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Genetic clustering of Borna disease virus natural animal isolates, laboratory and vaccine strains strongly reflects their regional geographical origin

Jolanta Kolodziejek,[1] Ralf Dürrwald,[2]† Sibylle Herzog,[3]† Felix Ehrensperger,[4]† Helga Lussy[1] and Norbert Nowotny[1,5]

Correspondence
Norbert Nowotny
Norbert.Nowotny@vu-wien.ac.at

[1]Zoonoses and Emerging Infections Group, Clinical Virology, Clinical Department of Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary Medicine, Vienna, Veterinärplatz 1, A-1210 Vienna, Austria

[2]Impfstoffwerk Dessau-Tornau GmbH (IDT), Rodleben, Germany

[3]Institute of Virology, Justus Liebig University, Giessen, Germany

[4]Institute of Veterinary Pathology, University of Zurich, Zurich, Switzerland

[5]Department of Medical Microbiology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

The aim of this study was to gain more detailed insights into the genetic evolution and variability of Borna disease virus (BDV). Phylogenetic analyses were performed on field viruses originating from naturally infected animals, the BDV vaccine strain 'Dessau', four widely used laboratory strains and the novel BDV subtype No/98. Four regions of the BDV genome were analysed: the complete p40, p10 and p24 genes and the 5′-untranslated region of the X/P transcript. BDV isolates from the same geographical area exhibited a clearly higher degree of identity to each other than to BDV isolates from other regions, independent of host species and year of isolation. Five different clusters could be established within endemic areas, corresponding to the geographical regions from which the viruses originated: (i) a Swiss, Austrian and Liechtenstein Rhine valley group, related closely to the geographically bordering Baden-Wurttemberg and Bavaria II group (ii) in the western part of Germany; (iii) a third group, called Bavaria I group, limited in occurrence to Bavaria; (iv) a southern Saxony-Anhalt and bordering northern Saxony group, bound to the territories of these federal states in the eastern part of Germany; and (v) a mixed group, consisting of samples from different areas of Germany; however, these were mainly from the federal states of Thuringia and Lower Saxony. The laboratory strains and the vaccine strain clustered within these groups according to their geographical origins. All field and laboratory strains, as well as the vaccine strain, clearly segregated from the recently described and highly divergent BDV strain No/98, which originated from an area in Austria where Borna disease is not endemic.

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INTRODUCTION

Clinical symptoms in horses similar to Borna disease (BD) were first described in the 17th and 18th centuries in southern Germany. The name 'Bornasche Krankheit' has been derived from the district around the city of Borna, near Leipzig, Saxony, Germany, where a large number of horses died during epidemic-like outbreaks in the 1890s and first decades of the 20th century.

The aetiological agent, Borna disease virus (BDV), exhibits several unique characteristics. Genome organization and nucleotide sequences of BDV are similar to those of other members of the order Mononegavirales; due to features such as nuclear localization of replication and transcription (Pyper *et al.*, 1998), the unusually high level of sequence conservation (Formella *et al.*, 2000) and a wide host range, probably also including humans (Rott *et al.*, 1985; Bode *et al.*, 1995), BDV has been classified as the prototypic and only member of the genus *Bornavirus* within the family *Bornaviridae*. BDV RNA contains six open reading frames (ORFs) that encode six structural proteins, i.e. the nucleoprotein p40, the phosphoprotein p24, the matrix protein

†These authors contributed equally to this paper.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide and protein sequences described in this paper are AY374519–AY374552.

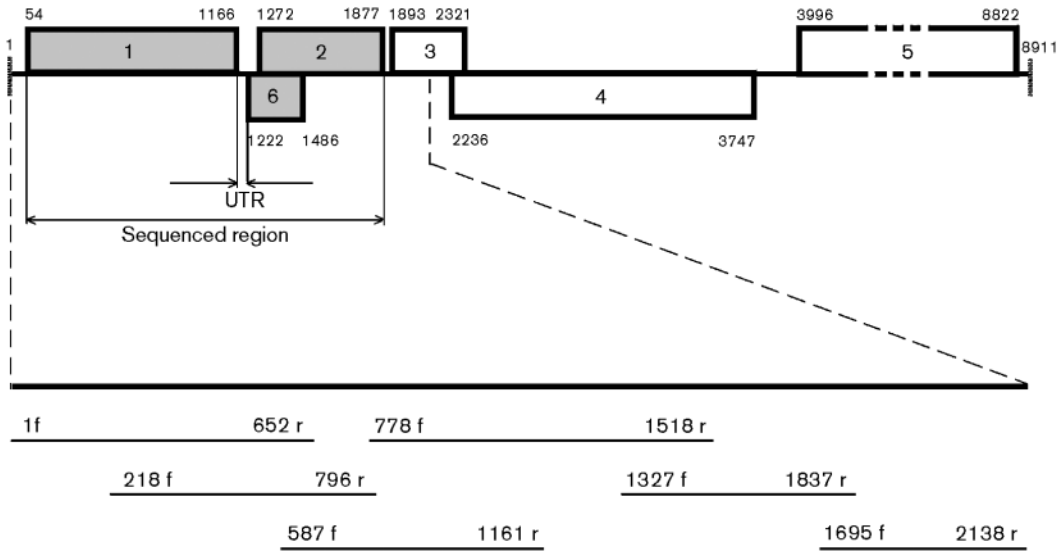

Fig. 1. Gene order of BDV. Overlapping RT-PCR products of various BDV isolates were sequenced in this study. The resulting 1824 bp fragments (nucleotide positions 54–1877) include the complete coding sequences for the viral proteins p40, p10 and p24, as well as the 5′-untranslated region (UTR) of the X/P transcript between p40 and p10. The sequenced region and the 5′-UTR of the X/P transcript are indicated by arrows. For each PCR product, the position and orientation of the primers are shown. All nucleotides are numbered according to the corresponding position on BDV strain V (GenBank accession no. U04608) (Briese *et al.*, 1994). 1, p40 (N protein, nucleoprotein, ORF I); 2, p24 (P protein, phosphoprotein, ORF II); 3, gp18 (M protein, matrix protein, ORF III); 4, gp84/94, p57 (G protein, glycoprotein/envelope, ORF IV); 5, p190 (L protein, L-polymerase, ORF V); 6, p10 (X protein, ORF VI). Abbreviations: f, forward; r, reverse.

gp18, the surface glycoprotein gp94, the viral RNA-dependent RNA polymerase p190 and the recently described sixth protein, named X protein or p10 (Briese *et al.*, 1994; Cubitt *et al.*, 1994; Schwemmle *et al.*, 1999; summarized by Boucher *et al.*, 1999) (Fig. 1).

Although BDV infection was first described only in horses and sheep in German areas (reviewed by Dürrwald & Ludwig, 1997), it is now known that BDV is able to infect a wide variety of animal species, and it seems to be geographically more widespread than thought previously (summarized by Staeheli *et al.*, 2000). BDV-endemic areas in central Europe include Germany (distinct regions within the federal states of Bavaria, Baden-Wurttemberg, Hesse, Lower Saxony, Saxony-Anhalt, Saxony and Thuringia), the eastern part of Switzerland (the cantons Graubuenden and Sankt Gallen), the principality of Liechtenstein, and – recently identified – the most western federal state of Austria (Vorarlberg) (Weissenböck *et al.*, 1998; Caplazi *et al.*, 1999; Suchy *et al.*, 2000; summarized by Staeheli *et al.*, 2000) (Fig. 2).

More recent research indicated that some BDV sequences from horses, sheep, cats and humans in Japan, Taiwan, Iran, the UK and the USA were almost identical to laboratory strains derived from central European isolates. These results affected the basics of BDV research deeply, as it remains possible that some reports on the detection of BDV in animals and humans may represent artefacts resulting from accidental contamination of samples with laboratory strains (Staeheli *et al.*, 2000).

Sequence comparisons of a limited number of field viruses from cases of classical BD revealed that almost all strains are related closely to each other, as demonstrated in several studies (summarized by Staeheli *et al.*, 2000). Their nucleic acid sequences differ by not more than 5 %. Nucleotide exchanges do not usually affect amino acid sequences, indicating that functional constraints might exist (Staeheli *et al.*, 2000). The only exception so far is the divergent strain No/98 (Nowotny *et al.*, 2000).

