. intermedia* were separated from the main *B. intermedia* cluster, which is consistent with a previous study that indicated that there is considerable overall genetic diversity amongst isolates with the phenotype of *B. intermedia* (Suriyaarachchi *et al.*, 2000). Some atypical isolates could not be identified to a species level by MLST.

Phylogenetic trees or dendrograms are useful for obtaining an overview of evolutionary relations. However, dendrograms provide almost no information on the evolutionary descent of isolates within a clonal complex (Feil *et al.*, 2004). Therefore, eBURST analysis was used to identify isolates with an epidemiological connection and isolates with a closely related evolutionary history (Fig. 4). The results support the previous conclusion that MLST data can be useful for epidemiological studies (Urwin & Maiden, 2003), and that eBURST analysis gives a more accurate epidemiological identification for some of the isolates than the dendrograms (Figs 1, 2, 3, 4). Limitations of eBURST may arise from too high a degree of allelic variations observed in the DNA sequences. Nevertheless, the relationships identified in this study by eBURST are highly likely to be correct because they were supported by independent epidemiological data. eBURST analysis of MLST data has been claimed to show only a fifth of the evolutionary relationships present within a cluster in a dendrogram (Didelot & Falush, 2006). However, AATs instead of DNA STs might give a clearer representation of closely related isolates, which in turn will result in a more accurate picture of the evolutionary relationships in the bacterial population. In this study, constructing a dendrogram based on amino acid sequences substantially unified the respective major *Brachyspira* species (Fig. 2b). A close evolutionary connection between mallard and porcine isolates of *B. hyodysenteriae* and '*B. suanatina*' (Fig. 4; thin lines) was identified by eBURST analysis with the same data. The epidemiological relationship between mallard and porcine isolates was strengthened by their similarity in RAPD banding patterns, and the known migration patterns of wild mallards (Fransson & Pettersson, 2001) that enhance opportunities for transmission to farmed pigs.

In conclusion, with few exceptions each of the *Brachyspira* species clustered separately in the MLST dendrograms, and the majority of the isolates pathogenic to pigs could be delineated and defined. This observation demonstrates the utility of the MLST scheme that was developed, although it could be improved further. A high level of genetic variability was observed amongst members of the genus.

reported with other MLST systems targeting multiple species (Miller *et al.*, 2005).

The clusters obtained in the MLST dendrograms (Figs 2 and 3) were generally in concordance with the identification of *Brachyspira* species based on culture and biochemical tests. This points to the potential of MLST as a tool for establishing the species affiliation and differentiation of *Brachyspira* strains, as previously described for other MLST systems (Diavatopoulos *et al.*, 2005; Ventura *et al.*, 2006). The sequence ambiguities observed could be associated with repeated gene copies, which previously have been suggested to occur in *B. hyodysenteriae* (Zuerner & Stanton, 1994).



**Fig. 4.** Population snapshot of 66 *Brachyspira* isolates obtained by using eBURST. The data were derived from the allelic variation in seven MLST loci. Isolates identical in six of seven loci were identified as closely related in evolutionary descent, which is represented with a line between the relevant isolates. Isolate designations are indicated in red for *B. hyodysenteriae*, in green for '*B. suanatina*', in lilac for *B. intermedia* and in blue for *B. pilosicoli*. Isolates recovered from sources other than pigs are indicated in italics, and field isolates originating from outside Sweden are marked with an asterisk (compare with Figs 2 and 3). The thick lines represent results obtained from DNA STs and AATs. Thin lines represent results obtained from AATs only. The positions and distance between the dots are incidental and do not include additional information. Missing sequence data or genes that were too variable limited the identification of closely related isolates; only the 44 isolates with complete sequence data for all loci can be connected if an epidemiological or evolutionary link has been predicted. Dots without isolate designations represent single isolates.

The MLST data were also shown to be useful for molecular epidemiological studies, and in particular the eBURST analysis was shown to be more discriminatory than the use of dendrograms for determining relationships. The use of amino acid sequence data revealed evolutionary connections between isolates within the same geographical area and between isolates from pigs and mallards.

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