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Highlights

- We have examined the diversity of flagellin genes of *B. bronchiseptica* strains
- The PCR-RFLP revealed eight *fla*A types, the sequence analysis showed four clusters
- All but one *B. bronchiseptica* strains from swine showed type B fragment pattern
- The Hungarian isolates of canine origin were uniform (type A)
- The diversity of strains from humans indicated the zoonotic impact of *B. bronchiseptica*

Flagellin typing of *Bordetella bronchiseptica* strains originating from different host species

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Short title: *fla*A typing of *B. bronchiseptica*

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Abstract

Bordetella bronchiseptica is a widespread Gram-negative pathogen occurring in different mammal species. It is known to play a role in the aetiology of infectious atrophic rhinitis of swine, canine kennel cough, respiratory syndromes of cats, rabbits and guinea pigs, and sporadic human cases have also been reported. In this study, ninety-three *B. bronchiseptica* strains were examined from a broad range of host species and different geographical regions using restriction fragment length polymorphism analysis of polymerase chain reaction products of *fla*A to reveal the possible host-specificity of the flagellin. Eight types (A-H) of *fla*A were identified, including five newly described ones (D-H). All but one of the twenty-two *B. bronchiseptica* strains from swine showed type B fragment pattern. The eighteen Hungarian isolates of canine origin were uniform (type A) while in other countries type B and D were also present in dogs. The sequence and phylogenetic analysis of 36 representative strains of *fla*A types revealed four clusters. These clusters correlated with *fla*A PCR-RFLP types and host species, especially in pigs and dogs. The revealed diversity of the strains isolated from human cases indicated possible zoonotic transmissions from various animal sources.

Keywords: *B. bronchiseptica*, *fla*A, PCR-RFLP, sequence and phylogenetic analysis

1. Introduction

Bordetella bronchiseptica is a common inhabitant of the respiratory tract of several animal species (pig, dog, cat, rabbit, guinea pig, horse). Basically, it is considered a veterinary pathogen; however, an increasing number of human infections have also been reported, mostly in immunocompromised patients (Goodnow, 1980; Wernli et al., 2011). *B. bronchiseptica* is a close relative of *B. pertussis* and *B. parapertussis* (Parkhill et al., 2003), but the only one with wide host range. These classical *Bordetellae* show some variation in the characteristics of their virulence determinants (adhesins, toxins) that may reflect to their host preference (Matoo and Cherry, 2005).

The expression of *Bordetella* virulence determinants is controlled by a two-component signal transduction system (BvgAS) that follows environmental changes by phase variation between virulent (bvg+) and avirulent (bvg−) phases. The bvg− phase is supposed to be important for the survival of the bacterium in the environment (Cotter and Miller, 1994).

Motility, ensured by peritrichous, multistranded, 18 to 22 nm thick flagella (Richter and Kress, 1967), is characteristic for the bvg− phase of *B. bronchiseptica* that seems crucial to reach a susceptible host and adhere to the target cells (Smyth, 1988; Savelkoul et al., 1996). Furthermore, flagella may have a role in additional microbial processes, such as the induction of proinflammatory mediators (López-Boado et al., 2005). Nicholson et al. (2012) demonstrated that flagella were necessary for initiating and enhancing the bacterium − cell-surface interaction. Therefore, flagella may have the potential to make a distinction between the various host species. In *B. bronchiseptica*, the bvgAS locus negatively controls the synthesis of flagella (Akerley et al., 1992). Flagellin, encoded by *fla*A, is a subunit protein, which polymerizes to form the filaments of bacterial flagella. Passerini de Rossi et al. (1997) proposed to use flagellin as a marker of the avirulent (bvg−) phenotype of *B. bronchiseptica*.

Because of their role in attachment, flagella may have the potential to recognise suitable hosts. Winstanley et al. (2001) analysed the *fla*A gene of thirty *B. bronchiseptica* strains, mostly obtained from cats, with polymerase chain reaction-restriction fragment length analysis (PCR-RFLP), and divided the isolates into three groups (A, B and C) using *Hae*III, *Msp*I, *Mbo*I and *Rsa*I restriction enzymes. Friedman et al. (2006) confirmed the existence of these *fla*A types by testing *B. bronchiseptica* strains from different hosts.

In the current study, we examined a larger number of *B. bronchiseptica* strains that represented a broad range of host species and different geographical regions using PCR-RFLP and sequence analysis of *fla*A to reveal the possible host-specificity of the flagellin.

2. Materials and Methods

2.1. Bacterial strains, culture conditions and biochemical tests

We used 93 strains of *B. bronchiseptica* obtained from different hosts and geographical areas (Table 1). Fifty-three strains were isolated in Hungary during a period of 30 years while forty strains were obtained from worldwide collections. The strains were cultivated on Columbia agar (LabM, Bury, United Kingdom) supplemented with 5% sheep blood and incubated under aerobic conditions at 37 °C for 24 hours. Primary identification was performed by conventional biochemical tests (oxidase, catalase, urease, nitrate and indole reactions, and utilisation of glucose, lactose and sucrose). The strains were stored in sterile skim milk (LabM, Bury, United Kingdom) at -70 °C.

2.2 DNA extraction and PCR

Bacterial colonies from pure cultures were suspended in nuclease-free water (50 µl), and boiled for 10 min to obtain DNA. The bacterial lysates were centrifuged at 12,000g for 2 min and aliquots of 1 µl of the supernatant was used as template DNA in the PCRs.