The aim of this study was the investigation of possible sequence variability of BDV isolates originating from different regions within BD-endemic areas. In addition, the live-attenuated vaccine strain 'Dessau' and the novel BDV subtype No/98 were included in the study. As possible sample or PCR contamination by BDV laboratory strains may play a crucial role in BDV research, the four most important BDV laboratory strains (V, He/80, RW98 and H1766) were also analysed. Phylogenetic analyses were performed on 33 BD field viruses originating from different animal species (horse, sheep and donkey), different central European countries (Germany, Switzerland and the principality of Liechtenstein) and different years (1985–1998). Four regions of the BDV genome were analysed: the complete p40, p10 and p24 genes, as well as the 5′-untranslated region of the X/P transcript. Furthermore,

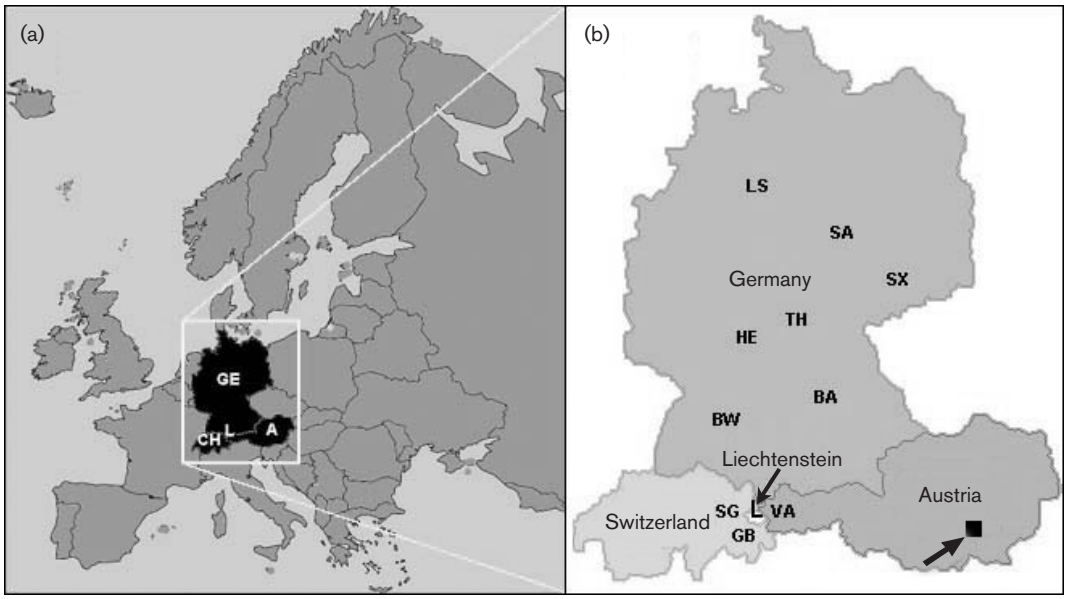

Fig. 2. (a) Map of Europe. The four countries with BD-endemic areas (A, Austria; CH, Switzerland; GE, Germany; L, Liechtenstein) are highlighted. (b) BD-endemic areas in Germany, Switzerland, Austria and the principality of Liechtenstein are indicated by letters. Abbreviations: BA, Bavaria; BW, Baden-Wurttemberg; GB, Graubuenden; HE, Hesse; L, Liechtenstein; LS, Lower Saxony; SA, Saxony-Anhalt; SG, Sankt Gallen; SX, Saxony; TH, Thuringia; VA, Vorarlberg. The square (marked by an arrow) indicates the place of the pony from which the novel BDV subtype No/98 was isolated [non-endemic area of Austria (federal state of Styria)].

established BDV sequences from Germany, mainly from the endemic regions in the federal states of the eastern part of Germany, were incorporated in additional phylogenetic analyses. Four selected phylogenetic trees are presented and discussed.

METHODS

Samples. BDV-infected brain tissues and BDV isolates from 25 horses, five sheep and two donkeys were analysed. All originated from natural spontaneous cases of BD in the western part of Germany (18 samples), Switzerland (15 samples) and the principality of Liechtenstein (two samples) between 1985 and 1998. BDV diagnosis was confirmed by histology and BDV-specific immunohistochemistry; for the German specimens, virus isolation in cell culture was performed.

In addition, strain No/98 was included in the study. This genetically highly divergent BDV strain was isolated in 1998 from a pony stallion originating from the Austrian federal state of Styria, where no cases of BD had been recorded previously. Initially, almost half of the No/98 genome was sequenced (GenBank accession no. AF136236; Nowotny *et al.*, 2000); subsequently, the entire genome was analysed (GenBank accession no. AJ311524; Pleschka *et al.*, 2001).

Furthermore, four laboratory strains were investigated: strains V, He/80, RW98 and H1766. For strain H1766 (GenBank accession no. AJ311523), the original brain suspension (from horse no. H1766) was also available. Strain V was derived at the end of the 1920s from a diseased horse originating from Lower Saxony, Germany, and subjected to several passages in rabbits by Zwick and co-workers in Giessen (all references by Zwick and co-workers are summarized by Dürrwald & Ludwig, 1997). Based on RNA from strain V, the first BDV sequence was established in 1994 (GenBank accession no. U04608) (Briese *et al.*, 1994). In 2001, the whole genome of strain V was sequenced again in Freiburg (designated strain V/Fr; GenBank accession no. AJ311521) (Pleschka *et al.*, 2001). Strain He/80 (the abbreviation stands for Herzog/80 and not, as commonly cited, for Hessen/80) was isolated in cell culture in 1980 from a horse originating from Baden-Wurttemberg, Germany (horse H7). The complete nucleotide sequence of strain He/80 was determined by Cubitt *et al.* (1994) (GenBank accession no. L27077). Sequencing was repeated 7 years later by Pleschka *et al.* (2001) (named He/80/Fr; GenBank accession no. AJ311522). Strain RW98 (GenBank accession nos AF158629–AF158631), previously described as a possible new human BDV strain isolated from the blood of a psychiatric patient (Planz *et al.*, 1999), has been interpreted as the fourth rat passage of Giessen strain He/80 and designated BDV-4p (Planz *et al.*, 2003). However, the original Giessen BDV-rat-4p strain, used since 1986, was isolated in 1985 from the Bavarian horse H24; it has also been named rat-BDV (GenBank accession nos AJ250177–AJ250178).

Additionally, the live-attenuated vaccine strain 'Dessau', which was used widely in East Germany from 1949 to 1992 (from 1949 to 1990, Germany was divided into two states; as a consequence, the vaccine was only distributed in the eastern part), was incorporated in the study (vaccine batch no. 198 11 90, the last but one batch out of 199, which was produced in Tornau, near Dessau, Germany, in November 1990). The 'Dessau' vaccine strain was originally derived from an equine isolate (a spontaneous case of BD from the East German endemic region that happened between 1949 and 1956), was subsequently highly adapted to rabbits (far more than 128 documented rabbit-brain passages until 1967, and an estimated number of 250 passages in total until the 1980s, when the seed-virus principle was introduced; Möhlmann & Maas, 1960; Schulz *et al.*, 1968; H. Müller & W. Bauer, personal communication).

Table 1. Characteristics of 33 BDV isolates, one vaccine strain, four known laboratory strains (plus two that were re-sequenced) and the variant strain No/98, used in the phylogenetic studies

Abbreviations: Baden-Wurtt, Baden-Wurttemberg; ND, not documented; t.p., this paper.

