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# Disease surveillance in wild Victorian cacatuids reveals co-infection with multiple agents and detection of novel avian viruses

Michelle Sutherland[a,*,1], Subir Sarker[b,f,**], Paola K. Vaz[c], Alistair R. Legione[c], Joanne M. Devlin[c], Patricia L. Macwhirter[d], Pamela L. Whiteley[e], Shane R. Raidal[f]

[a] Burwood Bird and Animal Hospital, 128 Highbury Rd, Burwood, Vic 3125, Australia
[b] Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Vic 3086, Australia
[c] Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Vic 3052, Australia
[d] Greencross Vets Springvale, 570 Springvale Rd, Springvale South, Vic 3172, Australia
[e] Wildlife Health Victoria: Surveillance, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Werribee, Vic 3030, Australia
[f] School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia

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ABSTRACT

Wild birds are known reservoirs of bacterial and viral pathogens, some of which have zoonotic potential. This poses a risk to both avian and human health, since spillover into domestic bird populations may occur. In Victoria, wild-caught cockatoos trapped under licence routinely enter commercial trade. The circovirus Beak and Feather Disease Virus (BFDV), herpesviruses, adenoviruses and *Chlamydia psittaci* have been identified as significant pathogens of parrots globally, with impacts on both aviculture and the conservation efforts of endangered species. In this study, we describe the results of surveillance for psittacid herpesviruses (PsHVs), psittacine adenovirus (PsAdV), BFDV and *C. psittaci* in wild cacatuids in Victoria, Australia. Samples were collected from 55 birds of four species, and tested using genus or family-wide polymerase chain reaction methods coupled with sequencing and phylogenetic analyses for detection and identification of known and novel pathogens. There were no clinically observed signs of illness in most of the live birds in this study (96.3%; n = 53). Beak and Feather Disease Virus was detected with a prevalence of 69.6% (95% CI 55.2–80.9). Low prevalences of PsHV (1.81%; 95% CI 0.3–9.6), PsAdV (1.81%; 95% CI 0.3–9.6), and *C. psittaci* (1.81%; 95% CI 0.3–9.6) was detected. Importantly, a novel avian alphaherpesvirus and a novel avian adenovirus were detected in a little corella (*Cacatua sanguinea*) co-infected with BFDV and *C. psittaci*. The presence of multiple potential pathogens detected in a single bird presents an example of the ease with which such infectious agents may enter the pet trade and how novel viruses circulating in wild populations have the potential for transmission into captive birds. Genomic identification of previously undescribed avian viruses is important to further our understanding of their epidemiology, facilitating management of biosecurity aspects of the domestic and international bird trade, and conservation efforts of vulnerable species.

## 1. Introduction

Australia is home to 56 parrot species, 51 of which are endemic, and 26 of which are listed as threatened or vulnerable in the wild (Garnett et al., 2010). The prevalence and potential spread of infectious diseases and the emergence of novel pathogens may therefore have devastating effects for both wild and captive Australian psittacine bird populations.

The role of wild birds as reservoirs of bacterial and viral pathogens has been studied previously (Reed et al., 2003; Jenkins et al., 2018; Jelocnik et al., 2017), and concurrent infection with multiple agents has been reported (Latimer et al., 1993; Raidal et al., 1998). The circovirus Beak and Feather Disease Virus (BFDV), psittacine herpesviruses (PsHVs), and avian adenoviruses have been identified as significant viral pathogens of parrots in Australia and worldwide (Scott et al.,

* Corresponding author.
** Corresponding author at: Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Vic 3086, Australia.
*E-mail addresses:* michelle@unusualpetvets.com.au (M. Sutherland), s.sarker@latrobe.edu.au (S. Sarker), pvaz@unimelb.edu.au (P.K. Vaz), legionea@unimelb.edu.au (A.R. Legione), devlinj@unimelb.edu.au (J.M. Devlin), patvet@bigpond.com (P.L. Macwhirter), pamw@unimelb.edu.au (P.L. Whiteley), shraidal@csu.edu.au (S.R. Raidal).
[1] Present address: The Unusual Pet Vets, 210 Karingal Drive, Frankston, Vic 3199, Australia.

