

Extensive recombination detected among beak and feather disease virus isolates from breeding facilities in Poland

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Abstract

Beak and feather disease virus (BFDV) causes the highly contagious, in some cases fatal, psittacine beak and feather disease in parrots. The European continent has no native parrots, yet in the past has been one of the world's biggest importers of wild-caught exotic parrot species. Following the banning of this practice in 2007, the demand for exotic pet parrots has largely been met by established European breeding facilities, which can also supply buyers outside Europe. However, the years of unregulated importation have provided numerous opportunities for BFDV to enter Europe, meaning the likelihood of birds within captive breeding facilities being BFDV positive is high. This study examined the BFDV status of such facilities in Poland, a country previously shown to have BFDV among captive birds. A total of 209 birds from over 50 captive breeding facilities across Poland were tested, and 43 birds from 18 different facilities tested positive for BFDV. The full BFDV genomes from these 43 positive birds were determined, and phylogenetic analysis revealed that these samples harboured a relatively high degree of diversity and that they were highly recombinant. It is evident that there have been multiple introductions of BFDV into Poland over a long period of time, and the close association of different species of birds in the captive environment has probably facilitated the evolution of new BFDV strains through recombination.

Introduction

Psittacine beak and feather disease (PBFD) is a highly contagious and often fatal disease of parrots. Characteristic symptoms include depression, lethargy and anaemia, progressing to abnormal feather growth and culminating in bilateral, symmetrical loss of feathers (Ritchie *et al.*, 1989). Although in some cases the beak may also be affected by necrosis, overgrowth or cracking, this does not appear to occur in all species (Pass & Perry, 1984; Ritchie *et al.*, 1989). Infection may be acute or chronic. Whilst the acute form often affects juvenile birds, causing sudden death even in the absence of symptoms (Doneley, 2003), the chronic form is not always fatal, and infected birds may survive for years (Ritchie *et al.*, 1989). However, as PBFD is also thought to target immune tissue, chronically infected birds will frequently eventually succumb to secondary infections due to PBFD-induced immune suppression (Latimer *et al.*, 1991; Ritchie *et al.*, 1989; Todd, 2000).

The main causative agent of Pbfd is beak and feather disease virus (BFDV), a non-enveloped icosahedral virus (~20 nm in diameter) with a 2 kb circular ssDNA genome, in the family *Circoviridae* (Ritchie *et al.*, 1989, 1990). The BFDV genome is transcribed bidirectionally with two major ORFs: ORF1 on the virion strand encoding a replication-associated protein (Rep), and ORF2 on the complementary strand of the double-stranded replicative intermediate encoding the capsid protein (CP) (Niagro *et al.*, 1998). The function of a third ORF located on the virion strand of all BFDV isolates is currently unknown.

Whilst feather loss among infected wild Australian parrots has been noted since early in the 20th century (Ashby, 1906), Pbfd was only described officially in a number of South Pacific psittacine birds in the 1970s (Pass & Perry, 1984). BFDV is highly contagious, highly environmentally stable and, besides being transmitted via faeces, feather dust and crop secretions, can also be transmitted to unhatched chicks by their infected mothers (Rahaus *et al.*, 2008; Ritchie *et al.*, 1991; Todd, 2000). Given that more than a quarter of all known parrot species are considered to be at risk of extinction (IUCN Red List of Threatened Species, version 2011, <http://www.iucnredlist.org/>), it is of some concern that the international parrot trade could potentially facilitate the spread of pathogenic BFDV variants that might ultimately endanger wild parrot populations.

Wild parrots are naturally found predominantly in the southern hemisphere, generally in tropical or subtropical regions (Forshaw, 2010), although they also occur in the more temperate regions of South America and Africa and on temperate islands such as New Zealand. There are no parrots native to continental Europe. However, during the time of unregulated parrot trafficking (prior to 2007), many different parrot species were imported either as pets or for trading purposes, and the occasional release of some of these birds into the wild has today resulted in the establishment of wild populations of some species in the more temperate areas of Europe. Accordingly, psittacine species now account for ~18 % of Europe's established wild populations of introduced birds (Strubbe & Matthysen, 2009). The ban on wild-caught parrot importation into Europe [Commission Regulation (EC) No. 318/2007] has not reduced the demand for pet parrots, and hence most of this demand is met from established European breeding facilities, which are also able to export birds to buyers in countries outside Europe. Whilst breeding facilities may indirectly have had a positive influence on the conservation of the various endangered parrot species, these facilities can provide an ideal environment for cross-species transmission of BFDV strains and, potentially, for the rapid evolution of novel BFDV variants through recombination in birds co-infected with genetically distinct variants of these viruses.