Designation	GenBank accession no.	Reference	Host	Country of origin	Area of BD case	Exact location	Year of isolation/ batch production/ sequencing
BDV samples used in this study							
H446	AY374534	t.p.	Horse	Germany	Hesse	Bebra	1992/–/2003
H544	AY374535	t.p.	Horse	Germany	Bavaria	Lindelburg	1993/–/2003
H639	AY374536	t.p.	Horse	Germany	Lower Saxony	Salzhausen	1993/–/2003
H640	AY374537	t.p.	Horse	Germany	Lower Saxony	Gödenstorf	1993/–/2003
H1499	AY374520	t.p.	Horse	Germany	Baden-Wurtt	Aalen	1994/–/2003
H1505	AY374521	t.p.	Horse	Germany	Bavaria	Kaltenberg	1994/–/2003
H1766	AY374522	t.p.	Horse	Germany	Lower Saxony	Hannover	1994/–/2003
H3053	AY374523	t.p.	Horse	Germany	Bavaria	Ergolding	1996/–/2003
H3321	AY374524	t.p.	Horse	Germany	Bavaria	Jettingen	1997/–/2003
H3452	AY374525	t.p.	Horse	Germany	Bavaria	Baierbach	1997/–/2003
H3515	AY374526	t.p.	Horse	Germany	Bavaria	Straßlach	1997/–/2003
H3519	AY374527	t.p.	Horse	Germany	Bavaria	Gaimersheim	1997/–/2003
H3575	AY374528	t.p.	Horse	Germany	Bavaria	Munich	1997/–/2003
H3915	AY374529	t.p.	Horse	Germany	Bavaria	Nürnberg	1998/–/2003
H3940	AY374530	t.p.	Horse	Germany	Bavaria	Neuburg*	1998/–/2003
H3950	AY374531	t.p.	Horse	Germany	Bavaria	Munich	1998/–/2003
H4026	AY374532	t.p.	Horse	Germany	Bavaria	Eppisburg	1998/–/2003
H4050	AY374533	t.p.	Horse	Germany	Bavaria	Jettingen	1998/–/2003
E85-0795	AY374552	t.p.	Horse	Switzerland	Graubuenden	Maienfeld	1985/–/2003
S88-2297	AY374551	t.p.	Horse	Switzerland	Graubuenden	Chur	1988/–/2003
S89-2224	AY374550	t.p.	Horse	Switzerland	Graubuenden	Jenins	1989/–/2003
S91-1307	AY374542	t.p.	Sheep	Liechtenstein		Vaduz	1991/–/2003
S91-1350	AY374543	t.p.	Sheep	Switzerland	Sankt Gallen	Buchs	1991/–/2003
S91-1460	AY374544	t.p.	Horse	Switzerland	Graubuenden	Malans	1991/–/2003
S91-1539	AY374546	t.p.	Sheep	Liechtenstein		Triesen	1991/–/2003
S91-1552	AY374547	t.p.	Horse	Switzerland	Graubuenden	Chur	1991/–/2003
S93-1186	AY374540	t.p.	Sheep	Switzerland	Sankt Gallen	Mels	1993/–/2003
S95-1114	AY374539	t.p.	Donkey	Switzerland	Graubuenden	Bonaduz	1995/–/2003
S95-1466	AY374545	t.p.	Donkey	Switzerland	Graubuenden	Arezen	1995/–/2003
S95-1924	AY374548	t.p.	Horse	Switzerland	Sankt Gallen	Bad Ragaz	1995/–/2003
S96-0868	AY374538	t.p.	Horse	Switzerland	Graubuenden	Bonaduz	1996/–/2003
S96-1202	AY374541	t.p.	Horse	Switzerland	Graubuenden	Valzeina	1996/–/2003
S98-2042	AY374549	t.p.	Sheep	Switzerland	Graubuenden	St Peter	1998/–/2003
BDV vaccine strain included							
Vaccine strain 'Dessau'	AY374519	t.p.	Horse	Germany	Saxony-Anhalt	ND†	1949–1950s/ 1990/2003
BDV laboratory strains included							
Strain V	U04608	Briese *et al.* (1994); Dürrwald & Ludwig (1997)	Horse‡§	Germany	Lower Saxony	ND	1929/–/1994
Strain V/Fr	AJ311521	Pleschka *et al.* (2001)	Horse§	Germany	Lower Saxony	ND	1929/–/2001
He/80	L27077	Narayan *et al.* (1983); Cubitt *et al.* (1994)	Horse§	Germany	Baden-Wurtt	Ulm	1980/–/1994
He/80/Fr	AJ311522	Pleschka *et al.* (2001)	Horse§	Germany	Baden-Wurtt	Ulm	1980/–/2001
RW98‖	AF158629–AF158633	Planz *et al.* (1999, 2003)	Horse‡¶	Germany	Bavaria	Munich	1985/–/1999
Strain H1766	AJ311523	Pleschka *et al.* (2001)	Horse§	Germany	Lower Saxony	Hannover	1994/–/2001
BDV outside endemic areas							
No/98	AJ311524	Nowotny *et al.* (2000); Pleschka *et al.* (2001)	Horse§	Austria	Styria	Burgfried, near Gnas	1998/–/2001

Table 1. cont.

*Neuburg, between Jettingen and Krumbach.
†Probably the southern part of Saxony-Anhalt (area around Halle/Saale, Köthen, Bernburg and Merseburg).
‡Passaged in rabbits.
§Cell culture-adapted.
||BDV-4p (a derivative of H24).
¶Passaged in rat brains.

Characteristics of all BD field viruses, the vaccine strain and laboratory strains analysed in this study are listed in Table 1.

Short BDV sequences from Austrian field cases (one dog and one horse from Vorarlberg) have been determined previously (GenBank accession nos AF054275 and AF054276, respectively) (Weissenböck *et al.*, 1998); although they were too short to be included in the phylogenetic analyses, these sequences were compared to corresponding sequences of the other BD viruses.

As original samples of the endemic regions in the federal states of the eastern part of Germany were not available for this study, several published sequences and others deposited in GenBank were used for comparative analysis. Most of these samples were collected and analysed by Dürrwald (1993), investigated by RT-PCR by Zimmermann *et al.* (1994) and later sequenced by Binz *et al.* (1994), Schneider *et al.* (1994) and Lüschow (1999). All of these additional sequences, established primarily in other studies, are listed in Table 2.

Isolation of RNA. Brain-tissue samples were homogenized by using sterile sand and resuspended in distilled, DEPC-treated water. The lyophilized vaccine was resuspended in DEPC-treated water. All of these suspensions, as well as cell-culture isolates, were frozen at −80 °C for 30 min, thawed and centrifuged at 1700 ***g*** for 5 min. A volume of 140 µl of each supernatant was used for RNA extraction, employing a QIAamp viral RNA purification kit (Qiagen) according to the manufacturer's instructions.

Primer design. Among different primer pairs tested, the one that proved to be best for the detection of classical BDV and a highly variant strain, described by Sorg & Metzler (1995), was used for screening PCR. Furthermore, by using the Primer Designer program (version 3.0; Scientific & Educational Software), six primer pairs were designed in order to amplify overlapping PCR products that comprised the complete N, X and P protein-encoding regions of the genome, as well as the 5′-untranslated region of the X/P transcript, and were synthesized by Invitrogen. The primer sequences were as follows: BDV1f: 5′-GTTGCGTTAACAACMAACCA-3′ and BDV652r: 5′-TGGCCGTTAATCCAATCTAT-3′ (652 bp), BDV218f: 5′-GAACGCAGTGGCATTGTTAG-3′ and BDV796r: 5′-CAYTCTGCGAGGTACTCCTT-3′ (579 bp), BDV587f: 5′-TGGTGAGACTGCTACACTAC-3′ and BDV1161r: 5′-TTAGACCAGTCACACCTATC-3′ (575 bp), BDV778f: 5′-AGGAGTACCTCGCAGAATG-3′ and BDV1518r: 5′-CCAGCTCCGTCACTARCTT-3′ (741 bp), BDV1327f: 5′-AGACACTACGACGGGAACGA-3′ and BDV1837r: 5′-TGGGAGCTGGGGATAAATGC-3′ (511 bp), and BDV1695f: 5′-GATCGCTCCATGAAGACAAT-3′ and BDV2138r: 5′-GAAGTCGTCAATCTGGAAGT-3′ (444 bp) (Fig. 1). Each primer name contains its position on the genome according to the corresponding position on BDV strain V (GenBank accession no. U04608).