The species-specific PCR was carried out as described previously (Hozbor et al., 1999). Amplifications were performed in a reaction mixture (25 µl final volume) containing 2.5 µl of 10× DreamTaq buffer (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA), 1.2 µl from 25 mM of $MgCl_2$, 200 µM of deoxynucleoside triphosphates, 15 pmol of forward and reverse primers (Sigma-GenoSys, Steinheim, Germany), 2 µl of dimethyl-sulphoxide and 1U of DreamTaq polymerase (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). *B. bronchiseptica* strains were typed using a pair of specific primers as described by Winstanley et al. (2001) and Friedman et al. (2006) to produce about an 1165 bp fragment of the *fla*A gene. Amplifications were performed in an Esco Swift Mini thermal cycler. The PCR products were analysed on a 1.5% agarose gel (SeaKem, Lonza, Basel, Switzerland) stained with GelRed (Biotium Inc., Hayward, USA) using standard procedures. The DNA fragments were visualised by UV illumination.

2.3. Restriction fragment length analysis

PCR amplicons of *fla*A (5 µl) were digested with the restriction enzymes *Msp*I, *Hinc*II and *Bgl*I (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) in three separate reactions according to the manufacturer's instructions incubated overnight at 37 °C. The enzyme-digested products were analysed by electrophoresis using 2.5% Metaphore agarose (Lonza, Basel, Switzerland) in 1× Tris borate EDTA (TBE) buffer, visualized with ethidium bromide (0.5 µg/ml) staining using UV light. The size of the restriction fragments was assigned by comparison with 100 bp DNA ladder (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA).

2.4. Sequence and phylogenetic analysis

The partial nucleotide sequences of the *fla*A genes (according to reference sequence RB50 between positions 61 bp and 1111 bp) were determined from amplicons in both directions using commercial sequencing facilities (Macrogen, Amsterdam, The Netherlands). The sequence data were analysed by the BioEdit Sequence Alignment Editor software (v. 7.1.3.0; Hall, 1999). Nucleotide and amino acid sequence identities were calculated by the pairwise distance algorithm (p-distance) with the MEGA version 6 software (Tamura et al., 2013). The multiple alignments of amino acid sequences were performed using the BioEdit CLUSTALW algorithm with BLOSUM protein weight matrix (Thompson et al., 1994). The phylogenetic analysis was conducted in MEGA6 (Tamura et al., 2013), the evolutionary history was inferred using the Neighbor-Joining method, and the evolutionary distances were computed using the Jukes-Cantor method. The analysis involved 45 nucleotide sequences (36 sequences in this study and 9 reference sequences from the GenBank). All positions containing gaps and missing data were eliminated. There were a total of 1042 positions in the final dataset. The GenBank accession numbers for the sequences reported in this paper are JX673952-JX673981 and KF211396-KF211401.

3. Results

3.1. Biochemical characterisation

All strains were catalase-, oxidase- and urease positive, negative in the indole reaction, and did not utilise the tested carbohydrates. On the other hand, the nitrate reduction profiles of the strains were variable, 9.7% of the strains (5 strains from pigs, 2 strains from dogs and a strain from guinea pig and rabbit) did not reduce nitrate to nitrite.

3.2. PCR-RFLP analysis

All strains produced a 237 bp band by species-specific PCR and about an 1165 bp product by *fla*A PCR, and the latter was analysed with three different restriction enzymes (*Msp*I, *Hinc*II and *Bgl*I). The size of the *Msp*I-digested PCR-RFLP fragments varied from 20 to 750 bp, the *Bgl*I-digested ones from 20 to 615 bp, and the *Hinc*II-digested ones from 25 to 790 bp (Figure 1). The smallest differentiation power of the RFLP patterns was observed with *Msp*I yielding only four different patterns, while digestion with *Hinc*II and *Bgl*I enzymes resulted 5 and 6 different RFLP patterns, respectively. The *fla*A RFLP types were generated from the combination of the results of the RFLP analysis, and thus eight different profiles were established among the 93 *B. bronchiseptica* strains designated A through H (Table 1). The most common profile was type A (41.9%), followed by type B (35.5%) and type C (12.9%). On the other hand, *fla*A type D, E and H did not occur among the Hungarian strains (Table 1). Type A, D and F showed identical patterns with *Msp*I, while type D and F belonged to the same RFLP group with *Bgl*I. Type C, E and H had similar patterns with *Hinc*II and *Msp*I restriction enzymes (Figure 1).

Three *B. bronchiseptica* strains isolated from human cases of various geographic origins and a strain from a dog showed type D flagellin profile. Only two strains, one from a man and one from a pig, represented type G. Three unique profiles (E, F, and H) were found in *B. bronchiseptica* strains of human (F, H) and turkey (E) origin. All but one *B. bronchiseptica* strains isolated from swine showed type B fragment patterns. The only exception (PV6) belonged to type G. In canine *B. bronchiseptica* strains three *fla*A types were found. The Hungarian isolates were uniform (type A) while in other countries type B and D were also present. All strains from guinea pig and koala belonged to group C, and strains from horse belonged to group A. Type A and type B profiles were present in equal proportion in strains isolated from rabbits.

3.3. Sequence and phylogenetic analysis

The multiple-sequence alignment of 45 nucleotide sequences (representing approximately 90% of *fla*A), including 36 *B. bronchiseptica fla*A partial sequences from this study and nine published *fla*A sequences from the GenBank (253 (dog): HE965806; RB50 (rabbit): BX470250; D445 (human): HE983627; Bbr77 (human): HE983628; MO149 (human): HE965807; 1289 (monkey): HE983626; AF232939-AF232941: Winstanley et al., 2001) showed that these sequences have two conserved regions in the N-terminal and C-terminal portions, whereas the central region is considerably variable and shows nucleotide substitutions, deletions and insertions. In the variable region, the majority of the nucleic acid substitutions (data not shown) resulted in amino acid change, indicating that most of the nucleotide changes were non-synonymous. The phylogenetic tree (Figure 2) based on evolutionary distances contains four distinct clusters; the genetic distances between clusters are listed in Table 2. The maximum pairwise genetic distance of nucleotide sequences (14.6%) was observed between strains MBORD 707 (turkey) and PV6 (pig), MBORD 901 (turkey), 5390 (human). The maximum pairwise genetic distance of deduced amino acid sequences (20.4%) was observed between strain MBORD 707 (turkey) and the members of cluster 1a.