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1986; Gomez-Villamandos et al., 1995; Mackie et al., 2003; Lazic et al., 2008; Das et al., 2016, 2017; Sutherland et al., 2017; Amery-Gale et al., 2017, 2018), with impacts on both aviculture and the conservation efforts of endangered species. *Chlamydia psittaci* frequently affects captive psittacine birds; avian chlamydiosis is considered endemic in many wild bird populations, and is of public concern as a zoonosis (Wain and Fielding, 2007; Vanrompay et al., 2007; Harkinezhad et al., 2009; Branley et al., 2016).

Wild-caught juvenile sulphur-crested cockatoos (*Cacatua galerita)*, long-billed corellas (*C. tenuirostris*), little corellas (*C. sanguinea*) and galahs (*Eolophus roseicapillus*), legally obtained under permit, routinely enter commercial trade in Australia. The handling and transport conditions of these birds facilitate the transmission of infectious diseases, and may serve as a source of exposure to both other birds and humans involved in their care (Raidal et al., 1993; Amery-Gale et al., 2018). Consequently, this trade may result in potential pathogens emerging in captive birds, their dispersal into disease-free regions and the transmission of zoonotic diseases.

The aim of this study was to investigate the epidemiology of BFDV, PsHVs, psittacine adenovirus (PsAdV), and *C. psittaci* in wild cacatuids in Victoria. We report the presence of a novel avian herpesvirus and a novel adenovirus detected in a little corella, that also tested positive for *C. psittaci* and BFDV on next generation sequencing (NGS) and PCR, respectively.

## 2. Materials and methods

### 2.1. Sample sources and extraction of genomic DNA

Samples were collected from wild sulphur-crested cockatoos, long-billed corellas, little corellas, and galahs between November 2015 and June 2016. The majority of birds sampled (n = 44, comprising sulphur-crested cockatoos [n = 13] and long-billed corellas [n = 31]) were trapped under license (Department of Economic Development Jobs Transport and Resources [DEDJTR] Victoria Scientific Procedures Fieldwork Licence 01180) from four regions in rural Victoria, Australia (Ararat [37°17′0″S, 142°55′0″E], Moriac [38°14′0″S, 144°10′0″E], Kaniva [36°22′0″S, 141°14′0″E] and Edenhope [37°03′0″S, 141°18′0″E] districts), and sampled prior to entering captivity. The remaining birds (n = 11, comprising little corellas [n = 5], sulphur-crested cockatoos [n = 4] and galahs [n = 2]) were wild birds originating from rural and semi-rural areas of Melbourne that were sampled on admission to a wildlife hospital (Animal Health Centre, Healesville Sanctuary, Healesville, Victoria, Australia) and an avian veterinary hospital (Burwood Bird and Animal Hospital, Burwood, Victoria, Australia). Some of these birds were euthanatised for other reasons, mainly untreatable injuries. This study was approved by the Wildlife and Small Institutions Ethics Committee (DEDJTR Victoria) under application number 01.15.

From each bird, blood was collected onto filter paper and a fresh blood smear prepared. A swab, moistened in sterile phosphate-buffered saline, of the choana and cloaca was also taken. Samples of organs (spleen, liver and brain) were taken from those birds that were euthanatised.

For herpesvirus, adenovirus and *C. psittaci* testing, genomic DNA was extracted from combined choanal/cloacal swabs from 55 birds (sulphur-crested cockatoos n = 17; long-billed corellas, n = 31; little corellas n = 5; galahs = 2), using a modified protocol for swab samples adapted from the ReliaPrep gDNA Tissue Miniprep System (Promega, USA) according to protocols in Sarker et al. (2018). Blood spots from the same sample population were used as the source of genomic DNA for BFDV testing. Genomic DNA extraction was performed using a ReliaPrep gDNA Blood Miniprep System (Promega, USA) following previously described protocols (Ypelaar et al., 1999; Khalesi et al., 2005; Bonne et al., 2008). All of the long-billed corellas were sampled post mortem after euthanasia with inhalant carbon dioxide as part of wildlife mitigation management performed by DEJTR (Victoria).