Detection of BDV RNA by RT-PCR. cDNA synthesis and PCR were carried out in a single step by using a commercially available kit (OneStep RT-PCR kit; Qiagen) according to the manufacturer's recommendations. An annealing temperature of 60 °C was employed for 45 PCR cycles. Each reaction contained 0·8 µM (final concentration) of each of the primers and 4 U RNAsin Plus RNase inhibitor (Promega). All amplifications were performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer) or in a Mastercycler gradient (Eppendorf).

Purification of the amplification products was done by using PCR Kleen spin columns (Bio-Rad) or a QIAquick Gel Extraction kit (Qiagen), following the manufacturers' protocols.

Sequencing and sequence analysis. An ABI PRISM BigDye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems), diluted in ABI PRISM BigDye 5× sequencing buffer (Applied Biosystems) in the ratio 2 : 3, was employed for sequencing PCR. The primer concentration used for this reaction was 4 pmol in 20 µl. For direct removal of unincorporated dye terminators from sequencing reactions, spin columns of a DyeEx 2.0 spin kit (Qiagen) were used. Clean reaction products were sequenced in both directions by employing an automatic ABI Prism 310 genetic analyser (Perkin Elmer).

The obtained BDV sequences were aligned by using the Align Plus program (version 3.0; Scientific & Educational Software serial no. 43071) and their genetic identity was verified by comparing them with sequences of published BDV laboratory strains (listed in Table 1).

Phylogenetic studies. Phylogenetic studies were performed by using the Phylogeny Inference Program package, PHYLIP (Felsenstein, 1993). Stability of the trees was tested by bootstrap resampling analysis of 100 replicates, computed with the SEQBOOT program. Genetic distances between each pair of sequences were calculated by using the DNADIST (for nucleotide sequences) and PROTDIST (for amino acid sequences) programs, based on the Kimura two-parameter model with a transition/transversion ratio of 2. From these distance matrices, phylogenetic trees were generated by the neighbour-joining method of the NEIGHBOR program and the best tree was displayed by the program DRAWGRAM.

Two phylogenetic trees [one including and one without the BDV variant strain No/98 (GenBank accession no. AJ311524)] were constructed with the sequences listed in Table 1 in each of the following genomic regions: the complete p40, p10 and p24 coding regions, as well as the 5′-untranslated region of the X/P transcript. In addition, phylogenetic trees that also included shorter BDV sequences, mainly from some federal states of the eastern part of Germany (Saxony-Anhalt, Thuringia and Saxony), were constructed based on partial p40 and almost-complete p24 coding regions (sequences listed in Tables 1 and 2). From the latter, the two phylogenetic trees without strain No/98 are presented in Figs 3 and 4; these phylogenies include most of the BDV sequences from BD-endemic regions in central Europe. Two selected phylogenetic trees (the other trees are not shown) of the viruses listed in Table 1, based on the nucleotide sequences of the whole p40 and p10 genes, are presented in Figs 5 and 6, respectively.

Figs 3, 4 and 5 represent phylogenetic trees that are based on nucleic acid sequences of the p40 (Figs 3 and 5) and p24 (Fig. 4) genes and

Table 2. Characteristics of BDV isolates for which already published sequences were available

Abbreviations: NA, Not available; ND, not documented; 1991/95 or 1994/95, collected between 1991 and 1995 or 1994 and 1995, respectively (exact year not documented).

Designation	GenBank accession no.	Reference	Host	Country of origin	Area of BD case	Exact location	Year of isolation
Partial BDV p40 gene sequences included in comparative phylogenetic analysis							
ALPHA	NA	Lüschow (1999)*	Horse	Germany	Bavaria	ND	1993
S1	U95356	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
TREPTOW	AF001615	Zimmermann *et al.* (unpublished)†	Horse	Germany	Schleswig-Holstein	ND	1995
A 188 94	NA	Lüschow (1999)*	Horse	Germany	Thuringia	Bachra	1994
A 66 B 3813	NA	Lüschow (1999)*	Horse	Germany	Thuringia	Wiehe	1998
133 med 91	NA	Lüschow (1999)*	Horse	Germany	Saxony-Anhalt	Schnellroda	1991
A 104 97	NA	Lüschow (1999)*	Horse	Germany	Thuringia	Hottelstedt	1997
18 med 92	NA	Lüschow (1999)*	Horse	Germany	Thuringia	Bachra	1992
No. 9/S-589	AY066023	Vahlenkamp *et al.* (2002)‡	Sheep	Germany	Bavaria	Upper Franconia	1999
HENKEL	NA	Lüschow (1999)*	Horse	Germany	Thuringia	ND	1993
A 88	NA	Lüschow (1999)*	Horse	Germany	Thuringia	ND	1995
S6	U95358	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
S2	U95357	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
82 med 91	NA	Lüschow (1999)*	Horse	Germany	Saxony-Anhalt	Borau-Kleben	1991
101 med 91	NA	Lüschow (1999)*	Horse	Germany	Saxony-Anhalt	Landsberg	1991
176 med 92	U94871	Zimmermann *et al.* (unpublished)†	Donkey	Germany	Saxony	Schkeuditz	1992
139 med 91	AF001611	Zimmermann *et al.* (unpublished)†	Horse	Germany	Saxony-Anhalt	Dannigkow	1991
E 219 91	NA	Lüschow (1999)*	Horse	Germany	Saxony	Räpitz	1991
116 med 91	NA	Lüschow (1999)*	Horse	Germany	Saxony-Anhalt	Hundeluft	1991
WT-1 = Halle B1/91	S67502	Schneider *et al.* (1994)	Horse	Germany	Saxony-Anhalt	Dalena	1991
191 med 91	NA	Lüschow (1999)*	Horse	Germany	Saxony-Anhalt	Gübs	1991
763/000	U94879	Zimmermann *et al.* (unpublished)†	Dog	Germany	Saxony-Anhalt	Wittenberg	1994
Y	U94875	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1994/95
111 med 91	NA	Lüschow (1999)*	Sheep	Germany	Saxony-Anhalt	Wiesig	1991
FANNY	NA	Lüschow (1999)*	Horse	Germany	Bavaria	Gunzenhausen	1994
Almost-complete BDV p24 gene sequences used for comparative phylogeny							
No. 9/S-589	AY066023	Vahlenkamp *et al.* (2002)‡	Sheep	Germany	Bavaria	Upper Franconia	1999
S2	U94884	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
S6	U94885	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
51 med 93 cerebrum/ kidney = horse1-2	NA	Binz *et al.* (1994)	Horse	Germany	Thuringia	Eckolstädt	1993
61 med 93 = horse4	NA	Binz *et al.* (1994)	Horse	Germany	Saxony-Anhalt	Kleingöhren, near Weißenfels	1993
18 med 92 brain pool = horse2-1	NA	Binz *et al.* (1994)	Horse	Germany	Thuringia	Bachra	1992
T 780 = horse3	NA	Binz *et al.* (1994)	Horse	Germany	Saxony-Anhalt	Käthen, near Stendal	1992

Table 2. cont.

Designation	GenBank accession no.	Reference	Host	Country of origin	Area of BD case	Exact location	Year of isolation
176 med 92	U94872	Zimmermann *et al.* (unpublished)†	Donkey	Germany	Saxony	Schkeuditz	1992
176 med 92 = donkey1	NA	Binz *et al.* (1994)	Donkey	Germany	Saxony	Schkeuditz	1992
763/000	U94880	Zimmermann *et al.* (unpublished)†	Dog	Germany	Saxony-Anhalt	Wittenberg	1994
S1	U94883	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1991/95
51 med 93 parotid-gland = horse1-1	NA	Binz *et al.* (1994)	Horse	Germany	Thuringia	Eckolstädt	1993
T 366 = sheep 1	NA	Binz *et al.* (1994)	Sheep	Germany	Saxony-Anhalt	Halle/Saale	1992
Y	U94876	Zimmermann *et al.* (unpublished)†	Sheep	Germany	Saxony-Anhalt	ND	1994/95
18 med 92 nucleus caudatus = horse2-2	NA	Binz *et al.* (1994)	Horse	Germany	Thuringia	Bachra	1992
WT-1 = Halle B1/91	S67507	Schneider *et al.* (1994)	Horse	Germany	Saxony-Anhalt	Dalena	1991

*Horses with numbers and letters were collected by Dürrwald (1993); horses with names (e.g. FANNY or ALPHA) were collected by the group of Bode in Berlin; final sequencing was done by Lüschow (1999).