Previous studies have reported on BFDV infection in European captive psittacine breeding facilities (Bert *et al.*, 2005; Henriques *et al.*, 2010; Piasecki & Wieliczko, 2010; Rahaus & Wolff, 2003; Tomasek & Tukac, 2007), but in the majority of cases, rather

than isolating and sequencing full viral genomes, only a fragment of Rep was amplified simply for the purpose of determining the presence of the viral infection. This is significant because various studies have shown that recombination is a major process contributing to the diversity of circoviruses in general (Cai *et al.*, 2012; Cheung, 2009; Heath *et al.*, 2004; Lefeuvre *et al.*, 2009; Massaro *et al.*, 2012; Varsani *et al.*, 2010, 2011) and BFDV in particular. For such species, analyses of full-length genome sequences can often provide greater evolutionary insights than studies of individual genes.

Here, we determined BFDV full-genome sequences isolated from a range of psittacine species sampled from breeding facilities throughout Poland. Whilst recombination analyses of the full-length genomes confirmed the evidence of frequent genetic exchanges between different BFDV lineages, phylogenetic analyses showed that there have probably been multiple introductions of BFDV into Poland.

Results and discussion

BFDV screening

Of 209 birds from 23 genera sampled from 50 breeding facilities across Poland, 43 birds (from 14 species in ten genera) tested positive for BFDV. These positive samples were from 18 different breeding facilities across Poland (Fig. 1 and Table S1, available in JGV Online). There were nine positive samples each from budgerigars (*Melopsittacus undulatus*; 9/13 birds tested, 69.2 %), ring-necked parakeets (*Psittacula krameri*; 9/32, 28.1 %) and African grey parrots (*Psittacus erithacus*; 9/52, 17.3 %). The other positive samples were from Australian king parrots (*Alisterus scapularis*; 2/4, 50 %), red-winged parrots (*Aprosmictus erythropterus*; 2/4, 50 %), crimson rosellas (*Platycercus elegans*; 2/4, 50 %), Senegal parrots (*Poicephalus senegalus*; 2/6, 33.3 %), Alexandrine parakeets (*Psittacula eupatria*; 2/ 3, 66.7 %), a blue-fronted Amazon (*Amazona aestiva*; 1/5, 20 %), an orange-winged Amazon (*Amazona amazonica*; 1/ 2, 50 %), a white cockatoo (*Cacatua alba*; 1/1, 100 %), a Pacific parrotlet (*Forpus coelestis*; 1/1, 100 %), an Eastern rosella (*Platycercus eximius*; 1/2, 50 %) and a Cape parrot (*Poicephalus robustus*; 1/1, 100%).

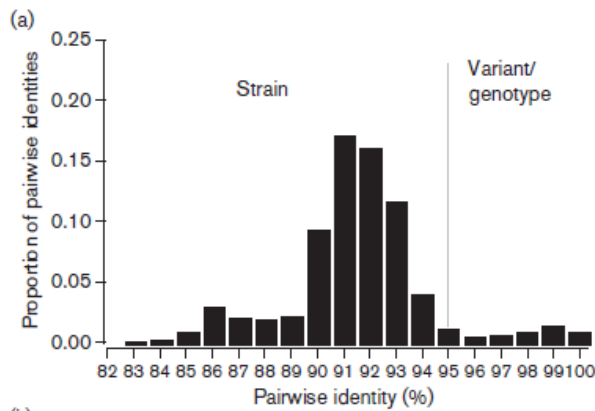
Full-genome pairwise genetic identity analysis

We performed analysis of the distribution of pairwise identities of the 43 genomes determined in this study together with 141 full BFDV genomes available in GenBank (a total of 184 genomes involving 16 836 pairwise comparisons) in order to rationally assign these to tentative strain groupings for the purposes of this study (Fig. 2a and Supplementary Data). The distribution indicated that the majority of the pairwise identity scores fell within the range 90–94 %.