### 2.2. PCR testing for the detection of pathogens

Samples were tested for the presence of herpesvirus DNA using a pan-*Herpesviridae* PCR assay utilising degenerate, deoxyinosine-substituted oligonucleotide primers specifically targeting a highly conserved region of the herpesvirus DNA polymerase gene, as previously described (Chmielewicz et al., 2003). Two attempts were made to detect herpesvirus DNA in the samples by PCR. Initially, the primers DFA, ILK, and KGI (5′-GAYTTYGCIAGYYTITAYC, 5′-TCCTGGACAAGCAGCARIYSGCIMTIAA, and 5′-GTCTTGCTCACCAGITCIACICCYTT, respectively) were used (Chmielewicz et al., 2003). Subsequently, we utilised the primers TGV and IYG (5′-TGTAACTCGGTGTAYGGITTYACIGGIGT and 5′- CACAGAGTCCGTRTCICCRTAIAT, respectively) (Chmielewicz et al., 2003). In each case, macropodid herpesvirus-1 was used as the positive control, while distilled $H_2O$ was used as the negative control, respectively.

Extracted DNA samples were initially tested for the presence of adenovirus DNA by consensus-nested PCR method using the primers polFouter: 5′- TNMGNGGNGGNMGNTGYTAYCC and polRouter: 5′-GTDGCRAANSHNCCRTABARNGMRTT, and subsequently with the primers PolFinner: 5′-GTNTWYGAYATHTGYGGHATGTAYGC and PolRinner: 5′ CCANCCBCDRTTRTGNARNGTRA, that target the DNA polymerase gene of adenoviruses, as described previously (Wellehan et al., 2004). In these PCR reactions, fowl adenovirus-8b DNA and distilled $H_2O$ were included as positive and negative controls, respectively.

The presence of *Chlamydiaceae* DNA in extracted DNA samples was tested for using a PCR with high-resolution melt curve analysis targeting the 16S rRNA region using the 16SG oligonucleotide set as described by Robertson et al. (2009). For the detection of BFDV, a PCR was performed on 46 DNA samples sourced from within the study population (sulphur-crested cockatoos n = 13; long-billed corellas n = 30; little corellas n = 3), using a set of primers (Forward 5′-AAC CCTACAGACGGCGAG-3′ and Reverse 5′-GTCACAGTCCTCCTTGT ACC-3′) that targets 717-bp section of the BFDV *Rep* gene according to established protocols (Ypelaar et al., 1999; Khalesi et al., 2005; Bonne et al., 2008). In these PCR reactions, BFDV known positive DNA and distilled $H_2O$ were included as positive and negative controls, respectively.

### 2.3. Library preparation and next-generation sequencing on selected DNA

When a sample (bird #46) was positive for PsHV and BFDV using established PCR techniques, a DNA library was prepared from a cloacal swab using an established protocol adapted from Illumina Nextera XT DNA Library Prep V3 Kit (Illumina, San Diego, USA) (Sarker et al., 2018), and sequencing of the DNA library was performed as paired-end on an Illumina® MiSeq chemistry according to the manufacturer's instructions.

### 2.4. Bioinformatics, annotation and phylogenetic analysis

Viral genomes were assembled *de novo* using SPAdes (Bankevich et al., 2012) and through reiterated mapping of read data as per Vaz et al. (2016). The final herpesvirus genome arrangement was assembled using Geneious software (version 10.2.3; Biomatters Ltd) using gallid alphaherpesvirus-1 as reference (Genbank accession JX646899). The translated amino acid sequence of the herpesvirus DNA polymerase gene was extracted, and aligned with other representative herpesvirus sequences using ClustalW 2.1 (Larkin et al., 2007) with free end gaps and with gapped sites removed. A maximum likelihood phylogenetic tree was constructed using a Le Gascuel (LG) substitution model, one substitution rate category, 100 bootstrap replicates, and topology searching using NNI and SPR algorithms.