†Unpublished, but deposited in GenBank under accession nos U94863–U94885, U95356–U95358 and AF001604–AF001617; the sequence of a dog with BD from Germany was taken from an epidemiological study by Dürrwald (unpublished), during which brain samples of different animal species (cats, dogs, cattle, red foxes, badgers, martens, hares, squirrels, wild boars, deer, house mice and brown and black rats) were collected within the central eastern German BD-endemic region between 1994 and 1995, of which the dog brain was the only BDV-positive sample (deposited in GenBank under accession nos U94879–U94882).

‡The BDV sequence of a sheep (GenBank accession no. AY066023) from a region in the northern part of Bavaria, close to Saxony (region of Upper Franconia), that contracted BD in July 1999 (T. W. Vahlenkamp, personal communication) was analysed by Vahlenkamp *et al.* (2002).

Fig. 6 shows a phylogenetic tree based on p10 amino acid sequences. In addition, in Fig. 6, the variable BDV strain No/98 (Nowotny *et al.*, 2000) was included, whereas in Figs 3–5, it was omitted in order to enhance the resolution of the trees and make the subclustering more visible. p10 was chosen as an example of an amino acid-based tree because strain No/98 exhibits the highest amino acid difference (19 %) to other BDV isolates in this region.

RESULTS

Amplification

A previously described primer pair (Sorg & Metzler, 1995) that was employed for BDV screening was able to detect classical BDV strains, as well as the highly variable strain No/98. The six primer pairs that were specifically designed for this study (Fig. 1) and amplified the complete p40, p10 and p24 genes of each BDV sample, as well as the 5′-untranslated region of the X/P transcript, yielded DNA amplicons of the expected sizes (data not shown). In total, a 1824 bp fragment, corresponding to 20·5 % of the whole BDV genome, was analysed in all samples (Fig. 1).

Sequence analysis

Multiple alignments of the 40 BD field viruses, vaccine and laboratory strains (without strain No/98) analysed showed overall nucleotide sequence identities of 96–99 % in the p40, 98–100 % in the p10 and 97–99 % in the p24 regions. These data corresponded to amino acid identities of 98–100 % in the p40, 96–100 % in the p10 and 98–100 % in the p24 protein. The sequence of the 5′-untranslated region of the X/P transcript was not available for strain RW98. Among the other BDV isolates that were sequenced in this region, the maximum divergence amounted to only 4 % (calculated without strain No/98). When No/98 served as the reference strain, identities were between 80 and 85 %.

Phylogenetic analyses

In all genomic regions investigated and at both the nucleic acid and amino acid levels, the samples segregated clearly into country-specific major clusters, German BD field viruses and laboratory strains on one hand and Swiss/Liechtenstein BDV isolates on the other hand (Figs 3–6).

Genetic clustering of German BD field viruses

German BDV isolates and laboratory strains localized in four major branches: one consisted of BDV isolates from Bavaria only, the second was composed of isolates from Baden-Wurttemberg and Bavaria, the third included samples mainly from Saxony-Anhalt and bordering northern Saxony and the fourth branch was a mixture of isolates from several German federal states, i.e. Thuringia, Lower

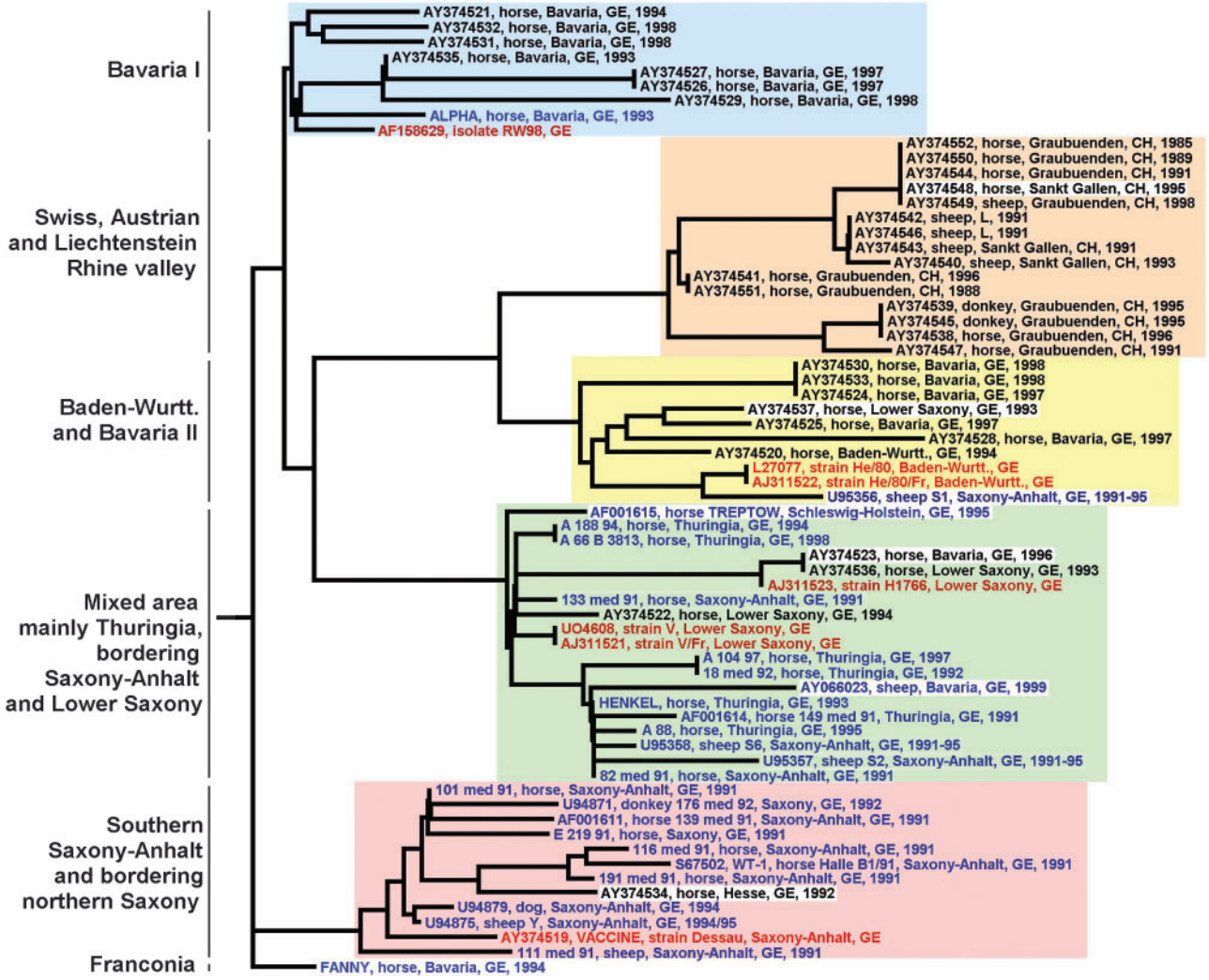

Fig. 3. Phylogenetic tree of 399 bp nucleic acid sequences (corresponding to nucleotide positions 312–710 of reference strain V, GenBank accession no. U04608) within the p40 gene of various BDV isolates [33 BDV specimens from naturally infected animals from the western part of Germany, Switzerland and Liechtenstein (indicated in black), one vaccine strain and four BDV laboratory strains (indicated in red); 25 already published sequences, mainly from the eastern part of Germany (indicated in blue) were also included]. Important BD-endemic areas are highlighted. For better resolution of the tree, the variant strain No/98 was omitted. Analyses were performed by using the PHYLIP phylogeny program package, version 3.57c (SEQBOOT, DNADIST and NEIGHBOR). The tree was outgrouped to the German BDV sequence of horse 'FANNY' from Bavaria. Description: Bavaria I, more frequent in Mid-Franconia and northern and central Upper Bavaria, but also prevalent in Swabia; Bavaria II, Swabia, Upper Bavaria and Lower Bavaria; 1991–95, collected between 1991 and 1995 (exact year not documented); 1994/95, collected in 1994 or 1995. Abbreviations: Baden-Wurtt, Baden-Wurttemberg; CH, Switzerland; GE, Germany; L, Liechtenstein.

