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Phylogeny of *beak and feather disease virus* in cockatoos demonstrates host generalism and multiple-variant infections within *Psittaciformes*

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ABSTRACT

Phylogenetic analyses of the highly genetically diverse but antigenically conserved, single-stranded circular, DNA genome of the avian circovirus, *beak and feather disease virus* (BFDV) from cockatoo species throughout Australia demonstrated a high mutation rate for BFDV (orders of magnitude fall in the range of 10^{-4} substitutions/site/year) along with strong support for recombination indicating active cross-species transmission in various subpopulations. Multiple variants of BFDV were demonstrated with at least 30 genotypic variants identified within nine individual birds, with one containing up to 7 variants. Single genetic variants were detected in feathers from 2 birds but splenic tissue provided further variants. The rich BFDV genetic diversity points to Australasia as the most likely geographical origin of this virus and supports flexible host switching. We propose this as evidence of Order-wide host generalism in the *Psittaciformes* characterised by high mutability that is buffered by frequent recombination and slow replication strategy.

Introduction

Psittacine beak and feather disease (PBFD) is a chronic but ultimately fatal viral disease of young parrots and cockatoos, characterised by long term incubation of up to 2 years followed by immunotolerance and massive viral excretion and enduring antibody negative status. All *Psittaciformes* are considered susceptible to infection since it has been reported in more than 60 species of cockatoos and parrots (Bassami et al., 2001; Ritchie et al., 2003; Todd, 2004) and has been known to occur naturally in wild Australian birds for more than 120 years (Ashby, 1907; Powell, 1903; Raidal et al., 1993b). The disease is recognised as a key threatening process for endangered psittacine birds in Australia and has spread globally, now affecting a wide range of psittacine species both in wild and captive populations (Bassami et al., 2001; Clout and Merton, 1998; Ha et al., 2007; Raidal et al., 1993b; Ritchie et al., 1990; Sanada et al., 1999). The aetiological agent of the disease, *beak and feather disease virus* (BFDV), a compact circular, ambisense single-stranded DNA (ssDNA) virus belonging to the genus *Circovirus* in the family of *Circoviridae* (Heath et al., 2004; Kondiah et al., 2006; Niagro et al., 1998) is perhaps the simplest pathogen known to infect vertebrates.

Circoviruses and the recently recognised cycloviruses (Li et al., 2010; Rosario et al., 2011) are the smallest known autonomously replicating viruses, with genomes of approximately 2000 nucleotides encoding a replicase (*Rep*) and a single capsid protein (*Cap*) and this simplicity makes them a good candidate to model host-generalist phylogenetics and intra-host genetic variation in disease ecology and drivers of virulence versus attenuation. Circoviruses may also represent an ancient form in viral evolution related to plant geminiviruses and nanoviruses (Gibbs and Weiller, 1999) with circovirus "fossil" sequences recently identified integrated on the chromosomes of a wide range of vertebrates, invertebrates, protozoans, plants, fungi, algae and bacteria (Delwart and Li, 2012). Recombination has been clearly shown as a key mechanism within BFDV evolution but there is also evidence that the

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C-terminal region of *Rep* originated from an RNA-binding protein taken from a progenitor calicivirus, a RNA virus species that only infects vertebrates (Gibbs et al., 2006; Gibbs and Weiller, 1999).

In extant hosts and compared with other non-enveloped DNA viruses, of which the highly conserved 5 kb circular dsDNA genomes of avian polyomaviruses are probably the best choice for comparison, BFDV is highly genetically diverse and prone to mutation (Duffy and Holmes, 2008; Julian et al., 2013; Sarker et al., 2014a) yet, somewhat paradoxically, remains relatively antigenically conserved with only subtle evidence of different serological strains so far detected (Khalesi et al., 2005a, 2005b; Raidal et al., 1993; Shearer et al., 2008). The order Psittaciformes contains more than 370 species, most of which are concentrated in the tropical parts of the Southern Hemisphere (White et al., 2011). Over 20% have been subjected to conservation efforts including 85 species listed as critically endangered, endangered or vulnerable, and 19 species are considered at risk of extinction globally by the International Union for Conservation of Nature (IUCN, 2013). In Australia, five endemic species of black cockatoos (*Calyptorhynchus* spp.), such as the red-tailed black-cockatoo (*Calyptorhynchus banksii*) and glossy black-cockatoo (*Calyptorhynchus lathami*) as well as two white-tailed black-cockatoos of Western Australia have been classified as endangered in the IUCN Red List of Threatened species (IUCN, 2013), whereas the gang-gang cockatoo (*Callocephalon fimbriatum*) has been listed as an endangered species in New South Wales (NSW). Whilst habitat loss and degradation is considered their major threat (Garnett et al., 1999; White et al., 2011) the spread of infectious diseases, in particular PBFD, is also considered a key threatening process.