Scientific name	Common name	Number tested	Number positive	Virus strain
<i>Agapornis</i> sp.	Lovebirds	6	0	
<i>Alisterus scapularis</i>	Australian king parrot	4	2	
<i>Amazona aestiva</i>	Blue-fronted Amazon	5	1	
<i>Amazona amazonica</i>	Orange-winged Amazon	2	1	
<i>Amazona barbadensis</i>	Yellow-shouldered Amazon	3	0	
<i>Amazona ochrocephala</i>	Yellow-fronted Amazon	2	0	
<i>Amazona</i> sp.	Amazon parrot	2	0	
<i>Aprosmictus erythropterus</i>	Red-winged parrot	4	2	
<i>Ara ararauna</i>	Blue and yellow macaw	17	0	
<i>Ara chloroptera</i>	Red and green macaw	8	0	
<i>Ara macao</i>	Scarlet macaw	3	0	
<i>Aratinga scuticaudata</i>	Blue-crowned conure	1	0	
<i>Barnardius barnardi</i>	Malles ring-necked parrot	8	0	
<i>Barnardius zonarius</i>	Port Lincoln parrot	2	0	
<i>Cacatus siba</i>	White cockatoo	1	1	
<i>Cyanoliscus patagonus</i>	Patagonian conure	1	0	
<i>Dicopsittacus nobilis</i>	Red-shouldered macaw	2	0	
<i>Ecluctus oratus</i>	Ecluctus parrot	1	0	
<i>Elaphus roseicapillus</i>	Galah	8	0	
<i>Forpus coelestis</i>	Pacific parrotlet	1	1	
<i>Melopsittacus undulatus</i>	Budgerigar	13	9	
<i>Pionites melanocephalus</i>	Black-headed caique	1	0	
<i>Platycercus elegans</i>	Crimson rosella	4	2	
<i>Platycercus eximius</i>	Eastern rosella	2	1	
<i>Poicephalus robustus</i>	Cape parrot	1	1	
<i>Poicephalus senegalus</i>	Senegal parrot	6	2	
<i>Probosciger aterrimus</i>	Palm cockatoo	1	0	
<i>Propyrrhus maracana</i>	Blue-winged macaw	2	0	
<i>Psephenus</i> sp.	Grass parrots	2	0	
<i>Psittacula alexandri</i>	Red-breasted parakeet	3	0	
<i>Psittacula cyanocephala</i>	Plum-headed parakeet	2	0	
<i>Psittacula derbiana</i>	Lord Derby's parakeet	1	0	
<i>Psittacula euphratis</i>	Alexandrine parakeet	3	2	
<i>Psittacula krameri</i>	Ring-necked parakeet	32	9	
<i>Psittacus erithacus</i>	African grey parrot	52	9	
<i>Trichoglossus haematodus</i>	Rainbow lorikeet	3	0	
Total		209	43	



Fig. 1. Details of parrots tested for BFDV are listed in the table on the left and the sampling locations of BFDV isolates with assigned strains are provided in the map on the right.



(b)

○	<i>Agapornis</i> sp.		ROL	43
	Black-cheeked lovebird	1	FRT	6
	Rosey-faced lovebird	2	DEU	1
	Lovebird	1	GBR	2
⊙	<i>Alisterus</i> sp.		ZAF	35
	Australian king parrot	2	ZMB	1
●	<i>Amazona</i> sp.		CHN	1
	Amazon parrot	2	THA	17
	Blue-fronted Amazon	1	JPN	7
	Orange-winged Amazon	1	AUS	12
▲	<i>Aprosmictus</i> sp.		NZL	26
	Red-winged parrot	2	USA	31
○	<i>Ara</i> sp.			
	Great green macaw	1		
	Blue and yellow macaw	1		
	Red and green macaw	1		
	Red-shouldered macaw	1		
	Severe macaw	1		
●	<i>Cacatua</i> sp.			
	White cockatoo	2		
	Major Mitchell's cockatoo	1		
	Salmon-crested cockatoo	1		
	Sulphur crested cockatoo	5		
	Eastern long-billed corella	1		
⊖	<i>Cyanoramphus</i> sp.			
	Yellow-crowned parakeet	8		
	Red fronted parakeet	16		
	New Caladonian parakeet	1		
⊕	<i>Eclectus</i> sp.			
	Eclectus parrot	9		
+	<i>Eolophus</i> sp.			
	Galah	1		
▲	<i>Fapsus</i> sp.			
	Pacific parrotlet	1		
●	<i>Melopsittacus</i> sp.			
	Budgerigar	19		
●	<i>Nymphicus</i> sp.			
	Cockatiel	3		
★	<i>Pionites</i> sp.			
	White-bellied caique	1		
★	<i>Platyercus</i> sp.			
	Eastern rosella	8		
	Crimson rosella	2		
○	<i>Poicephalus</i> sp.			
	Jardine parrot	5		
	Cape parrot	14		
	Ruppell's parrot	1		
	African red-bellied parrot	1		
	Senegal parrot	2		
☺	<i>Probosciger</i> sp.			
	Palm cockatoo	1		
●	<i>Psephotus</i> sp.			
	Bluebonnet	1		
	Red-rumped parrot	1		
●	<i>Psittacula</i> sp.			
	Alexandrian parrot	5		
	Ring necked parakeet	14		
●	<i>Psittacus</i> sp.			
	African grey parrot	23		
⊗	<i>Trichoglossus</i> sp.			
	Rainbow lorikeet	2		
	Deplanche's lorikeet	17		
⊕	Unknown sp.			
	Unknown (pooled)	1		

Fig. 2. (a) Distribution of pairwise identities (with pairwise deletion of gaps) of 184 full-length BFDV genomes (16836 pairwise comparisons). The vertical line indicates the 95% cut-off value for assignment of strains. (b) Summary of strains assigned to the full genomes of BFDV from this study and those available in GenBank. Boxed letters indicate the virus strains and colours indicate the country of isolation.