Saxony, Bavaria and Schleswig-Holstein (Figs 3–5). On the other hand, phylogenetic analysis of the p40 gene indicated that German BDV isolates may, to some extent, be grouped around the laboratory strains V, He/80 and RW98 and the vaccine strain (Figs 3 and 5).

'Strain V group'. The majority of viruses that were investigated in this study and placed in this cluster originated from the BD-endemic area of Lower Saxony, independent of the year of isolation (1993–1996) (Fig. 5). The identities of the field isolates in this cluster to strain V amounted to 98, 98–100 and 98 % for the nucleotide sequences of the p40, p10 and p24 regions, respectively, and to 100, 97 and 99 % for the corresponding amino acid sequences. Furthermore, there are clades within the 'strain V group', one of which contains only BDV isolates from Thuringia and regions in Saxony-Anhalt not far from the border with Thuringia, and of a sheep from a northern Bavarian location close to Saxony; the other clades are located between sequences of BDV isolates

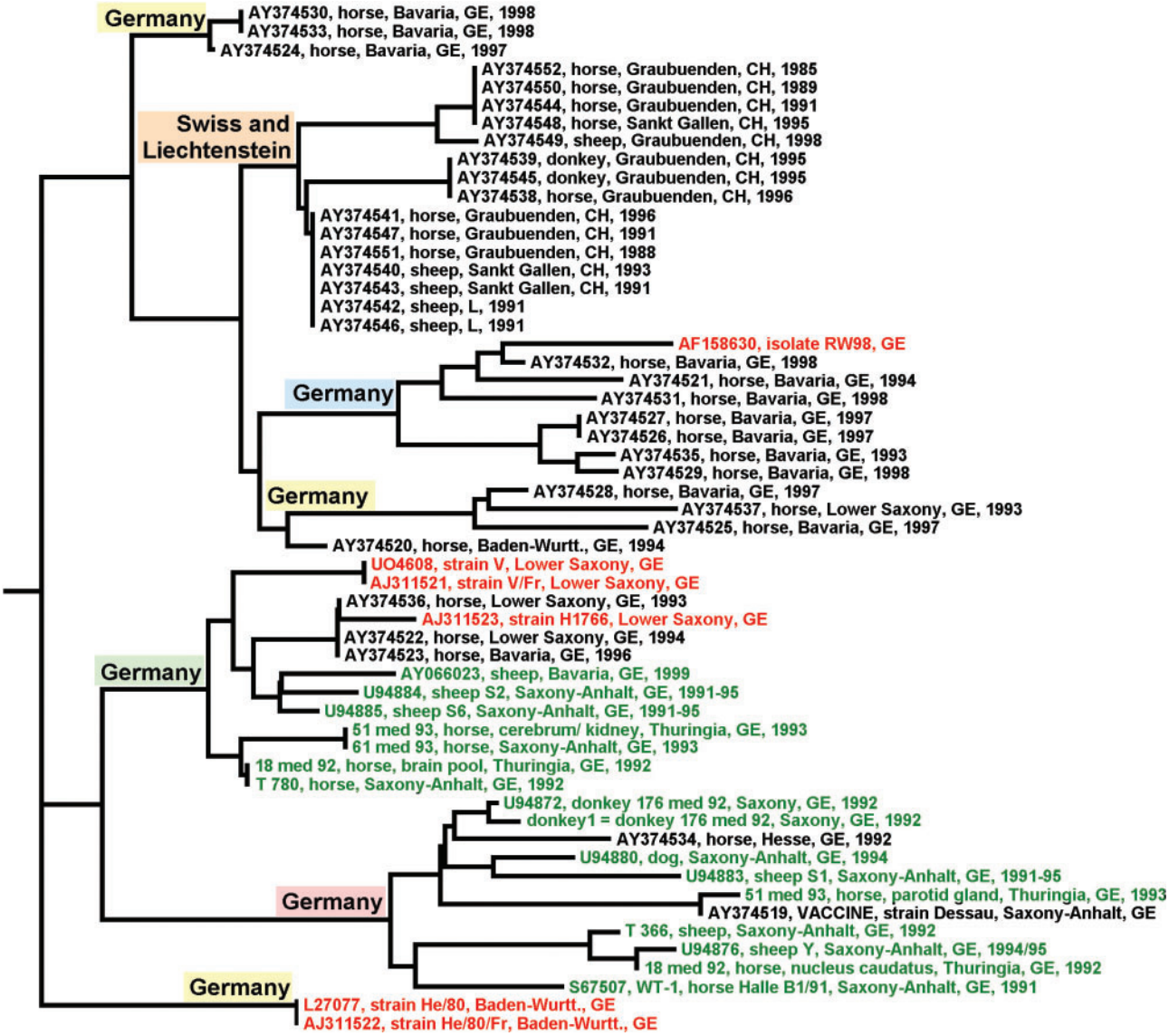

Fig. 4. Phylogenetic tree of 604 bp nucleic acid sequences (corresponding to nucleotide positions 1272–1875 of reference strain V, GenBank accession no. U04608) within the p24 gene of various BDV isolates [33 BDV specimens from naturally infected animals from the western part of Germany, Switzerland and Liechtenstein (indicated in black), one vaccine strain and four BDV laboratory strains (indicated in red), as well as 16 specimens that were sequenced in other studies, mainly from the eastern part of Germany (indicated in green)]. For better resolution of the tree, the variant strain No/98 was excluded. The tree was constructed as described for Fig. 3, except that the tree was outgrouped to the BDV sequence of the German laboratory strain He/80=He/80/FR (GenBank accession nos L27077 and AJ311522). The He/80 group is divided in this tree, whereas it is a uniform group within the p40 gene. Description: 1991–95, collected between 1991 and 1995; 1994/95, collected 1994 or 1995. Abbreviations: Baden-Wurtt, Baden-Wurttemberg; CH, Switzerland; GE, Germany, L, Liechtenstein.

from Lower Saxony and Schleswig-Holstein and one case from Bavaria. Some of the sequences that were derived from different animals from the same area proved to be (almost) identical, although these animals did not develop BD at the same time, but within a period of up to 5 years (Fig. 3).

'He/80 group'. In the genetic cluster around strain He/80, which itself originates from Baden-Wurttemberg, equine isolates from different German endemic areas are localized: Bavaria ($n=5$), Lower Saxony ($n=1$) and Baden-Wurttemberg ($n=1$), isolated between 1993 and 1998 (Fig. 5). The identities of this cluster to strain He/80 amounted to 98–100, 97–99 and 97–98 % for nucleotide sequences in the p40, p10 and p24 regions, respectively, and to 99–100, 95–98 and 97–98 % for the corresponding amino acid sequences. In addition, this cluster contains one sequence from another study, a BDV isolate originating

