Psittacine beak and feather disease virus (BFDV) genotypes in Australia

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Introduction

Differences in the clinical and pathological manifestation of PBFD in the wide number of psittacine bird species that are known to be susceptible to infection with beak and feather disease virus (BFDV) have been thought to be due to host factors rather than antigenic or genetic variation in BFDV (Ritchie *et al.,* 1990). However, the latter has not been investigated thoroughly. Different isolates of BFDV behave antigenically similarly at least in haemagglutination and haemagglutination inhibition assays (Raidal *et al.,* 1993) but the use of genetic based techniques for either diagnosis and/or research on PBFD requires an understanding of any genomic variation that may occur between isolates. It is possible that adaptation of particular genotypes to specific species may have occurred, or regional differences in strains may have developed. Such potential differences would have significance in the development of universal PCR assays that would detect all strains of BFDV. They would also have significance in our future understanding of the replication of BFDV, and in the potential gene-coding assignments of the virus.

Concurrent with the sequence analysis of the BFDV reported previously (Bassami *et al.,* 1998) and herein designated BFDV-AUS, sequence analysis of an additional isolate of BFDV was reported in the USA (Niagro *et al.,* 1998). Differences were present in the sequence of this compared to the Australian isolate but the differences were not major and the overall similarity suggested that there may be minimal sequence diversity between strains of BFDV from throughout the world. However, in the related porcine circovirus (PCV) 2 distinct genotypes had been reported (Meehan *et al.,* 1997; Hamel *et al.,* 1998) and it was potentially possible that similar genotypic differences have occurred in BFDV and might be detected if additional isolates of the virus were examined. An investigation of potential sequence diversity in BFDV was undertaken. The complete DNA sequence of 8 BFDV strains from various regions throughout Australia and from a variety of psittacine bird species were compared and the significance of the results discussed.

Materials and methods

Source and preparation of samples

Heparinised or EDTA-containing peripheral blood or dystrophic feathers from 8 BFDV-affected psittacine birds were collected from throughout Australia as shown in Table 1. The virus genomic material contained within the DNA extracted from the PBMC or feathers of each of these birds was defined as a virus "isolate"; care was taken that no individual BFDV-affected bird in this collection had any known contact with other birds from which samples were obtained. Sample preparation and DNA extraction procedures from PBMC and feathers were as previously described (Bassami *et al.,* 1998).

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PCR amplification and sequence analysis of circovirus DNA

Two sets of primers, Iprm3.F and Iprm2.R in one direction, and Prm2.F and Iprm3.R primers in the reverse direction (Table 2), were used to amplify the entire genome of the 8 isolates; the predicted size of the products were 717 bp and 1298 bp. The primers were designed using the sequence of the single BFDV reported previously (Bassami *et al.*, 1998). The PCR products were ligated into the pCR2.1 cloning vector and subsequently sequenced as described previously (Bassami *et al.*, 1998). Direct sequencing of the PCR products was also undertaken.

For each isolate a combination of direct sequencing of PCR products and sequencing of products in pCR2.1 was performed in both directions. The sequence data obtained for each isolate therefore were from at least one pCR2.1 insert and one direct sequencing reaction, in both cases covering the entire genome.

Sequences were edited and assembled using SeqEd (SeqEd TM version 1.0.3, Applied Biosystems Inc) and analysed using a range of software and programs provided by the Australian National Genomic Information Services (ANGIS) and MacVector 6.5 software. For multiple sequence alignment and phylogenetic analysis of sequences the programs PileUp, Distances and Growtree of the Wisconsin Package, Genetics Computer Group (GCG), Madison, Wisconsin, provided by ANGIS were used.