Cluster 1a was composed of *B. bronchiseptica* strains carrying type A *fla*A alleles, and representing different host species (Figure 2). Cluster 1b contained *B. bronchiseptica* strains having *fla*A type D and F, and originated mostly from humans. Cluster 2 strains belonged to *fla*A type B, and originated mostly from pigs. Cluster 3 comprised strains of *fla*A type C isolated from different hosts.

The strains belonging to cluster 1a and 1b contained only one amino acid deletion at position 132 (Figure 3), while strains from cluster 2 and 3 possessed three amino acid deletions. Cluster 2 strains showed deletions at positions 109, 168-169, while cluster 3 strains lacked amino acids at positions 109, 132 and 175 (Figure 3).

The *B. bronchiseptica* strains of human origin proved to be quite heterogeneous by the PCR-RFLP analysis of the flagellin gene: five types (A, C, D, F and H) occurred among the seven strains. The most frequently detected type was D (57%). The pairwise genetic distance between the nucleotide sequences of human strains ranged from 0.0% to 13.8%, and between deduced amino acid sequences ranged from 0.0% to 19.6%. The phylogenetic tree showed that the human isolates belonged into three distinct lineages (Figure 2).

4. Discussion and conclusions

In this study, we examined ninety-three *B. bronchiseptica* strains with *fla*A PCR-RFLP and sequence analysis. We could improve the discriminative potential of *fla*A PCR-RFLP analysis by the combined use of *Msp*I (Winstanley et al., 2001), *Hinc*II and *Bgl*I (Friedman et al., 2006) enzymes that led to the distinction of eight different *fla*A types. The most common types were A, B and C that is in agreement with the formerly described analysis of isolates from cats (Winstanley et al., 2001) and other host species (pig, rabbit, dog, cat and human) (Friedman et al., 2006). The newly established types originated from dog (type D), turkey (type E and G), human (type F and H) and pig (type G). Regarding the isolates from Hungary, the most common profiles were also type A (47.2%), B (41.5%) and C (9.4%). On the other hand, the distribution of the *fla*A types showed some correlation with the host species. Our isolates from dogs were uniformly type A, while strains isolated from dogs in other countries showed variability (type A, B and D). In pigs, type B proved to be dominant. The only exception was *B. bronchiseptica* PV6 that belonged to type G. This strain, however, was isolated from a freshly contaminated SPF herd in Hungary, and the source of infection remained unknown. *B. bronchiseptica* PV6 is a non-DNT-producing strain (Magyar et al.,

1988) with a highly unusual *Pvu*II ribotype, unique pertactin and filamentous hemagglutinin types not typical for other isolates from swine (Brockmeier and Register, 2007). The similarity between PV6 and MBORD 901 (turkey) with *fla*A PCR-RFLP and sequence analysis confirmed that *B. bronchiseptica* PV6 is a strain unusual in pigs, and the source of infection must have been another host or carrier species. However, our findings differ from the data reported by Friedman et al. (2006) who found three types (type A, B and C) among strains from dogs and two types (type B and C) among strains from pigs. Previously, *B. bronchiseptica* strains from Hungary and from other countries were ribotyped to five clusters (Register and Magyar, 1999). Most of the strains belonged to cluster "I" which included seven ribotypes. The majority of the strains from pigs belonged to ribotype 3, while strains from other host species represented various other types (Register and Magyar, 1999). Ribotyping is based upon analysis of the ribosomal genes, which are highly conserved within different bacterial species, whereas PCR-RFLP focuses on restriction enzyme cleavage sites of an amplified region (*fla*A). The porcine strains represented uniform types by both methods that may indicate some relationship between the host (pig) and the genotype, at least in a certain geographical region (Hungary).

Our results suggested that *fla*A PCR-RFLP type C is most prevalent in strains from guinea pig, cat and koala although the numbers of strains from these species were rather limited to draw far-reaching conclusions in this respect. Furthermore, we demonstrated the *B. bronchiseptica* strains from rabbits belonged to type A or B, nearly in equal proportions. These observations were consistent with the data previously reported by Friedman et al. (2006). High degree of variation (type A, C, D, F and H) occurred among the strains of human origin. Although *B. bronchiseptica* is primarily a veterinary pathogen, it occurs occasionally in humans as well, typically causing respiratory infections in young, elderly or immunocompromised patients (Mattoo and Cherry, 2005). The recovery of *B. bronchiseptica* from humans is well-documented (Wernli et al., 2011; Register et al., 2012). In some cases, the zoonotic transmission could be traced (Guierard et al., 1995). The high divergence we found within *B. bronchiseptica* strains from human cases reflected the overall diversity of the strains from various animal species. This finding strengthens the zoonotic importance of *B. bronchiseptica* indicating that humans can be infected from a wide range of animal sources rather than having own type or types of *B. bronchiseptica*.

The sequence analysis of *fla*A genes of *B. bronchiseptica* strains with different PCR-RFLP profiles showed that the N-terminal and C-terminal regions, which are responsible for secretion and polymerization, are highly conserved, whereas the central region is greatly variable. Other bacterial species, e.g. *Salmonella* spp., *Campylobacter* spp., *Heliobacter pylori* also have this variability in the central region of the flagellin gene (Winstanley and Morgan, 1997). The phylogenetic tree based on partial *fla*A sequences contains four distinct clusters (Figure 2). The *fla*A sequences of *B. bronchiseptica* strains of cluster 1a show a closer proximity with the *fla*A genes of cluster 1b than with that of cluster 2 or 3.