In the present study we analysed BFDV genomes from a range of host species primarily to ascertain threats to endangered cockatoos particularly in relation to predictable host-switch events involving more common competitors such as the sulphur-crested cockatoo (*Cacatua galerita*), galah (*Eolophus roseicapillus*), long-billed corella (*Cacatua tenuirostris*) and Major Mitchell's cockatoo (*Lophochroa leadbeateri*) in captive and wild populations throughout Australia. Our aim was to evaluate the phylogenetic and biogeographic relationships of cockatoo-derived BFDV genomes comparing 38 new genomes with representative BFDV genomes, *Rep* and *Cap* gene data from existing geographical and genotype clades. We also tested the hypotheses that phylogenies based on *Cap* and *Rep* gene alone are not congruent with whole genome analysis; that cockatoo derived BFDV clades represent evidence of flexible host generalism; and that genetic diversity and levels of divergence within BFDV clades are similar within and not constrained by host availability.

Results

Analyses of BFDV genome sequences for patterns of relatedness

A Bayesian phylogenetic tree of 38 entire BFDV genome sequences (GenBank accession numbers: KF385399–KF385436) from seven different cockatoo species along with another 81 BFDV genome sequences publicly available on GenBank exhibited a pattern of strong geographic clustering to Australia, but within Australia all psittacine bird species appeared potentially susceptible to each genotype (Fig. 1) (see ML tree with original bootstraps support as Supplementary Fig. S1 and Table S1 for more detail information). Phylogenetic analysis of *Rep* alone matched closely ($R^2=0.814$, $P<0.001$ Supplementary Fig. S2) with whole genome analysis but *Cap* was much less able to predict whole genome phylogeny ($R^2=0.733$, $P<0.001$ Supplementary Fig. S3). Regression analyses failed to demonstrate any significant correlations between BFDV genetic distances, host genetic distances, spatial distances, temporal or wild status. Analysis using BaTS showed no significant association between host species or geographical location and phylogeny within Australia.

BFDV genomes from two red-tailed black cockatoos (GenBank accession numbers: KF385399 and KF385400) in Western Australia shared $>89\%$ pairwise nucleotide identity but Bayesian phylogenetic analysis revealed that the potential sources of BFDV infection were different (Fig. 1). For example, one BFDV genome (KF385400) demonstrated strongest clade support (100%) with spatially distant genotypes of BFDV from a cockatiel (Shearer et al., 2008), sulphur-crested cockatoo (KF385415) from Victoria, one long-billed corella BFDV genome (KF385428) from Victoria and one galah BFDV genome (KF385435) from Queensland. Whereas, another genome from a wild caught red-tailed black cockatoo (KF385399) showed $>79\%$ clade identity with a temporally (13 years), and spatially (4000 km) distant rainbow lorikeet genome (AF311299) from Victoria.

A similar scenario was documented in the case of the endangered glossy black cockatoo. Even though, the BFDV genomes (KF385408–KF385412) from five glossy black cockatoo shared 90.3–99.1% sequence identity, Bayesian phylogenetic tree indicated that the BFDV originated in this individual from a different ancestor (Fig. 1). BFDV genomes (KF385409–KF385412) from four glossy black cockatoo shared $>88\%$ clade support with other Australian BFDV genomes; whereas another BFDV genome from glossy black cockatoo (KF385408) formed a separate and well-supported monophyletic clade with other BFDV genomes (KF385399, AF311299, AF31195–AF31196). On the other hand, five gang-gang cockatoo BFDV genomes formed a well supported clade (99%) indicating that these birds may support a unique genotype, but closely related ($>83\%$ clade identity) to other Australian BFDV genotypes.

Unexpectedly, a BFDV genome obtained from a captive orange-bellied parrot (KF188691) from Victoria had strong clade ($>96\%$) support with seven BFDV genomes (KF385420–KF385425) from Victorian long-billed corella and one long-billed corella BFDV genome (KF385429) from Queensland.

Estimation of mutation rate

Using uncorrelated relaxed lognormal and strict molecular clock the estimated mutation rates for entire genome, *Rep* and *Cap* gene varied slightly and falling in the same order of magnitude (10^{-4} subs/site/year) (Table 3), which is much higher than any other DNA virus. The mean rate of mutation estimated for BFDV full genome data sets ranged from 8.18×10^{-4} to 9.67×10^{-4} subs/site/year. These were outside the 95% HDPs (8.32×10^{-5}–3.94×10^{-4}) of randomised data sets (Table S2) and therefore supportive of a temporal structure in the data. Higher mutation rates were also consistently estimated in *Cap* versus *Rep* (Table 3). However, using Path-O-Gen, there was little support for a good temporal signal for BFDV genome data sets ($r^2=0.122$, residual mean square $=1.43\times10^{-3}$ and correlation-coefficient $=0.320$).

Frequent recombination in BFDV genomes

Using RDP4, SBP and GARD recombination was detected in the BFDV genomes from cockatoo species (Figs. 2 and S4). The strongest support for recombination was detected in the c-terminal portion of the capsid gene between a wild-caught long-billed corella (KF385429) and a wild red-tailed black cockatoo bred (KF385399) originally sourced from Queensland and Western Australia respectively (specific recombination breakpoint location: 1277; $P\leq0.001$ using SBP and GARD). A second recombination was detected in the intergenic region of the genome between two glossy black cockatoo BFDV genomes from the New South Wales.