Based on this distribution of pairwise identity scores, sequences sharing .95 % identity were considered here to belong to different strains of BFDV. We assigned each of the 26 discrete strain groupings a letter between A and Z. We further categorized the sequences within each strain grouping into descriptive subtype groupings with isolates sharing .98 % pairwise identity considered to belong to the same subtype. Subtypes were assigned a number given as a subscript to the strain name (e.g. subtype 1 in strain A is referred to as A₁). Besides these descriptive classifications, we retained previously proposed nomenclatures ([Julian *et al.*, 2012](#); [Massaro *et al.*, 2012](#); [Ortiz-Catedral *et al.*, 2010](#); [Varsani *et al.*, 2010, 2011](#)), except that the budgerigar isolates were renamed (Fig. 2b and Table S1).

Under this tentative classification, the newly sequenced BFDV genomes from Poland included those belonging to five new strains: BFDV-Q (*n*54), BFDV-R (*n*51), BFDV-S (*n*52), BFDV-T (*n*510) and BFDV-U (*n*51). There were two subtypes of BFDV-Q, with subtype Q₁ isolated from two budgerigars and a crimson rosella, and subtype Q₂ isolated from a Pacific parrotlet. BFDV-Q₁ isolates shared .98 % pairwise identity among themselves and ~94 % identity with BFDV-Q₂ isolates (Supplementary Data). A BFDV-R₁ isolate from a blue-fronted Amazon shared ~92–94 % identity with BFDV-B isolates sampled from cockatoos, cockatiels and galahs in Australia and Japan. BFDV-S₁ isolates from two red-winged parrots shared between ~92 and 94 % pairwise sequence identity with BFDV-A isolates, whilst the two isolates shared 98 % identity with each other. BFDV-T isolates formed two subtypes: three BFDV-T₁ isolates from African grey parrots, three from ring-necked parakeets, two from budgerigars and one from an orange-winged Amazon. These BFDV-T₁ isolates shared .98.7 % identity with each other and ~96–97 % identity with a BFDV-T₂ isolate from an African grey parrot. As a group, the BFDV-T isolates shared between 92 and 94 % pairwise sequence identity with BFDV-L and -M isolates from South Africa and Zambia. Finally, BFDV-U₁ was isolated from an Australian king parrot and shared 92.9–95.7 % identity with BFDV-K isolates from Australia, Thailand, the UK and the USA. The remaining 25 isolates belonged to existing strains, although new subtypes of these strains were also detected (Table S1).

Of the 18 breeding facilities with BFDV-infected birds, 11 had birds that were all infected with a single strain. In the other seven facilities, however, two or more strains were detected (Fig. 1). The presence of multiple strains within a single facility increases the chances of birds becoming co-infected with viruses belonging to more than one genetically divergent BFDV lineage. This in turn increases the chances within these facilities of novel divergent BFDV variants arising through recombination.

Polish BFDV isolates are highly recombinant

Evidence of recombination was detected in a large number of Polish BFDV isolates, and a summary is provided in Fig. 3. Similar to observations made by [Lefeuvre *et al.* \(2009\)](#)

and [Varsani et al. \(2011\)](#), we noted that the intergenic regions were apparently recombination hotspots. Striking among all of the detected recombination events was that the genomes of the budgerigar and ring-necked parakeet BFDV isolates (BFDV-X, W, -Y and -Z from Poland, Japan and China) were all highly recombinant. Within these genomes, we found evidence of three to five independent recombination events.

BFDV-X₂ isolates from four ring-necked parakeets were all found to be descended from a highly recombinant common ancestral genome carrying evidence of at least five separate recombination events. Event 1 in Fig. 3, spanning a portion of the intergenic region and the N-terminal region of the *rep* gene, involved an ~600 bp fragment derived from a BFDV-W₁-like virus. Completely embedded within this ~600 bp region was a second ~285 bp fragment derived from an unknown (i.e. currently unsampled) BFDV strain (event 7 in Fig. 3). Events 9 and 13 overlapped within the *rep* gene, with event 9 involving an ~250 bp fragment derived from a BFDV-Y₂-like isolate, and event 13 involving an ~370 bp fragment from a BFDV-Y-like isolate. Finally, with event 10 in Fig. 3, a fragment of ~310 bp spanning the 39-intergenic region and the C-terminal portion of the *cp* gene was derived from a virus resembling BFDV-N, -Q and -Z isolates. Interestingly, BFDV-X₁, -W₁ and -Y, all isolates from budgerigars (Japan and China), also carried evidence of some of these recombination events (events 1, 7 and 10; Fig. 3). This suggested either that these recombination events predated the divergence of these groups or that larger genome fragments carrying these recombinationally derived sequences had themselves been spread by more recent recombination among multiple BFDV lineages. It is plausible that mixed infections involving different strains within budgerigars may have yielded a prototype BFDV-X₂ genome with altered host specificity. In addition to evidence of recombination events in common with the BFDV-X₂ isolates, the BFDV-X₁ and -W₁ isolates shared evidence of two other recombination events (events 5 and 12 in Fig. 3).