Phylogenetic analysis of the novel corella adenovirus was performed using representative adenovirus hexon amino acid sequences. Sequences were aligned using MAFFT, then realigned with ClustalW 2.1 with free end gaps and all gapped sites masked. Maximum likelihood trees were constructed with an LG substitution model with 100 bootstrap replicates, 4 substitution rate categories, and topology searching using NNI and SPR. A second tree that included the shorter PsHV-1 and BuAdV hexon sequences was constructed as above.

Illumina read data was used to undertake *C. psittaci* analysis. Mapping to the 6BC reference genome (Genbank accession number: NC_017287) was undertaken using minimap2 (Li, 2018). Multilocus sequence typing (MLST) (Pannekoek et al., 2008) was performed using the software tool 'SRST2' (Inouye et al., 2014) in conjunction with the PubMLST database (Jolley et al., 2018). The software tool 'RealPhy' (Bertels et al., 2014) was used to align the DNA read data to 54 published *C. psittaci* genomes to identify nucleotide differences present (Supplementary Data). The generated alignment was used for phylogenetic analysis. A phylogenetic tree was produced with MRBayes v3.2.6 (Huelsenbeck and Ronquist, 2001) with four chains with 1,000,000 chain length, subsampled every 1000 iterations, with a 10% burn in. The general time reversible substitution model with gamma distribution and a proportion of invariable sites (GTR + I + G) was used based on assessment with model selection software with ModelTest-NG v0.15 (Huelsenbeck and Ronquist, 2001).

## 3. Results

There were no clinically observed signs of illness in most of the live birds in this study (96.3%; n = 53/55). The two birds with clinical signs of disease were both little corellas that presented to an avian veterinary hospital. Psittacid herpesvirus DNA was detected by a pan-herpes consensus PCR (Chmielewicz et al., 2003) in 1/55 samples (1.81% prevalence; 95% CI 0.3–9.6) (Table 1). The positive sample was detected in a little corella originating from semi-rural Melbourne (37°66′89″S, 145°09′48″E). This bird (bird #46) showed non-specific signs of illness on clinical examination (fluffed feathers, with eyes closed). Though neither adenovirus nor *C. psittaci* were detected during initial PCR screening, PsAdV DNA and *C. psittaci* DNA were detected by NGS in the sample from the same little corella as detailed previously (bird #46), giving an overall prevalence of 1.81% (95% CI 0.3–9.6) for both PsAdV and *C. psittaci* in the study population.

**Table 1**
Estimated prevalence of Beak and Feather Disease Virus (BFDV), Psittacine Herpesvirus (PsHV), Psittacine Adenovirus (PsAdV) and *C. psittaci* detected by PCR and NGS in wild psittacines of four species tested in Victoria, Australia.

| Species | BFDV positive birds/n (%) [95% CI] | PsHV positive birds/n (%) [95% CI] | PsAdV positive birds/n (%) [95% CI] | *C. psittaci* positive birds/n (%) [95% CI] |
|---|---|---|---|---|
| Sulphur-crested cockatoo (*Cacatua galerita*) | 12/13 (92.30%) [66.7-98.6] | 0/17 (0%) [0-1.84] | 0/17 (0%) [0-1.84] | 0/17 (0%) [0-1.84] |
| Long-billed corella (*Cacatua tenuirostris*) | 17/30 (56.70%) [39.2-72.6] | 0/31 (0%) [0-1.1] | 0/31 (0%) [0-1.1] | 0/31 (0%) [0-1.1] |
| Little corella (*Cacatua sanguinea*) | 3/3 (100%) [43.9-100] | 1/5 (20%) [3.6-62.4] | 1/5 (20.0%) [3.6-62.4] | 1/5 (20.0%) [3.6-62.4] |
| Galah (*Eolophus roseicapillus*) | 0/0 | 0/2 (0%) [0-6.57] | 0/2 (0%) [0-6.57] | 0/2 (0%) [0-6.57] |
| **Total no. positive birds/n Estimated prevalence (%) [95% CI]** | **32/46 (69.56%) [55.2-80.9]** | **1/55 (1.81%) [0.3-9.6]** | **1/55 (1.81%) [0.3-9.6]** | **1/55 (1.81%) [0.3-9.6]** |