Phylogenetic analysis was undertaken of the complete nt sequence and the predicted amino acid sequences of the proteins encoded by ORF1 and ORF2 of all isolates for which sequence data were available. This included the nt sequences of BFDV-AUS (Bassami *et al.*, 1998) and BFDV-USA (Niagro *et al.*, 1998); the corresponding ORFs of porcine circovirus types 1 and 2 (PCV1 and PCV2; GenBank accession numbers U49186 and AF071878, respectively), and a columbid circovirus (CoCV; GenBank accession number AF252610) were also included for comparison. The data were edited and aligned using the PileUp program (ANGIS). Multiple aligned sequence files were used as input data to generate distances between them using Distances program. A variety of methods were used to compare the results, including the Kimura Two-Parameter Distance (Kimura, 1980) and Jukes-Cantor Distance (Jukes and Cantor, 1969) methods. The calculated distances were used to construct phylogenetic trees using the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) of the Growtree program. The data generated for each phylogenetic analysis were bootstrapped using the ESeqBoot program, a modified version of the PHYLIP software provided in the GCG package.

Results

Comparison of total nucleotide sequence of different isolates

The size of the genome of the 8 BFDV isolates ranged from 1992 to 2018 nt. Two isolates, SCC1-WA and Galah-WA, were 1993 nt, the same as BFDV-AUS (Bassami *et al.*, 1998). The size of the genome of SCC-NT, MMC-WA, and ELBC-SA was 1994, 1995 and 1992 nt, respectively. The genome of one isolate LK-VIC was 2007 nt and LB-WA and BB-WA were 2018 nt (Table 3). Overall nt identity of the isolates compared to BFDV-AUS ranged from 84% to 97% and BFDV-AUS shared 92% nt identity with BFDV-USA.

Comparative organisation of the genome of different isolates

The organisation of the genome of the 8 isolates was similar to that of BFDV-AUS. The similarities included the position of the ORFs, the hairpin structure between ORF1 and ORF2, the nonanucleotide

motif (TAGTATTAC) therein, the 3 motifs of the Rep protein involved in rolling circle replication, and the P-loop motif.

Some point mutations of 1 or 2 nt size were evident in the 8 isolates compared to BFDV-AUS. In addition, various deletions and insertions of more than 2 nt were detected by comparison to BFDV-AUS. In LK-VIC an insertion of 14 nt, an insertion of 4 nt and two insertions of 3 nt occurred, starting at nt 83, 53, 965 and 1936, respectively. In this isolate two deletions of 6 and 3 nt were detected starting at nt 1186 and 1946. In SCC-NT and MMC-WA, there was an insertion of 4 nt at position 53. In addition to this insertion, an insertion of 6 nt at position 1883 for SCC-NT and 1884 for MMC-WA was observed. In these isolates, a deletion of 4 nt commencing at position 89 in both isolates, and a deletion of 3 nt at position 1715 for SCC-NT and 1716 for MMC-WA, were identified. In BB-WA and LB-WA there was an insertion of 17 nt starting at nt 1196, and two insertions of 3 nt at positions 1931 and 1952.

Open reading frame 1

The start codon of ORF1 in isolate LK-VIC was positioned at nt 148, while for other isolates the start codon was at nt 131. The size of ORF1 in all isolates, except LK-VIC that was 870 nt, was the same as BFDV-AUS, 867 nt. In LK-VIC, an insertion of 3 nt (one amino acid) was observed at position 818 of ORF1. The size of ORF1 in BFDV-USA (Niagro *et al.*, 1998) was reported to be 897 nt; however, they indicated the start codon for this ORF as a TCT which is 30 nt upstream of another more likely start codon ATG, which would make the size of the ORF1 of BFDV-USA the same as that of the Australian isolates.

The inter-isolate variation of the nt sequence of ORF1 of BFDV-AUS compared to the 8 isolates and BFDV-USA ranged from 86% to 99% (Table 4). Similarly, the inter-isolate variation of the deduced amino acid sequences ranged from 86% to 99% (Table 4), indicating a high level of conservation in this putative protein.