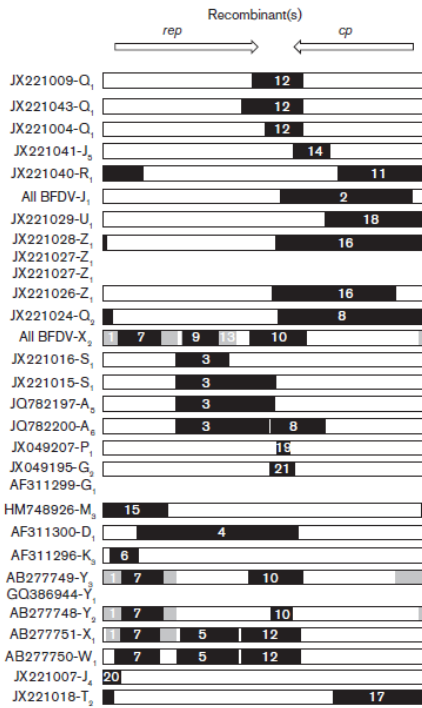
The particularly high degrees of recombination observable among some of the Polish BFDV isolates could conceivably be the direct consequence of Europe's long history of parrot importation. The captive breeding facilities that these birds are generally contained in provide the ideal environment for the rapid evolution of BFDV through recombination. The six previously unsampled BFDV strains (Q, R, S, T, U and Z) that are all unique to Poland all carry evidence of recombination events. These viruses might in fact represent direct evidence for the emergence of novel recombinant BFDV strains within the network of Polish breeding facilities. The possibility that movements of breeding stock between these facilities might, by fostering an environment within Poland that is conducive to the spreading and mixing of BFDV lineages, eventually lead to the emergence of novel pathogenic recombinant BFDV variants certainly warrants further investigation. Furthermore, it is noteworthy that, given the predominance of Polish recombinant viruses among the European BFDV isolates that have been analysed here, it is possible that situations similar to those in Poland exist in other European countries. BFDV sampling and full-genome

sequencing studies should perhaps also be carried out in these other countries to assess properly whether the exotic bird trade is accelerating the recombinational evolution of BFDV.

Phylogenetic evidence for multiple BFDV introductions into Poland

A maximum-likelihood (ML) phylogenetic tree was constructed using an alignment of all available full-length BFDV genome sequences from which all detectable evidence of recombination was removed (by replacing recombinationally derived tracts of sequence within the detected recombinant sequences with gap characters in the alignment from which the phylogenetic tree was constructed; Fig. 4). In contrast to the ML phylogenetic tree inference using the complete genomes (with recombinationally derived sequence left in the alignment; Fig. S1), in the recombination-free ML phylogenetic tree, we noted that BFDV-Y isolates from budgerigars collected from Japan and China were positioned basally on the tree relative to all currently sampled BFDV isolates. Furthermore, it was evident from Figs 2 and 4 that the analysed BFDV strains displayed some degree of host specificity; for example, BFDV-P and -G are one another's nearest relatives, and viruses from both strains have only ever been isolated from rainbow lorikeets (both wild and captive).

Most pertinent to the current study, however, was that the ML phylogenetic tree (Fig. 4 and Fig. S1) revealed that there have probably been at least five introductions of BFDV into Poland. The origins of the European BFDV-I, -Q and -Z isolates is not obvious, and it is plausible that the Polish populations of these strains could have originated from anywhere in the rest of the world. However, the BFDV-R and -T viruses circulating in Poland clustered within a diverse clade of predominantly southern African BFDV isolates and were therefore probably introduced into Europe and Poland at least at one point directly or indirectly from southern Africa. Similarly, the BFDV-J₁ isolates were nested within a clade of Australasian viruses and could plausibly have been introduced into Europe from this region.