Winstanley et al. (2001) revealed the genetic distance between their three *fla*A groups from 11% to 13%, whereas our results suggested genetic distance between the four clusters from 2.5% to 14.6% based on pairwise alignment of nucleic acid sequences and from 2.3% to 20.4% on pairwise alignment of amino acid sequences. The clusters of the phylogenetic tree correlate with the *fla*A PCR-RFLP types; cluster 1a includes type A strains, while cluster 2 involves type B strains. Clusters 1b and 3 are more heterogeneous since the newly described *fla*A PCR-RFLP types appear here. The structure of the phylogenetic tree shows a correlation between clusters and host species, just as the *fla*A PCR-RFLP types do. Remarkably, the pig strains are located on a distinct branch (cluster 2) and this branch is the only one not containing strains from man. Alignment of deduced amino acid sequences between the four clusters indicates a clonal population structure of *B. bronchiseptica*, as suggested earlier by

Musser et al. (1987). In the central region of *fla*A, the rapid accumulation of point mutations and/or recombination events may result in variations in the amino acid sequences. It requires additional molecular evolutionary analyses to evaluate the strength of positive selection by determining heterogeneity of the *fla*A central region.

In conclusion, by using the *fla*A RFLP-PCR and sequence analysis, we have demonstrated that the *B. bronchiseptica* strains show high *fla*A diversity, mainly in the central region. The observed PCR-RFLP types of *fla*A show correlation with the host species, especially in pigs and dogs. The revealed diversity of the strains of human origin indicates possible zoonotic transmissions from various animal sources. The *fla*A RFLP technique may be a useful epidemiological marker for *B. bronchiseptica*.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with people or organisations that could inappropriately influence or bias the content of this study.

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Figure captions

Figure 1.:

RFLP patterns of *fla*A obtained by *Msp*I, *Hinc*II or *Bgl*I. The *fla*A types were generated from the combination of RFLP profiles establishing eight different types as shown in Table 1. (A: 1-1-1, B: 2-2-2, C: 3-3-3, D: 1-4-4, E: 3-3-5, F: 1-5-4, G: 4-5-4, H: 3-3-6). Lane numbers indicate the unique RFLP patterns produced by the corresponding restriction endonuclease. M: GeneRuler 100 bp DNA Ladder (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA)

Figure 2.:

Phylogenetic tree based on partial nucleotide sequence (1042 bp) of *fla*A gene of 45 *B. bronchiseptica* strains. 36 sequences were listed in this study, and 9 sequences demonstrated reference strains from GenBank (253 (dog): HE965806, RB50 (rabbit): BX470250, D445 (human): HE983627, Bbr77 (human): HE983628, MO149 (human): HE965807, 1289 (monkey): HE983626, AF232939-AF232941: Winstanley et al., 2001). The optimal tree with the sum of branch length = 0.23043532 is shown. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

*: strains from cats obtained from GenBank (Winstanley et al., 2001).

-: reference sequences from Genbank

Figure 3.:

Multiple alignment based on deduced amino acid sequences of *fla*A of 45 *B. bronchsieptica* strains. 36 sequences were listed in this study, and 9 sequences demonstrated reference strains from GenBank (253 (dog): HE965806; RB50 (rabbit): BX470250; D445 (human): HE983627; Bbr77 (human): HE983628; MO149 (human): HE965807; 1289 (monkey): HE983626; AF232939-AF232941: Winstanley et al., 2001). The figure shows only the variable region, according to reference sequences between amino acid residues 121 and 321. Dashes indicate gaps, and dots indicate identity.

Table 1.

List of *B. bronchiseptica* strains analysed in this study. Strains written in bold were sequenced. The GenBank accession numbers for the sequences reported in this paper are JX673952-JX673981 and KF211396-KF211401.

Strain	Host	Country of origin	Year of isolation	Type of RFLP patterns			*fla*A type
				*Msp*I	*Hinc*II	*Bgl*I	
5339	Dog	Hungary	2005	1	1	1	A
5340	Dog	Hungary	2005	1	1	1	A
5347	Dog	Hungary	2006	1	1	1	A
5348	Dog	Hungary	2006	1	1	1	A
5362	Dog	Hungary	2006	1	1	1	A
5460	Dog	Hungary	2007	1	1	1	A
5462	Dog	Hungary	2007	1	1	1	A
5533	Dog	Hungary	2009	1	1	1	A
5534	Dog	Hungary	2009	1	1	1	A
5587	Dog	Hungary	2009	1	1	1	A
5593	Dog	Hungary	2009	1	1	1	A
5605	Dog	Hungary	2010	1	1	1	A
5625	Dog	Hungary	2010	1	1	1	A
5626	Dog	Hungary	2010	1	1	1	A
5628	Dog	Hungary	2009	1	1	1	A
5629	Dog	Hungary	2008	1	1	1	A
5639	Dog	Hungary	2005	1	1	1	A
Bö/11	Dog	Hungary	2004	1	1	1	A
Bb-11	Dog	United Kingdom	unknown	2	2	2	B
Bb 335	Dog	United Kingdom	unknown	1	1	1	A
MBORD 591	Dog	United States	unknown	1	4	4	D
MBORD 685	Dog	United States	unknown	2	2	2	B
MBORD 750	Dog	Denmark	unknown	1	1	1	A
MBORD 787	Dog	The Netherlands	unknown	1	1	1	A
MBORD 843	Dog	Switzerland	unknown	1	1	1	A
NCTC 452	Dog	United States	1910s	1	1	1	A
5240	Pig	Hungary	1996	2	2	2	B
5269	Pig	Hungary	2003	2	2	2	B
5323	Pig	Hungary	2005	2	2	2	B
5356	Pig	Hungary	2006	2	2	2	B
5463	Pig	Hungary	2007	2	2	2	B
5493	Pig	Hungary	2008	2	2	2	B
5500	Pig	Hungary	2008	2	2	2	B
5505	Pig	Hungary	2008	2	2	2	B
5594	Pig	Hungary	2009	2	2	2	B
B 58	Pig	Hungary	1988	2	2	2	B
CE	Pig	Hungary	1985	2	2	2	B
KM22	Pig	Hungary	1993	2	2	2	B
PV6	Pig	Hungary	1983	4	5	4	G