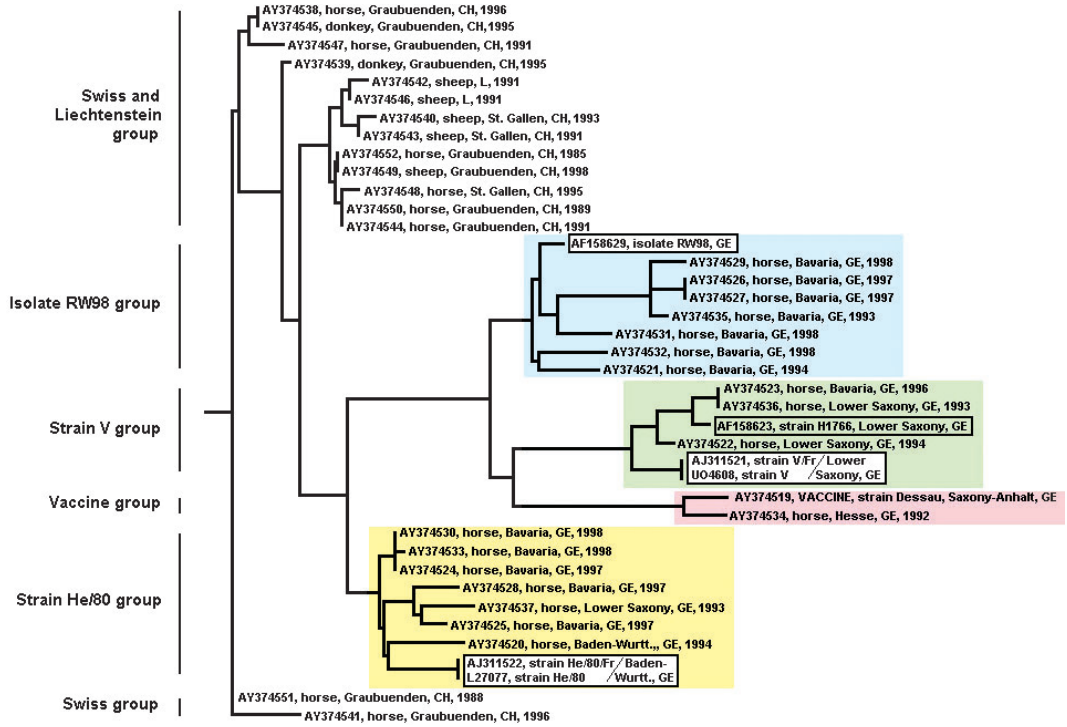

Fig. 5. Phylogenetic tree of complete p40 gene nucleic acid sequences (1113 bp in length) of various BDV isolates [33 BDV specimens from naturally infected animals, one vaccine strain and four BDV laboratory strains, without the variant strain No/98 (in order to enhance the resolution of the phylogenetic analysis)]. The tree was constructed as described for Fig. 3, except that it was outgrouped to the Swiss BDV sequence designated S96-1202 (GenBank accession no. AY374541). Groups with close relationships to laboratory strains and to the vaccine strain are highlighted.

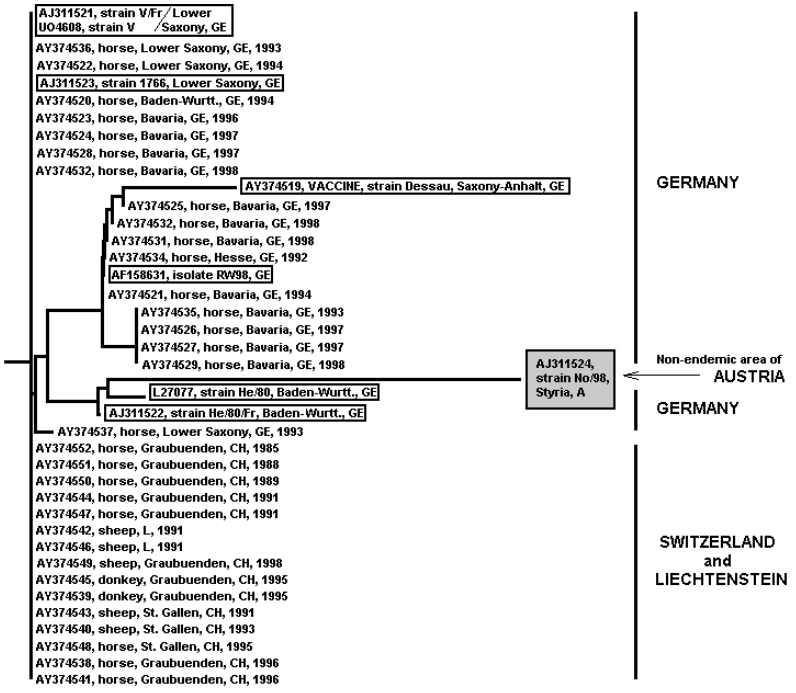

Fig. 6. Phylogenetic tree of complete p10 amino acid sequences of various BDV isolates (33 BDV samples from naturally infected animals, one vaccine strain, four BDV laboratory strains and the novel BDV subtype No/98). The tree was constructed as described in Fig. 3, except that the PROTDIST program was used (instead of DNADIST) and the tree was outgrouped to the Swiss BDV sequence designated S96-1202 (GenBank accession no. AY374541).

from Saxony-Anhalt. He/80 forms a uniform group in the p40 region (Figs 3 and 5), whereas the group is split in the p24 region into three subclusters, which are related to other Bavarian sequences and to those from Switzerland and Liechtenstein (Fig. 4).

'RW98 group'. RW98 is localized in a subgroup of samples that were, without exception, collected in the endemic area of Bavaria between 1993 and 1998 (Figs 3–5). Nucleotide identity rates in this group were 98–99 % for p40, 99–100 % for p10 and 97–99 % for the p24 region; amino acid identity rates were 99–100, 98–100 and 99 %, respectively.

'Strain Dessau group'. The 'strain Dessau' branch comprises BDV isolates that were collected mainly from the federal state of Saxony-Anhalt between 1991 and 1995. A few sequences of this group were from the federal states of Thuringia and Saxony, where the BDV vaccine was in use until the early 1990s. One sequence in this group originated from a horse from Hesse, where the Dessau BDV vaccine was never used. The sequences related most closely to the vaccine strain originated from locations near the cities of Halle/Saale, Leipzig, Köthen, Wittenberg, Roßlau and Burg (in the southern part of Saxony-Anhalt and bordering the area of Leipzig in the north-western part of Saxony), a region where the ancestor of the vaccine strain was probably isolated nearly half a century before (Figs 3–5).

Genetic clustering of BD field viruses from Switzerland and Liechtenstein

Two subgroups of BD field viruses from Switzerland and Liechtenstein were observed (Fig. 4). The first subgroup (with two clades) incorporates BDV isolates originating from the Swiss endemic area of Graubuenden, with the exception of one sample that originated from Sankt Gallen (however, from the city of Bad Ragaz, which is located in the bordering area to Graubuenden); these were isolated between 1985 and 1998 from five horses, two donkeys and one sheep. Identities within this clade were particularly high: 98–100, 100 and 98–99 % for the nucleotide sequences of the p40, p10 and p24 regions, respectively, and 100 % for all three corresponding amino acid sequences.

The second subgroup contains BDV isolates of different host species (horse and sheep) and years of isolation (1988–1996), but also of different geographical regions (Graubuenden, St Gallen and Liechtenstein). Nucleotide identity rates in this group were 98–99 % for p40, 99–100 % for p10 and 99–100 % for the p24 region; the amino acid sequences exhibited 99, 100 and 100 % identity rates, respectively.

Genetic clustering of Austrian BD field viruses

Comparative analyses of short internal sequences of ORF1 (encoding p40) of BDV isolates from an Austrian dog (GenBank accession no. AF054275) and an Austrian horse (GenBank accession no. AF054276), both from the federal state of Vorarlberg that borders the endemic areas of Switzerland and Liechtenstein, revealed a clear distinction from laboratory strains and a position in the cluster of Swiss and Liechtenstein-derived viruses (data not shown). The Austrian strain No/98 (GenBank accession no. AJ311524) always occupied a unique position in the phylogenetic trees (e.g. in Fig. 6; the other trees are not shown) and exhibited identities at the nucleotide level of only 84–85, 88–90 and 85–88 % in the p40, p10 and p24 genes, respectively, corresponding to amino acid identities of 97–98, 80–82 and 96–97 % in the p40, p10 and p24 proteins, respectively, compared to other BDV isolates.

DISCUSSION

In general, it is noteworthy that the genetic clustering proved to be independent of the animal species (horse, sheep, donkey or dog) from which the sample originated and the year in which it was collected (1985–1998). Pairwise genetic distances calculated for all aligned sequences of BDV isolates from endemic areas clearly show specific clusters corresponding to territorial origin and allow the establishment of different geographical groups: a Swiss, Austrian and Liechtenstein Rhine valley group, a Baden-Wurttemberg and Bavaria II (or He/80) group in the western part of Germany, a third group that is limited in occurrence to Bavaria, called the Bavaria I (or RW98) group, a southern Saxony-Anhalt and bordering northern Saxony (or strain 'Dessau') group, bound to the territories of these federal states in the eastern part of Germany, and a mixed group that consists of samples from different areas of Germany (however, mainly from the federal states of Thuringia and Lower Saxony) (strain V group) (Figs 3–5).