Event	Recombinant region	Potential major parent	Potential minor parent	Detection method	<i>P</i> value
1	1826–448	Unknown	W	RGBMCST	2.95 × 10⁻³¹
2	1162–1988	K ₁ , K ₂ , K ₃ , K ₄ , M ₁ , M ₂ , U	J ₂ , J ₄ , J ₅	RGBMCST	5.91 × 10⁻²⁰
3	431–1055	A ₁ , A ₂ , A ₃ , A ₄	J ₂ , J ₄ , J ₅	RGBMCST	5.82 × 10⁻²⁶
4	158–1200	D ₂	B	RGBMCT	3.31 × 10⁻¹⁷
5	449–851	X	J ₄ , Z	RGBMT	2.44 × 10⁻¹⁷
6	49–180	K ₁ , K ₂	J ₁ , L ₁ , M ₁ , M ₂ , M ₃ , M ₄ , U, T	RGBMCT	1.94 × 10⁻¹⁶
7	62–347	E, I, J ₂ , J ₃ , J ₄ , J ₅ , L, N, O, U ₁	Unknown	RGBMCST	4.48 × 10⁻¹⁰
8	1135–69	B ₂ , K ₁ , K ₂ , K ₃ , K ₄ , T, L, M ₁ , M ₂ , M ₃ , M ₄ , U	O ₁	RGBMCT	3.22 × 10⁻¹⁰
9	475–730	O ₁	Y ₂ , Y ₃	RGBMCST	7.90 × 10⁻¹⁴
10	919–1232	Unknown	N, O, Z	RGBMCST	1.59 × 10⁻¹³
11	1445–223	K ₁ , K ₂ , K ₃ , K ₄ , L, M ₁ , M ₂ , M ₃ , M ₄ , T, U	B	RGBMCST	4.48 × 10⁻¹²
12	874–1234	Unknown	N	RGBMCT	6.21 × 10⁻¹¹
13	468–840	A ₁ , A ₂ , A ₃ , A ₄ , B ₂ , B ₃ , B ₄ , B ₅ , C ₁ , D ₂ , E, F, H, I, J ₂ , J ₃ , J ₄ , J ₅ , K, M ₁ , M ₂ , M ₃ , N, O, P, T ₁	Y	GBMCT	1.02 × 10⁻¹¹
14	1055–1388	A ₁ , A ₂ , A ₃ , A ₄ , S	U, J, [JX049215]	RGBMST	9.56 × 10⁻¹⁰
15	1984–399	M ₁ , M ₂ , M ₄	I ₃	RGBMT	1.14 × 10⁻¹⁰
16	1085–1866	N ₂ , N ₃ , N ₄	E, F, H, P	GMC	9.37 × 10⁻⁹
17	1412–56	T ₁	Unknown	GMC	1.08 × 10⁻⁸
18	1360–2002	K ₁ , K ₂ , K ₃ , K ₄	T ₁	RGBMCT	7.96 × 10⁻⁷
19	638–708	All other P ₁	Unknown	RGB	8.72 × 10⁻⁶
20	420–540	J ₄ [JX221008, JX221006]	Unknown	RGBT	1.89 × 10⁻⁶
21	1060–1237	Unknown	F, P	RGB	5.88 × 10⁻⁴

Fig. 3. Schematic illustration and details of recombination events detected within the 184 full-length BFDV genomes analysed in this study using the RDP (R), GENECONV (G), BOOTSCAN (B), MAXCHI (M), CHIMAERA (C), SISCAN (S) and 3SEQ (T) methods implemented in the computer program RDP4. Only detection methods with associated *P* values of <0.05 are shown. The *P* value for the detection method is shown in bold.