Open reading frame 2

The size of ORF2 varied, as the actual start codon varied. In BFDV-AUS (Bassami *et al.*, 1998) it was predicted that the start codon for ORF2 was a CTG (Prats *et al.*, 1989) positioned at nt 16 in the complementary strand, while for BFDV-USA, a TCT situated at nt 28, but also in the complementary strand, was reported as the start codon for ORF2 (Niagro *et al.*, 1998). In the isolates sequenced in the current study, the start codon for 2 isolates (SCC-NT and MMC-WA) was proposed to be an ATG positioned at nt 39 in the complementary strand, whereas the start codon for Galah-WA, SCC1-WA and LK-VIC, as well as SCC-ORIG, could have been either CTG at position of 16 or TCT at position of 28 in the complementary strand. The start codon of ORF2 in BB-WA, LB-WA and ELBC-SA was probably a TCT located at nt 29 for BB-WA, LB-WA and 28 in ELBC-SA.

The identity of the nt sequence of ORF2 compared to BFDV-AUS varied from 80% to 99%, while the identity of the deduced amino acid sequences varied from 73% to 99% (Table 5).

Open reading frames 3 to 7

Apart from ORF1 and 2, the only other ORF shared in all isolates (Table 3) was the ORF previously designated ORF 5 (Bassami *et al.*, 1998). The size of this ORF5 varied in the different isolates: 303 nt in BFDV-AUS, Galah-WA, MMC-WA and ELBC-SA isolates, potentially encoding a protein of 101 amino acids, and 474 nt in the other isolates and including BFDV-USA, potentially encoding a protein

of 158 amino acids. The first 303 nt at the 5' end of those isolates with an ORF5 of 474 nt and the entire sequence of ORF5 of the other isolates was common in the 2 groups of isolates, with some point variations. ORF5 along with the inconsistently identified ORFs failed to demonstrate any significant identity with genomic sequences in the GenBank sequence database (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis of isolates

For phylogenetic analysis of the isolates, the nt sequence and the predicted amino acid sequences of the proteins encoded by ORF1 and ORF2 were edited as there were differences in the start codon of ORF2 in the different Australian isolates and the ORF1 of BFDV-USA; the first 10 amino acids (first 30 nt) of ORF1 of BFDV-USA, the first 8 amino acids (23 nt) of SSC-ORIG, SCC1-NT, Galah-WA and LK-VIC, and the first 4 amino acids (13 nt) of BB-WA and LB- WA were removed.

Phylograms of the entire nucleotide sequence (data not shown) and the predicted amino acid sequence of the proteins encoded by ORF1 and ORF2 of the 10 BFDV isolates for which sequence data were available showed that the isolates were in 4 clusters (Figure 1) but there were no regional differences or differences related to the psittacine species of origin.

Discussion

All 8 BFDV isolates for which complete sequence data were obtained in this study had the same basic structure as the BFDV-AUS isolate described previously (Bassami *et al.,* 1998), including the position of the ORFs, the location of a hairpin (stem-loop) structure (presumed to contain the origin of rolling circle replication) located between ORF1 and ORF2, the nonanucleotide motif (TAGTATTAC) therein, the 3 motifs within ORF1 involved in rolling circle replication, and the P-loop motif (Bassami *et al.,* 1998). The size of the BFDV genome in the 10 isolates for which sequence data are now available, varied from 1992 to 2018 nt. The Australian isolates were 1993 nt (3), 1992 (1), 1994 (1), 1995 nt (1), 2007 nt (1) and 2018 nt (2), and BFDV-USA described by Niagro *et al.* (1998) was 1993 nt. The differences were due to a number of small deletions and insertions compared to the BFDV-AUS described by Bassami et al. (1998). The insertions and deletions were mainly of 1 to 4 nt in size but a larger 14 nt insertion occurred in the non-coding regions of LK-VIC and a 17 nt insertion occurred in BB-WA and LB-WA. As the larger 14 and 17 nt insertions occurred in the non-coding regions, they are probably of little significance. The 1 to 4 nt insertions and deletions occurred not only in non-coding but also in ORF1 and ORF2 but the significance of these changes is unknown.