Table 1. (continued 1)

Strain	Host	Country of origin	Year of isolation	Type of RFLP patterns *Msp*I	*Hinc*II	*Bgl*I	*fla*A type
4609	Pig	United States	unknown	2	2	2	B
5599	Minipig	Denmark	2010	2	2	2	B
Bb-12	Pig	United Kingdom	unknown	2	2	2	B
Bg1	Pig	United Kingdom	unknown	2	2	2	B
BOXTEL	Pig	The Netherlands	unknown	2	2	2	B
DAN	Pig	Denmark	1999	2	2	2	B
GF 8	Pig	United Kingdom	unknown	2	2	2	B
IM 5	Pig	United Kingdom	unknown	2	2	2	B
MBORD 676	Pig	Australia	unknown	2	2	2	B
5008	Rabbit	Hungary	1988	1	1	1	A
5024	Rabbit	Hungary	1988	2	2	2	B
5122	Rabbit	Hungary	1990	2	2	2	B
5308	Rabbit	Hungary	2005	1	1	1	A
5601	Rabbit	Hungary	2010	2	2	2	B
5602	Rabbit	Hungary	2010	1	1	1	A
5612	Rabbit	Hungary	2010	2	2	2	B
5614	Rabbit	Hungary	2010	1	1	1	A
5622	Rabbit	Hungary	2010	1	1	1	A
5630	Rabbit	Hungary	2007	1	1	1	A
5631	Rabbit	Hungary	2006	2	2	2	B
5633	Rabbit	Hungary	2006	2	2	2	B
5636	Rabbit	Hungary	2006	2	2	2	B
5648	Rabbit	Hungary	2011	1	1	1	A
5653	Rabbit	Hungary	2011	2	2	2	B
RB 4032	Rabbit	Hungary	1984	2	2	2	B
Bb 9.73	Rabbit	France	unknown	1	1	1	A
Bb LC 2	Rabbit	United Kingdom	unknown	1	1	1	A
MBORD 704	Rabbit	United States	unknown	2	2	2	B
MBORD 730	Rabbit	Denmark	unknown	1	1	1	A
5491	Guinea pig	Hungary	2008	3	3	3	C
5495	Guinea pig	Hungary	2008	3	3	3	C
5497	Guinea pig	Hungary	2008	3	3	3	C
MBORD 669	Guinea pig	United States	unknown	3	3	3	C
MBORD 762	Guinea pig	Ireland	unknown	3	3	3	C
NCTC 8750	Guinea pig	United Kingdom	1950	3	3	3	C
BbCVI	Horse	United Kingdom	unknown	1	1	1	A
Bb-CV-2	Horse	United Kingdom	unknown	1	1	1	A
MBORD 628	Horse	United States	unknown	1	1	1	A
MBORD 898	Horse	Germany	unknown	1	1	1	A
M9	Cat	Hungary	1994	3	3	3	C
M48	Cat	Hungary	1994	3	3	3	C
MBORD 635	Cat	United States	unknown	3	3	3	C
MBORD 970	Cat	The Netherlands	unknown	1	1	1	A
MBORD 707	Turkey	United States	unknown	3	3	5	E
MBORD 901	Turkey	Germany	unknown	4	5	4	G
MBORD 681	Koala	Australia	unknown	3	3	3	C
MBORD 698	Koala	Australia	unknown	3	3	3	C
5390	Human	Hungary	2007	1	5	4	F

Table 1 (continued 2)

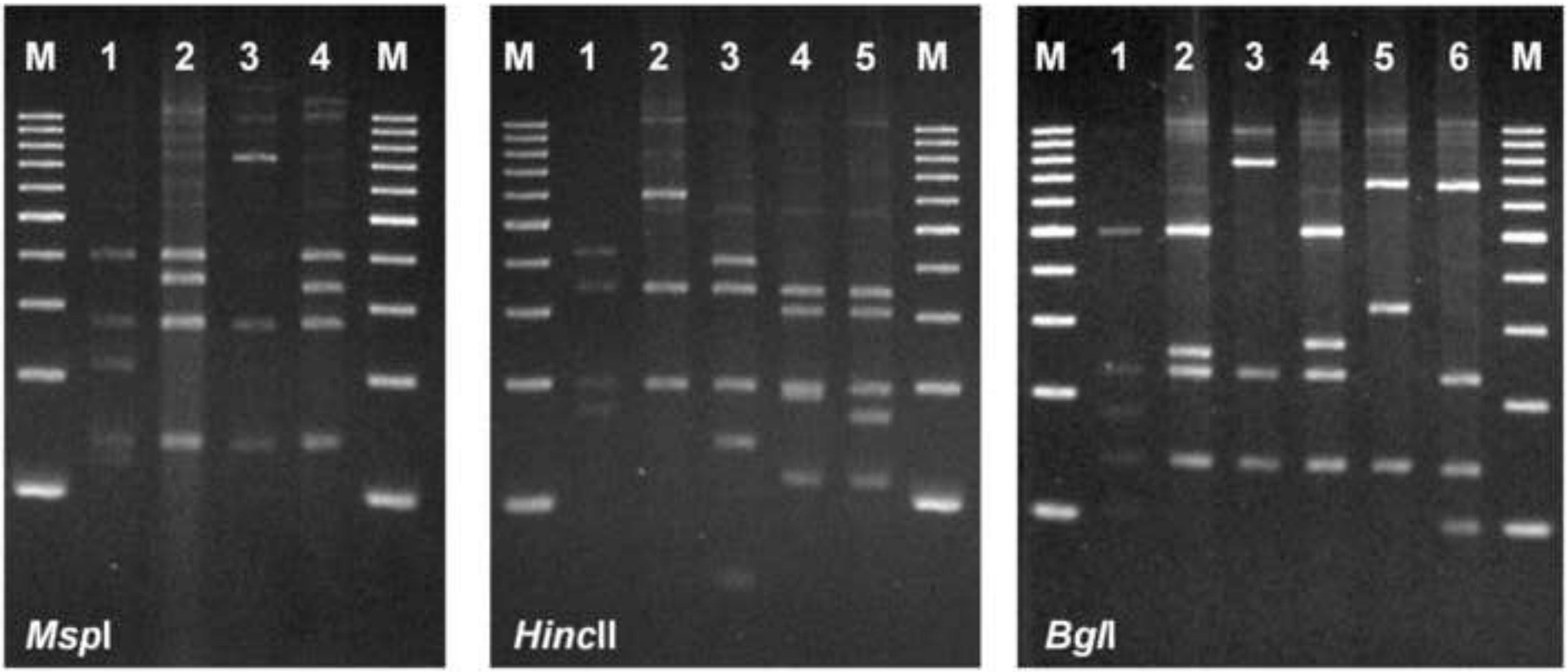
Strain	Host	Country of origin	Year of isolation	Type of RFLP patterns			*fla*A type
				*Msp*I	*Hinc*II	*Bgl*I	
Bb-ALI	Human	United Kingdom	unknown	3	3	6	H
Bb DANG	Human	United Kingdom	unknown	1	1	1	A
Bb DEL	Human	United Kingdom	unknown	1	4	4	D
Bb REM	Human	United Kingdom	unknown	3	3	3	C
Bb VAL	Human	France	unknown	1	4	4	D
MBORD 675	Human	Germany	unknown	1	4	4	D