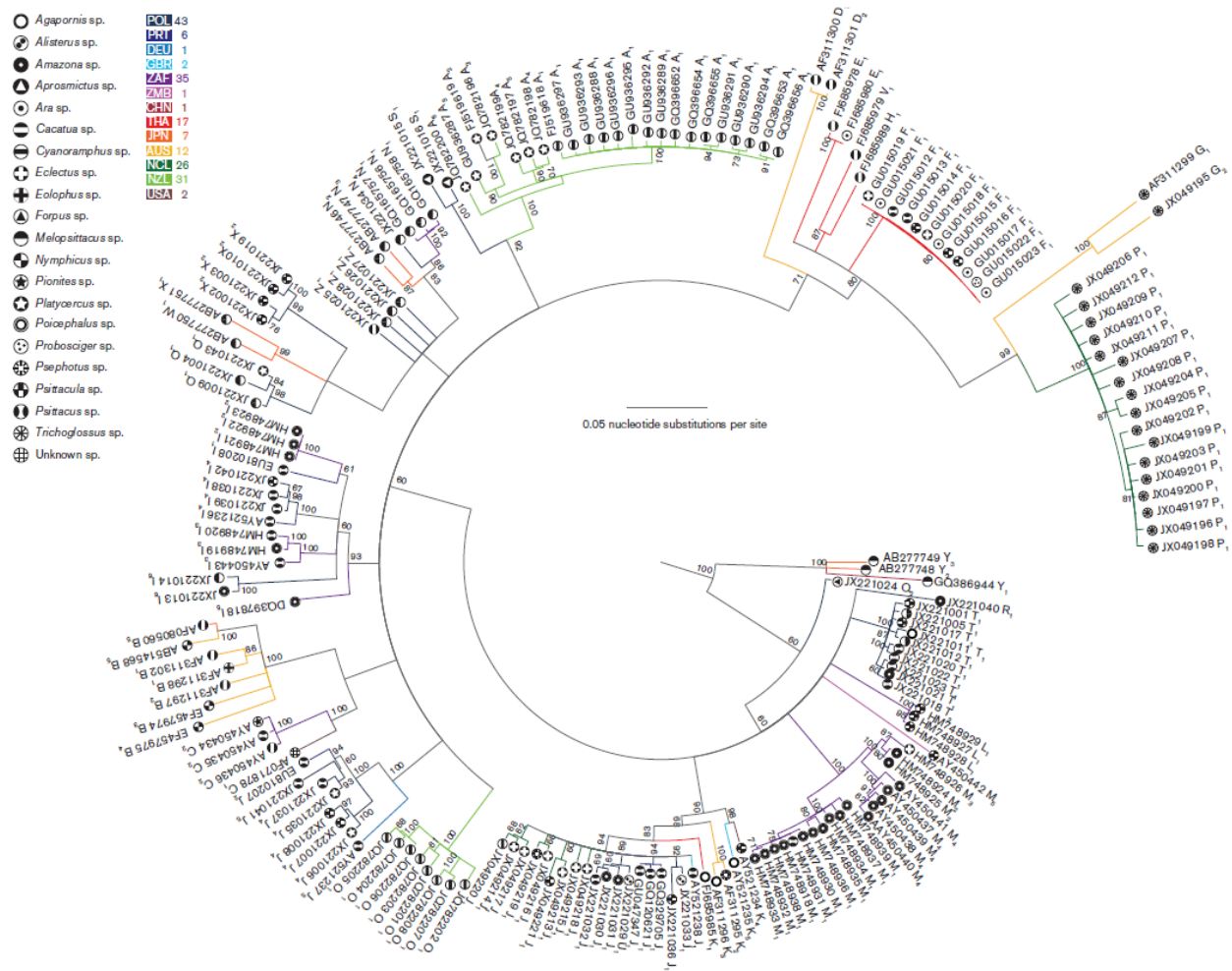


Fig. 4. ML phylogenetic tree showing the relationships of the full-genome sequences (minus recombinant regions) of BFDV isolates from this study together with all other available full BFDV genome sequences in GenBank. The tree was constructed in PhyML using the model GTR+I+G4, with 1000 non-parametric bootstrap replicates (shown on branches).

Rep and CP analysis

The ML tree constructed from the deduced Rep amino acid sequences of the 184 studied BFDV isolates revealed that that the highly recombinant budgerigar isolates were positioned basally relative to all the other BFDV Rep isolates (Fig. S2). This is not much different from the inference drawn from the full-genome ML tree ignoring recombination (Fig. S1).

Unlike the phylogenetic trees based on either the Rep amino acid sequence or the recombination-free full-genome nucleotide sequences (Fig. 4), in the phylogenetic tree based on the deduced CP amino acid sequences of these viruses, the cockatoo isolates were basal to all the other BFDVs (Fig. S3). This could be attributed the fact that the complementary sense of the genomes of these isolates are recombination free (Fig. 3). Furthermore, in comparison with Rep, CP displays a greater diversity.

It is noteworthy that, based on these trees, it is evident that Rep is evolving significantly more slowly than CP. To determine whether this could be accounted for by stronger purifying selection (i.e. selection disfavours amino acid substitutions) acting on Rep rather than on CP, we used the FEL method (Kosakovsky Pond & Frost, 2005; Kosakovsky Pond *et al.*, 2006) implemented in DATAMONKEY (<http://www.datamonkey.org/>) (Delpont *et al.*, 2010) to analyse the normalized synonymous (d_S) and non-synonymous (d_N) substitution rate ratios evident from codon alignments of the genes encoding these proteins. As expected, both *rep* and *cp* were inferred to be predominantly evolving under purifying selection. Consistent with our hypothesis that Rep is evolving under stronger purifying selection than CP, *rep* displayed a lower d_N/d_S score (0.62) than *cp* (0.88). Although we also detected six codon sites in *cp* and seven in *rep* that are apparently evolving under positive selection (i.e. selection favouring change), we noted that the vast majority of sites within the conserved Rep motifs of BFDV (i.e. rolling-circle motifs I, II and III, and Walker motifs A, B and C, common among all ssDNA viruses; reviewed by Rosario *et al.*, 2012) are all evolving under purifying selection.

Concluding comments

We have presented evidence that the highly diverse Polish BFDV population contains numerous recombinant virus lineages that have probably been introduced into the country on at least five separate occasions. Given the widespread trade in parrots among European countries, it is unlikely that the diversity of Polish BFDV population is unique in this continent. In fact, despite the availability in public databases of only nine full-length BFDV genomes from elsewhere in Europe, it is evident that these too have a high degree of diversity. Furthermore, we cannot ignore the fact that, despite restrictions on the importation of parrots into Europe, there may be ongoing illegal movements of BFDV strains into this continent from the rest of the world.

Despite Europe having no native parrot species and there being no real concern that BFDV will spread into and threaten wild-bird populations, the global export of these bred parrots is potentially contributing to the global spread of BFDV and is placing endangered parrots in other parts of the world at increased risk. It is somewhat ironic that the long-term fascination that Europeans have had with exotic parrot species may have resulted in the continent becoming a melting pot of BFDV diversity that might ultimately undermine efforts to save many of these species from extinction.