Overall nt identity of the isolates, compared to BFDV-AUS (Bassami *et al.,* 1998), ranged from 84% to 97% and there was no evidence that distinctly different genotypes occurred as have been described in PCV (Meehan *et al.,* 1997). The BFDV isolate reported from the USA (Niagro *et al.,* 1998) had 92% nt identity with BFDV-AUS.

Up to 7 ORFs were detected that were potentially able to encode proteins of greater than 8.7 kDa in BFDV-AUS (Bassami *et al.,* 1998), but not all these ORFs were present in all isolates. In the 8 isolates sequenced and described in this report, the number of ORFs potentially capable of encoding proteins greater than 8.7 kDa ranged from 4 to 7. The BFDV-USA isolate was reported to contain only 3 ORFs (Niagro *et al.,* 1998) but re-examination of the sequence indicated there were 4 ORFs potentially encoding proteins greater than 8.7 kDa. Of likely significance, however, is that the only ORFs consistently detected in all isolates, including BFDV-AUS and BFDV-USA were the ORFs described as 1, 2 and 5 in BFDV-AUS (Bassami *et al.,* 1998). The start codons for these ORFs was in all cases ATG except for ORF2 where in 2 cases it was ATG, in 4 cases CTG, and in 4 cases either CTG or TCT. Start codons other than ATG have been reported in other systems: CTG has been reported as a

start codon for the synthesis of a cell surface antigen coded by the murine leukemia virus (Prats *et al.,* 1989), and TCT was reported as a start codon for ORF1 by Niagro *et al.* (1998). However, Niagro *et al.* (1998) failed to detect the ATG start codon of the ORF1 of BFDV-USA, possibly because the G of the ATG was mistakenly identified as C; these authors therefore identified the start codon of ORF1 as an TCT, an additional 30 nt upstream of the correct start codon.

The size of ORF1 in all isolates, except LK-VIC which was 870 nt, were the same as BFDV-AUS, 867 nt. In LK-VIC an insertion of 3 nt (one amino acid) was observed at nt 818 of ORF1. The size of this ORF in BFDV-USA was reported to be 897 nt but the extra 30 nt were because Niagro *et al.* (1998) predicted that a TCT codon, 30 nt upstream of real start codon (ATG), acted as the start codon. The nt sequence variation in ORF1 ranged from 86% to 99% compared to BFDV-AUS. The degree of conservation in this ORF was greater than in any other ORF of the isolates sequenced.

As only 3 ORFs were consistently detected in all isolates of BFDV, and ORF1 is very likely to encode the Rep protein required for rolling circle replication (Mankertz *et al.,* 1997), then it follows that either ORF2 or ORF5 encodes the capsid protein. Based on the identity of the deduced amino acid sequence of ORF2 of BFDV to the deduced amino acid sequence of the protein encoded by ORF2 of PCV (Bassami *et al.,* 1998) it is likely that the ORF2 of BFDV encodes the capsid protein. This was also suggested by Niagro *et al.* (1998).

In only 2 isolates was the start codon for ORF2 an ATG; in the other isolates and including the USA isolate (Niagro *et al.,* 1998) it was CTG or TCT. There was up to 73% variation in the deduced amino acid sequences of the protein encoded by ORF2 of the various BFDV isolates; the different start codons and the position of these start codons contributed to differences in the nt identity and amino acid identity of the ORF2 in different isolates. Although there is currently no evidence of intra-species antigenic variation, this degree of amino acid variation in ORF2 suggests that intra-species antigenic variation might occur in the encoded capsid proteins, and further study of this possibility seems warranted.