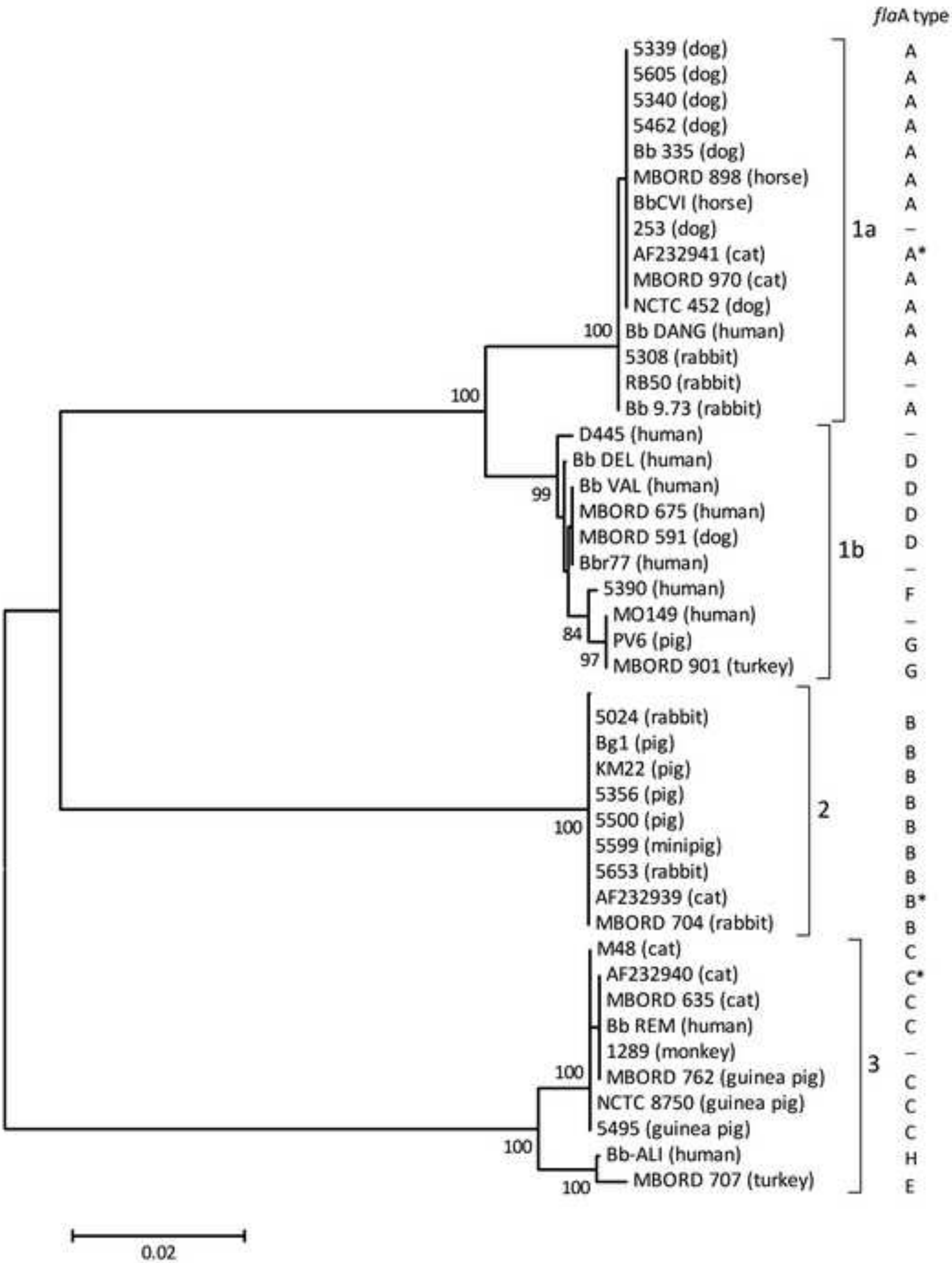
Table 2.