Methods

Sample collection, total DNA extraction and screening for BFDV. Blood and/or feather samples were collected from 209 birds encompassing 23 genera from more than 50 captive breeding facilities in Poland. Total DNA was extracted from samples using an iGenomic blood DNA extraction kit (Intron Biotechnology) according to the manufacturer's instructions. Four microlitres of total extracted DNA was then

used for PCR-based screening using a KAPA Blood PCR kit Mix B (KAPA Biosystems) and primers targeting the ~605 bp region of the Rep gene (59-TTAACAACCCTACAGACGGCGA-39 and 59-GGCGGAGCATCTCGCAATAAG-39) (Ritchie *et al.*, 2003). The PCR program was as follows: 94 uC for 5 min; 25 cycles of 94 uC for 30 s, 56 uC for 30 s and 72 uC for 45 s; and a final extension step at 72 uC for 1 min. The PCR products were resolved on a 1 % agarose gel stained with SYBR Safe DNA stain.

Rolling-circle amplification (RCA) and isolation of full-length BFDV genomes. Samples that were BFDV positive using the above-described method were then subjected to RCA using TempliPhi (GE Healthcare), as described previously (Julian *et al.*, 2012; Massaro *et al.*, 2012; Varsani *et al.*, 2011), to non-specifically amplify circular DNA. In brief, 1 ml total extracted DNA was added to 4 ml TempliPhi sample buffer and heated for 2 min at 94 uC. Once cooled to room temperature, 5 ml TempliPhi reaction buffer and 0.2 ml enzyme mix containing Q29 DNA polymerase were added, and the non-specific amplification reaction was allowed to proceed for 20 h at 30 uC. Two different approaches were used to obtain full-length viral genomes. In the first, 1 ml TempliPhi-amplified product was digested with *Bam*HI restriction enzyme. In the second, 1 ml TempliPhi product was then used in a touchdown PCR to amplify the full-length BFDV genome using back-to-back primers (AV-BFDV-F, 59-CYTACYCTKGGC-ATTGTGGC-39, and AV-BFDV-R, 59-TATHACRTCBCCYTCYTTACTGCA-39, targeted to the conserved domain of the Rep gene) as described by Julian *et al.* (2012). Kapa HiFi HotStart DNA polymerase (Kapa Biosystems) was used with the PCR protocol as follows: 94 uC for 2 min; ten cycles of 98 uC for 20 s, 62 uC for 20 s and 72 uC for 2 min; 20 cycles of 98 uC for 20 s, 52 uC for 20 s and 72 uC for 2 min; and a final extension of 72 uC for 2 min. The restriction enzyme-digested or PCR-amplified products were resolved on 0.7 % agarose gels and bands of ~2 kb were excised and cleaned using a MEGA-spin Agarose Gel Extraction kit (Intron Biotechnology). The restriction enzyme-digested products were ligated to *Bam*HI-cut pUC19 vector DNA and the PCR products were ligated to pJET1.2 vector DNA (CloneJET PCR cloning kit; Fermentas). The plasmid obtained from a single transformed *Escherichia coli* colony was isolated and sequenced by primer walking by MacroGen (Korea).

Sequence data preparation and analyses. Full-genome sequences of BFDV were assembled from contiguous sequencing reads using DNAMAN version 5.2.9 (Lynnon Biosoft) and aligned using MUSCLE (Edgar, 2004), along with other full-genome sequences downloaded from GenBank in September of 2012. MEGA5 (Tamura *et al.*, 2011) was used for manual editing of sequence alignments. Nucleotide sequence similarities (*p*-distances with pairwise deletion of gaps) of the full-genome sequences were compared using SDT version 1.0 (Muhire *et al.*, 2013).

Recombination among the BFDV isolates was analysed as described by Varsani *et al.* (2011) and Heath *et al.* (2004) using RDP4 with default settings, and the methods

RDP (Martin & Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005), MAXCHI (Smith, 1992), CHIMAERA (Posada & Crandall, 2001), SISCAN (Gibbs *et al.*, 2000) and 3SEQ (Boni *et al.*, 2007). Only recombination events detected by three or more of these methods, with associated *P* values ≥ 0.05 and phylogenetic support for recombination, were accepted as credible events. In general, the inferred parental sequences were assigned as the 'minor parent' based on the origin of the recombinant region and the 'major parent' based on the origin of the non-recombinant region of the viral genomes.

ML phylogenies were inferred using PhyML (Guindon *et al.*, 2010) with 1000 non-parametric bootstrap replicates and the model GTR+I+G4 for the full-genome sequences, as determined by RDP4 (Ortiz-Catedral *et al.*, 2010), or, for amino acid sequences of Rep or CP, the LG model in PhyML (Guindon & Gascuel, 2003) with aLRT branch support (Anisimova & Gascuel, 2006). Branches with $\geq 60\%$ support were collapsed using Mesquite version 2.75.

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