The third ORF, in addition to ORF1 and ORF2, common to all isolates, was ORF5. This ORF was of 2 size classes in different isolates, 303 nt and 474 nt, and it was the first 303 nt of the ORFs of 474 nt that was common in the other isolates. Whether ORF5 encodes a protein, and if it does then the function of this protein, is unknown and needs to be determined. Proteins in addition to the Rep and capsid proteins have been identified in other circoviruses. CAV, although it has minimal genomic similarity to the other animal circoviruses, has 3 partially overlapping major reading frames encoding putative proteins of 51.6, 24.0 and 13.6 kDa (Noteborn *et al.,* 1991), the largest of which is capsid and the smallest of which is apoptin, an early protein responsible for apoptosis (Douglas *et al.,* 1995). The 24 kDa protein of CAV (Douglas *et al.,* 1995) may act as a scaffold protein during virion assembly (Noteborn *et al.,* 1998). The plant nanovirus BBTV encodes a Rep protein (Harding *et al.,* 1993), a capsid protein (Wanitchakorn *et al.,* 1997), a putative 5 kDa protein (Beetham *et al.,* 1997) and a 10 kDa protein (Beetham, *et al.,* 1999) of unknown function. Another plant nanovirus, faba bean necrotic yellows virus (FBNYV), encodes a Rep (Katul *et al.,* 1995) and a capsid protein (Katul *et al.,* 1997), in addition to a putative protein of 17.4 kDa of unknown function but highly conserved between FBNYV and the other nanoviruses (Katul *et al.,* 1998). Geminiviruses also encode proteins in addition to the Rep, capsid and movement proteins: the geminivirus AC2 gene product transactivates the expression of the coat protein, perhaps mediated by unknown *cis*-acting elements (Ruiz-Medrano, 1999); the geminivirus AL2 gene product probably transactivates expression of the capsid protein and a movement protein gene (Horvath *et al.,* 1998).

The phylogenetic relationship between the predicted amino acid sequences of the proteins encoded by ORF1 and ORF2 of the 10 isolates for which sequence data were available, revealed that the virus isolates could be grouped into genetic clusters. Although some differences were detected when different methods were used to analyse the data, the isolates were consistently grouped in the same patterns by different methods. While most isolates were in one cluster and appeared to have a more recent common origin, 5 isolates clustered separately from the other isolates, including one cluster with 2 isolates LB-WA and BB-WA, another cluster with two isolates SCC-NT and MMC-WA, and third cluster with a single isolate from a rainbow lorikeet (LK-VIC). The significance of these differences between the isolates is unknown. There is little evidence to support a relationship between the genetic differences and the regional distribution of the isolates, or that there are differences in pathogenicity, antigenicity or any other physicochemical characteristics of BFDV. Nevertheless, the genetic variation detected in the different isolates needs to be considered when designing or interpreting the results of DNA-based assays such as PCR.

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Table 1. Samples from PBFD-affected birds of various species and geographical origins in Australia that were used as a source of genomic DNA for DNA sequence analysis of BFDV.

Isolate	Species	Origin
BB-WA	Blue bonnet *(Psephotus haematogaster*)	Private collection, Esperance
LK-VIC	Rainbow lorikeet (*Trichoglossus haematodus*)	Private collection, Melbourne
MMC-WA	Major Mitchell's cockatoo (*Cacatua leabeateri*)	Private collection, Perth
SCC1	Sulphur crested cockatoo (*Cacatua galerita*)	Wild bird, Perth
SCC-NT	Sulphur crested cockatoo	Wild bird, Darwin
LB-WA	Lovebird (*Agapornis roseicollis*)	Private collection, Perth
Galah-WA	Galah (*Eolophus roseicapillus)*	Wild bird, Perth
ELBC-SA	Eastern long-billed corella *(Cacatua tenuirostris)*	Private collection, Adelaide

Table 2. PCR primers used to amplify the complete nucleotide sequence of the 8 BFDV isolates. Primer pairs Prm2.F and Iprm2.R were used in one direction, and Iprm3.F and Iprm3.R in the reverse direction.