Genetic distances between *fla*A clusters based on nucleotide and deduced amino acid (shown in italics) sequences.

cluster	1a	1b	2	3
1a	0.0-0.1%	2.5-3.0%	13.0-13.1%	13.8-14.2%
	0.0%	*2.3-2.9%*	*15.0%*	*18.9-20.4%*
1b	2.5-3.0%	0.0-0.7%	11.5-12.0%	14.0-14.6%
	2.3-2.9%	*0.0-1.4%*	*13.9-15.0%*	*18.5-20.4%*
2	13-13.1%	11.5-12.0%	0.0%	13.5-14.3%
	15%	*13.9-15.0%*	*0.0%*	*14.7-15.4%*
3	13.8-14.2%	14.0-14.6%	13.5-14.3%	0.0-1.6%
	18.9-20.4%	*18.5-20.4%*	*14.7-15.4%*	*0-1.5%*

Figure




flaA type
5339 (dog) A
5605 (dog) A
5340 (dog) A
5462 (dog) A
Bb 335 (dog) A
MBORD 898 (horse) A
BbCVI (horse) A
253 (dog) –
AF232941 (cat) A*
MBORD 970 (cat) A
NCTC 452 (dog) A
Bb DANG (human) A
5308 (rabbit) A
RB50 (rabbit) –
Bb 9.73 (rabbit) A
1a
D445 (human) –
Bb DEL (human) D
Bb VAL (human) D
MBORD 675 (human) D
MBORD 591 (dog) D
Bbr77 (human) –
5390 (human) F
MO149 (human) –
PV6 (pig) G
MBORD 901 (turkey) G
1b
5024 (rabbit) B
Bg1 (pig) B
KM22 (pig) B
5356 (pig) B
5500 (pig) B
5599 (minipig) B
5653 (rabbit) B
AF232939 (cat) B*
MBORD 704 (rabbit) B
2
M48 (cat) C
AF232940 (cat) C*
MBORD 635 (cat) C
Bb REM (human) C
1289 (monkey) –
MBORD 762 (guinea pig) C
NCTC 8750 (guinea pig) C
5495 (guinea pig) C
Bb-ALI (human) H
MBORD 707 (turkey) E
3
100
100
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97
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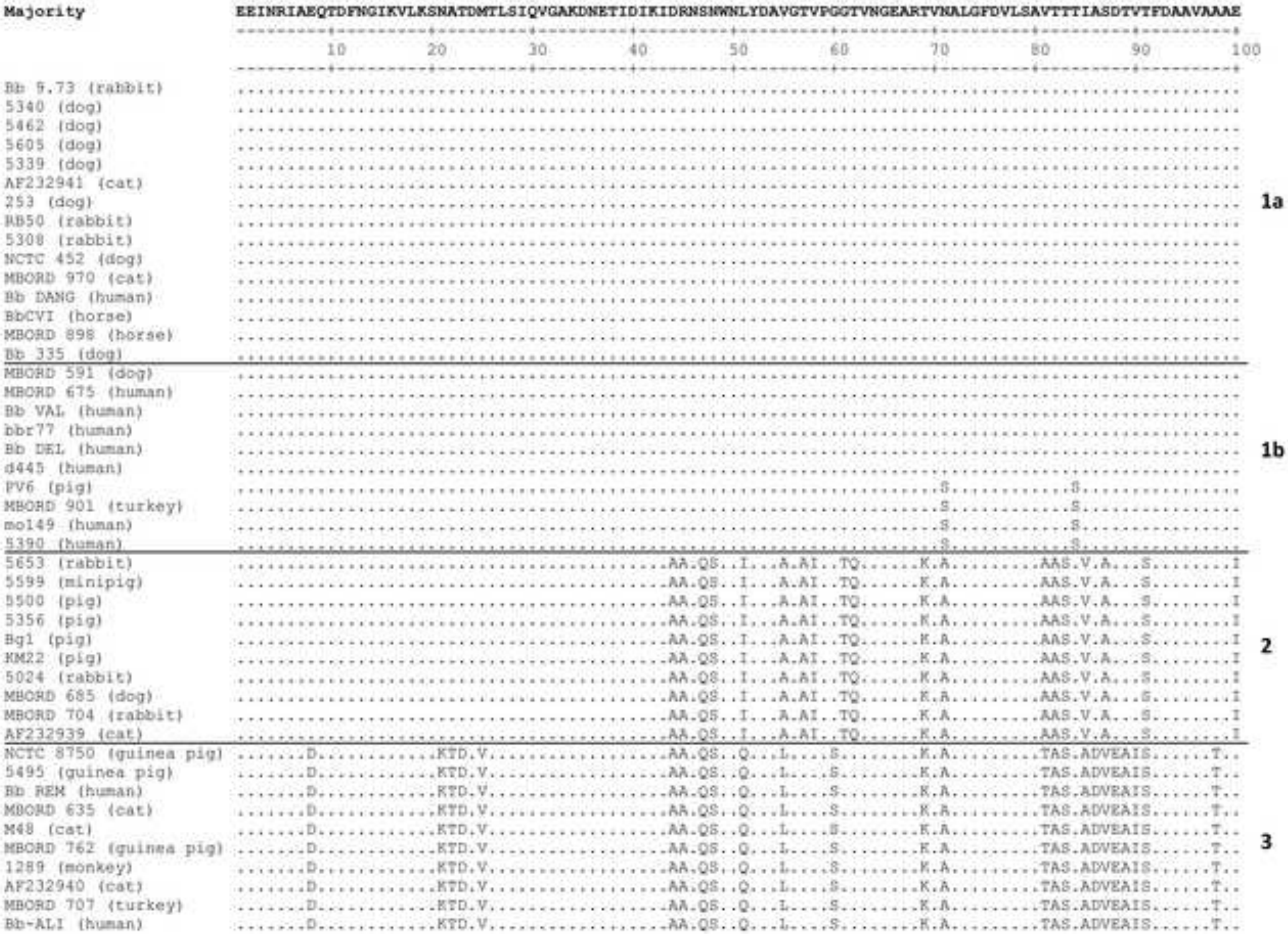
100
100
100
0.02