One feature that all BDV isolates from different regions and clusters have in common is high sequence identity of sequences from cases of the same location or neighbouring locations, independent of time. Three Bavarian BDV isolates within the Bavarian II group are related closely (Figs 3 and 5): two of them (GenBank accession nos AY374524 and AY374533) were isolated from horses of the same farm in 1997 and 1998, and the third one (accession no. AY374530) was isolated in 1998 in a neighbouring village. The region where these BDVs were isolated is located northwest of Augsburg, whereas the other Bavarian isolates clustering within the RW98 group originated from an area southwest of Augsburg. These three sequences form one out of three segregated subclusters in the p24 tree within the otherwise (p40) uniform He/80 or Bavarian II group, indicating that the sequence variation in the p24 gene may be a result of locally determined strain adaptation. In the eastern part of Germany, similar cases of sequence identity within the same region were also observed (horse 18 med 92 with horse A 104 97 and horse A 66 B 3813 with horse A 188 94) (Fig. 3).

The geographical bond further underlines the hypothesis of vector-borne transmission involving reservoir species, which has been raised in several epidemiological studies [Dürrwald, 1993; summarized by Staeheli *et al.* (2000) and Ikuta *et al.* (2002)]. The reasons for mixed geographical clustering could be different indigenous reservoir species and/or vectors, or different subpopulations of these species in different regions with a sympatric (overlapping) occurrence of some of them in certain territories.

Analyses of the exact locations of Bavarian and Baden-Wurttemberg-derived BD cases revealed a large area between Nuremberg, Regensburg, Stuttgart, Munich and the Lake of Constance where BD is prevalent. The sequence clusters of strains RW98 and He/80 occur in a mixed manner without clear segregation only within this area, indicating that distinct but different reservoir or vector species may exist there. South of this region, the river Rhine flows into the Lake of Constance. Upstream of the Rhine are the Austrian, Liechtenstein and Swiss BD-endemic areas. Without any exception, all Liechtenstein-derived and Swiss BD cases analysed in this study originated from places along the Rhine valley and tributaries of the Rhine. The two BD cases of a dog and a horse from Vorarlberg in Austria are also from lowland areas that are connected to the Bavarian and Swiss–Liechtenstein endemic areas by valleys (dog W1582/94 from Mäder in the Rhine valley and horse S5987/97 from Thüringen, near Bludenz in the Ill valley, a tributary of the Rhine). The Bavarian sequence cluster around He/80 (Bavaria II, Fig. 3) is related more closely to the Swiss, Liechtenstein and Austrian group than to those from other German regions, pointing towards a spread of BD along the Rhine valley from the Bavarian endemic region in the second half of the 20th century. This probably reflects movements of the He/80 reservoir or vector species. Changes in geographical distribution of BD have been observed by Dürrwald (1993), who reported a remarkable decrease in BD incidence within the formerly very important endemic region of central Saxony during the 1970s and an increasing occurrence of BD in the northern part of Saxony-Anhalt after World War II. Furthermore, the close connection of BD cases to the Rhine valley indicates a limitation of BD to distinct heights above sea level, as already observed by Zimmermann (1953), who found out in his study (comprising 3642 BD cases from Bavaria in 1948–1952) that, with only one exception (766 m), BD only appeared at heights of <700 m above sea level. In Thuringia, BD is not prevalent in higher places of the Thuringian Forest.

Within the endemic regions in the eastern part of Germany, there is a remarkable segregation of sequences from Thuringia, including areas bordering Saxony-Anhalt, and of those from Saxony-Anhalt. The Thuringia BDV cluster within the 'strain V group' comprises cases from a region between the cities of Sömmerda, Erfurt, Weimar, Zeitz and Weißenfels and a line north of the river Unstrut in Saxony-Anhalt, whereas the Saxony-Anhalt cluster around strain 'Dessau' contains sequences from a broad geographical region in the southern part of Saxony-Anhalt, with frontier areas south of the cities of Halle/Saale and Leipzig and north of the city of Magdeburg. Between these two regions, there is no obvious geographical sign that could explain the strong segregation. This may point towards a strong territorial bond of reservoir host species.

Strain V-related sequences are distributed most widely, mainly in Thuringia and Lower Saxony, but also in Bavaria. This could be due to a wide but rare distribution of reservoirs.

The BD case from Hesse (GenBank accession no. AY374534) originated from a location close to the former border with East Germany, raising the possibility of horse importation from Saxony-Anhalt, which could explain the sequence similarity to sequences of the 'Strain Dessau group'. Interestingly, all sequences that were related most closely to the vaccine strain originated from the same area, from which the ancestor of the vaccine strain probably came. These data could sustain the possibility that the vaccine strain was involved in certain natural cases of BD in Germany; however, not at all at such a high incidence as suggested by other investigators (Danner, 1978, 1982), indicating that place of origin may be a more reasonable explanation for these sequence similarities than spread of the vaccine strain.

Strain No/98 (horse S1131/98) (Nowotny *et al.*, 2000) originated from Burgfried, near Gnas (southeast of Graz) in Styria, Austria. This region is at lowland level and is strongly divided geographically from endemic regions by the Alps; also, this place is several hundred kilometres apart from any BD-endemic region. This may explain the highly divergent genome of strain No/98 compared to all other known BD viruses.

The demonstrated sequence segregation of BDV corresponding to geographical areas may indicate the existence of at least six types of transmission cycles or reservoir populations: the Swiss–Liechtenstein–Austrian Rhine valley type and the related He/80 or Bavaria II type, the RW98 or Bavaria I type, the segregated 'Dessau' strain or southern Saxony-Anhalt type, the widely distributed strain V type, which mainly occurs in Thuringia and Lower Saxony, and, finally, the very distinct No/98 or Styria type, which represents the only member so far of a novel second BDV subtype (Nowotny *et al.*, 2000). The mixed distribution of three types in Bavaria and the clear segregation of the two types from the eastern part of Germany in a region without any geographical barrier point towards the existence of different species of reservoirs or vectors within the same and closely connected territories, respectively. The reservoir hypothesis is further underlined by the fact that final hosts (horses and sheep) show no particularities that may be able to explain the geographically bound sequence clustering.

The vaccine strain 'Dessau' and the laboratory strains are

placed within different groups according to their geographical origin, indicating that there have been no or only few changes since their original isolation. This supports the results of other investigations that have described remarkable genetic stabilities of BDV that are unusual for an RNA virus (Formella *et al.*, 2000). This could indicate that even a single strain of BDV can be highly constrained by cellular mechanisms or selection pressures. These constraints might be stronger than those reported for other RNA viruses (Weaver *et al.*, 1992, 1999; Holmes *et al.*, 2002).

Classical BD is rare nowadays. Fewer than 100 cases occur annually. These are distributed widely over the endemic regions in Europe, making it difficult to collect sufficient numbers of samples for phylogenetic studies. This study comprises the largest collection of sequences from confirmed cases of classical BD in animals, of laboratory and vaccine strains (66 sequences have been included in the 'largest' tree; in total, sequences from 70 BD cases, laboratory or vaccine strains were analysed). This enabled us for the first time to establish geographically separated subgroups of sequence clusters. These sequence clusters allow, in general, a more comprehensive interpretation of the sequencing results. They show that, despite the unusually high genome stability of BDV, sequence variations exist within different geographical areas. Sequences of BD cases from the same or neighbouring locations proved to be particularly stable over years. The given detailed origins of the samples, as listed in Tables 1 and 2, provide a solid basis for future sequence interpretation. A BDV isolate from outside an endemic region (No/98) proved to be clearly separated genetically from all known BDV isolates of endemic areas. Therefore, BDV sequences from outside endemic areas should be interpreted with great caution if they are similar to laboratory strains from endemic regions. A summarized explanation for the geographically bound clustering of BDV isolates could be the possible existence of territorially bound reservoir or vector populations. In conclusion, extensive and detailed studies should be carried out to identify the natural animal host(s) and/or vector(s) of BDV.

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