Primer	Sequence: (5' - 3')	Size	Position in virion strand	Position in complementary strand
Prm2.F	TGGTACAAGGAGGACTGTGAC	21	878-898	
Iprm2.R	GTCACAGTCCTCCTTGTACC	20		1097-1116
Iprm3.F	(p)AACCCTACAGACGGCGAG	18	182-199	
Iprm3.R	(P)GTTAAGGGTGAAACACCAA CG	21		1814-1834

Table 3. Comparison of the genome size (nt length) and predicted ORFs encoding proteins greater than 8.7 kDa of the different isolates that were sequenced and including our original BFDV-AUS isolate (Bassami *et al.*, 1998; GenBank accession No. AF08060). The BFDV-USA isolate described by Niagro *et al*. (1998; GenBank accession No. AF071878) is shown for comparison.

Isolate	Genome size (nt)	Total ORFs	ORF 1	ORF 2	ORF 3	ORF 4	ORF 5	ORF 6	ORF 7
BFDV-AUS	1993	7	867	741	480	318	303	264	258
Galah-WA	1993	7	867	741	495	-	303	264	318
MMC-WA	1995	7	867	720	273	336	303	264	279
ELBC-SA	1992	5	867	729	495	-	303	264	-
BB-WA	2018	4	867	735	495	-	474	-	-
LB-WA	2018	4	867	735	456	-	474	-	297
LK-VIC	2007	5	870	741	498	-	474	-	282
SCC1-WA	1993	6	867	741	495	318	474	-	306
SCC-NT	1994	5	867	720	-	372	474	-	-
BFDV-USA	1993	4	897	732	471	-	474	-	-

Table 4. Pairwise comparison of nt and amino acid sequences of ORF1 (Rep) using Clustal W program (MacVector 6.5 software). The origin of isolates was as shown in Table 1; BFDV-AUS indicates the isolate described by Bassami et al. (1998; GenBank accession No. AF08060) and BFDV-USA the isolate described by Niagro *et al*. (1998; GenBank accession No. AF071878).

Isolate	BFDV-AUS	SCC1-WA	SCC-NT	MMC-WA	Galah-WA	ELBC-SA	LK-VIC	LB-WA	BB-WA	BFDV-USA
									Nucleotide identity (%)	
BFDV-AUS		95	94	95	95	96	88	93	94	91
SCC1-WA	96		93	96	99	96	90	93	94	91
SCC-NT	93	93		94	94	94	88	91	92	90
MMC-WA	96	97	93		96	97	90	94	95	92
Galah-WA	96	99	94	97		96	90	94	94	91
ELBC-SA	95	95	93	96	95		89	93	94	96
LK-VIC	88	89	87	89	89	88		88	89	86
LB-WA	94	93	90	94	94	92	86		99	90
BB-WA	96	95	92	95	95	93	88	98		91
BFDV-USA	92	92	89	92	93	91	86	90	91	
	Amino acid identity (%)									

Table 5. Pairwise comparison of nt and amino acid sequences of ORF2 (Capsid) using Clustal W program (MacVector 6.5 software). The origin of isolates was as shown in Table 1; BFDV-USA indicates the isolate described by Niagro *et al*. (1998; GenBank accession No. AF071878).

Isolate	BFDV-AUS	SCC1-WA	SCC-NT	MMC-WA	Galah-WA	ELBC-SA	LK-VIC	LB-WA	BB-WA	BFDV-USA
							Nucleotide identity (%)			
BFDV-AUS		99	80	80	96	94	82	85	85	88
SCC1-WA	90		80	80	95	94	82	85	85	88
SCC-NT	75	75		99	80	81	80	83	82	83
MMC-WA	75	75	99		80	81	80	82	82	83
Galah-WA	95	95	73	74		96	83	85	85	88
ELBC-SA	92	92	74	75	94		81	85	86	89
LK-VIC	78	78	74	74	78	76		85	85	84
LB-WA	81	80	75	76	82	81	80		98	88
BB-WA	82	82	76	76	83	82	80	95		88
BFDV-USA	85	85	76	77	85	85	87	81	82	
	Amino acid identity (%)									