Figure



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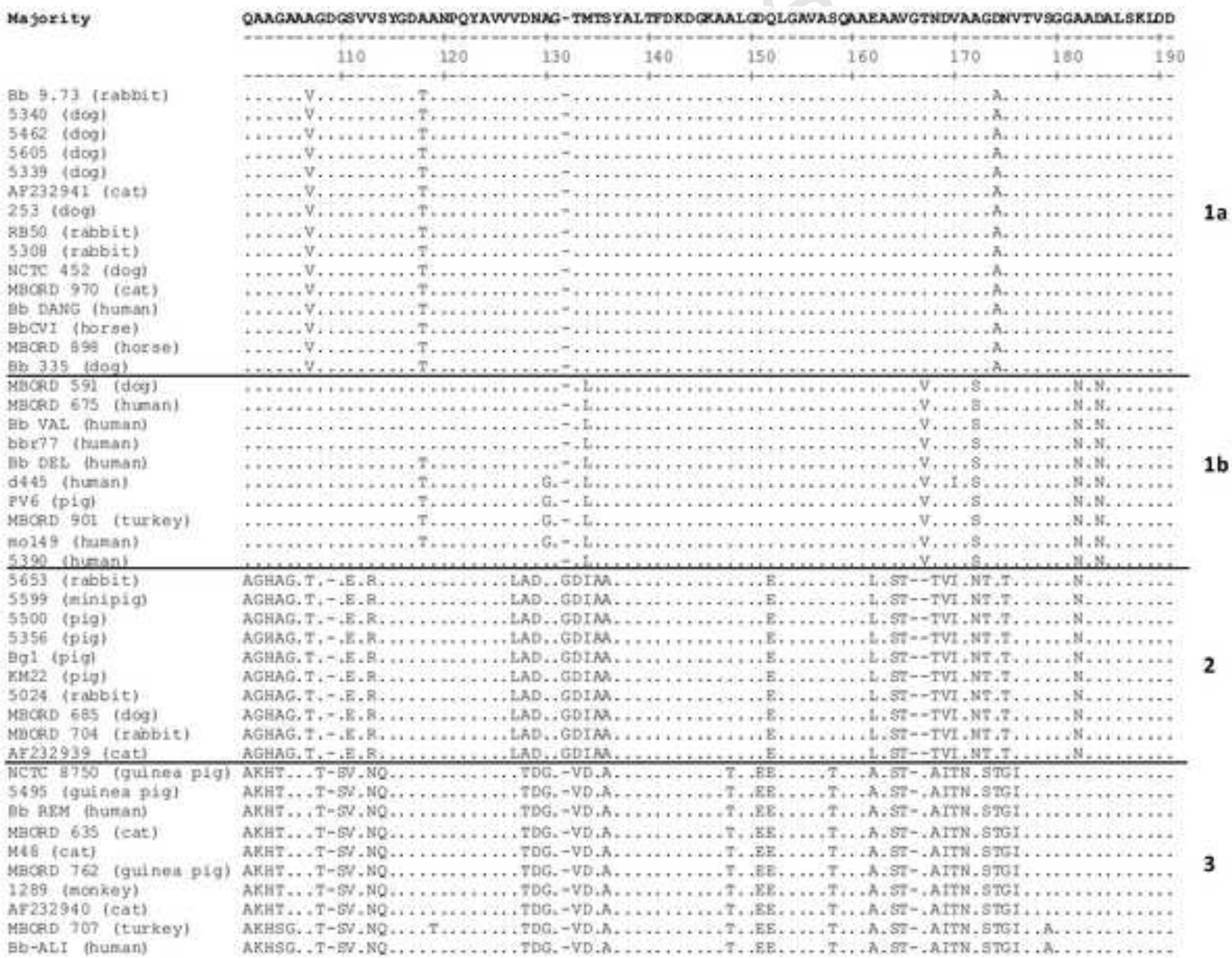
10 20 30 40 50 60 70 80 90 100
Bb 9.73 (rabbit)
5340 (dog)
5462 (dog)
5605 (dog)
5339 (dog)
AF232941 (cat)
253 (dog)
RB50 (rabbit)
5308 (rabbit)
NCTC 452 (dog)
MBORD 970 (cat)
Bb DANG (human)
BbCVI (horse)
MBORD 898 (horse)
Bb 335 (dog)
MBORD 591 (dog)
MBORD 675 (human)
Bb VAL (human)
bbr77 (human)
Bb DEL (human)
d445 (human)
PV6 (pig)
MBORD 901 (turkey)
mo149 (human)
5390 (human)
5653 (rabbit)
5599 (minipig)
5500 (pig)
5356 (pig)
Bg1 (pig)
KM22 (pig)
5024 (rabbit)
MBORD 685 (dog)
MBORD 704 (rabbit)
AF232939 (cat)
NCTC 8750 (guinea pig)
5495 (guinea pig)
Bb REM (human)
MBORD 635 (cat)
M48 (cat)
MBORD 762 (guinea pig)
1289 (monkey)
AF232940 (cat)
MBORD 707 (turkey)
Bb-ALI (human)
1a
1b
2
3

Figure



Majority
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110
120
130
140
150
160
170
180
190
Bb 9.73 (rabbit)
5340 (dog)
5462 (dog)
5605 (dog)
5339 (dog)
AF232941 (cat)
253 (dog)
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5308 (rabbit)
NCTC 452 (dog)
MBORD 970 (cat)
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Bb 335 (dog)
1a
MBORD 591 (dog)
MBORD 675 (human)
Bb VAL (human)
bbr77 (human)
Bb DEL (human)
d445 (human)
PV6 (pig)
MBORD 901 (turkey)
mo149 (human)
5390 (human)
1b
5653 (rabbit)
5599 (minipig)
5500 (pig)
5356 (pig)
Bg1 (pig)
KM22 (pig)
5024 (rabbit)
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NCTC 8750 (guinea pig)
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M48 (cat)
MBORD 762 (guinea pig)
1289 (monkey)
AF232940 (cat)
MBORD 707 (turkey)
Bb-ALI (human)
3