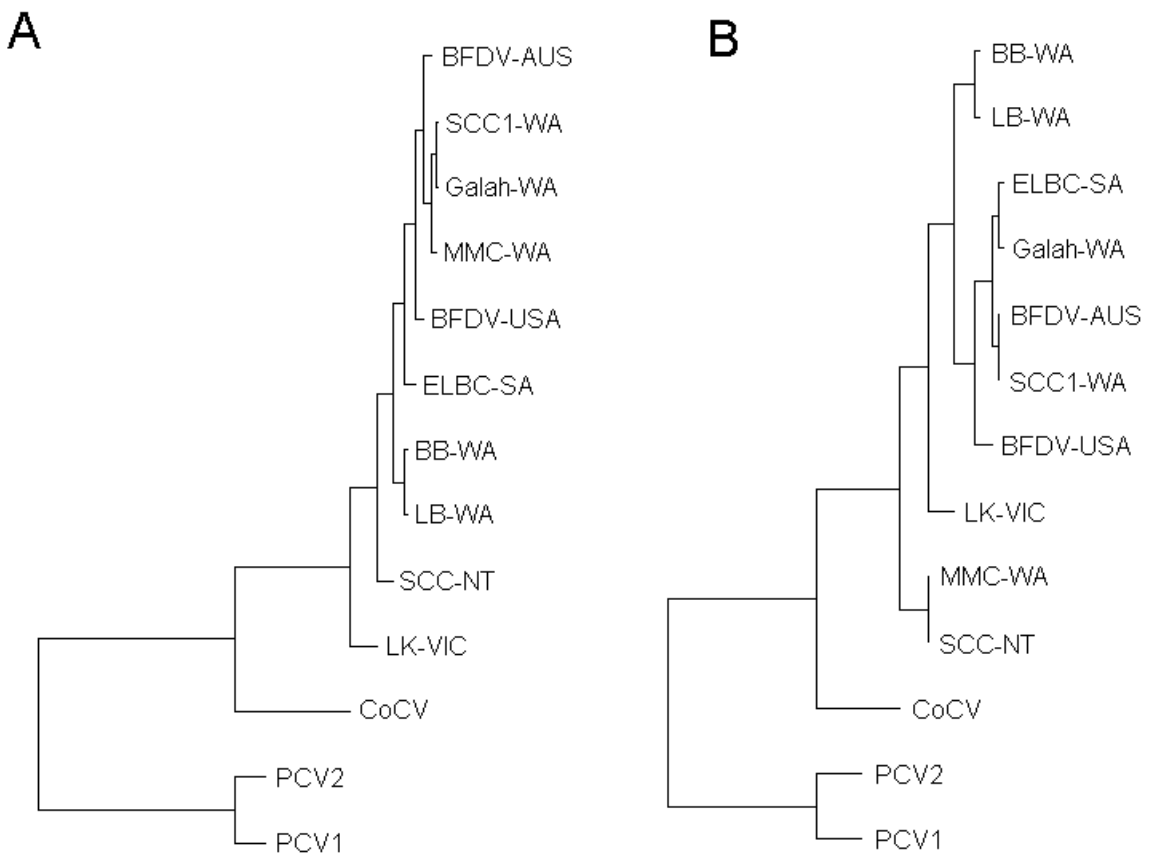

Figure 1. Unrooted phylograms of 10 isolates of BFDV based on the deduced amino acid sequence of the putative Rep and capsid proteins encoded by ORF1 (A) and ORF2 (B), respectively. The corresponding ORFs of porcine circovirus types 1 and 2 (PCV1 and PCV2; GenBank accession numbers U49186 and AF071878, respectively), and a columbid circovirus (CoCV; GenBank accession number AF252610) have been included for comparision. The phylograms were generated by UPGMA method based on the distance matrix calculated by Kimura and Jukes-Cantor methods, respectively. The abbreviations used to denote the isolates and their origins are as described in Table 1; BFDV-AUS refers to the isolate described previously by Bassami *et al.,* (1998) and BFDV-USA to the isolate described by Niagro *et al*., (1998).