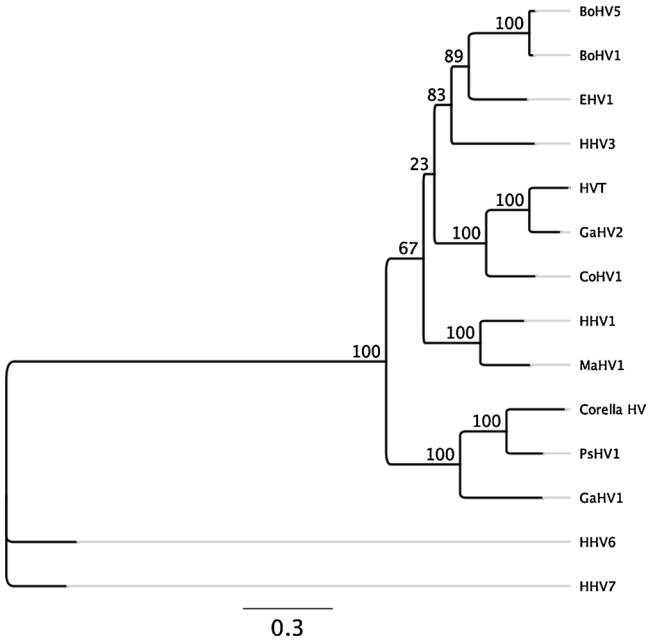


**Fig. 1.** Phylogenetic tree illustrating the relationship of a novel psittacid herpesvirus detected in a little corella (*Cacatua sanguinea*) to other representative alphaherpesviruses. The proposed name for the novel virus is Cacatuid herpesvirus-2 (CaHV-2). Accession numbers available in Supplementary Material.

### 3.1. A novel psittacid herpesvirus was detected on phylogenetic analysis

Phylogenetic analysis of the predicted amino acid sequence of the DNA polymerase gene with other representative herpesviruses demonstrated that the novel PsHV from the little corella sample clusters most closely with the iltoviruses, PsHV-1 and gallid herpesvirus1 (GaHV1) (Fig. 1). The proposed name for this novel PsHV is Cacatuid herpesvirus-2 (CaHV-2). The CaHV-2 draft genome is approximately 142 kbp in length, excluding terminal repeats, and has been deposited in GenBank under the accession number MK360902.

### 3.2. A novel psittacine adenovirus was detected on phylogenetic analysis

Phylogenetic analysis of the predicted amino acid sequence of the Hexon gene with other representative sequences of adenovirus genera confirmed the presence of a novel psittacid siadenovirus (Fig. 2). Clustering suggests it is most closely related to Raptor siadenovirus-A, Skua siadenovirus-A and Frog adenovirus-1. The proposed name for this novel virus is Corella adenovirus-1 (CorAdV-1). The CorAdV-1 genome obtained from the little corella was approximately 27.2 kbp in length and has been deposited in GenBank with the accession number MK227353.

### 3.3. Beak and Feather Disease Virus was detected by PCR in all species tested

Overall, 32 birds tested PCR positive for BFDV, giving a prevalence of 69.6% (95% CI 55.2–80.9) in the study population (Table 1). None of the sulphur-crested cockatoos (n = 13) tested exhibited clinical signs of illness on clinical examination. However, BFDV was detected in 92.3% (95% CI 66.7–98.6; n = 12) of these birds (Table 1).

Of the long-billed corellas (n = 30) sampled, 56.6% (95% CI 39.2–72.6; n = 17) were PCR positive for BFDV. All little corellas (n = 5) tested were PCR positive for BFDV. Only one of these birds exhibited clinical signs of illness, appearing fluffed and with its eyes closed. This bird (bird #46) also tested positive for PsHV, PsAdV and *C. psittaci* on NGS. To determine the sequence of the BFDV present in the

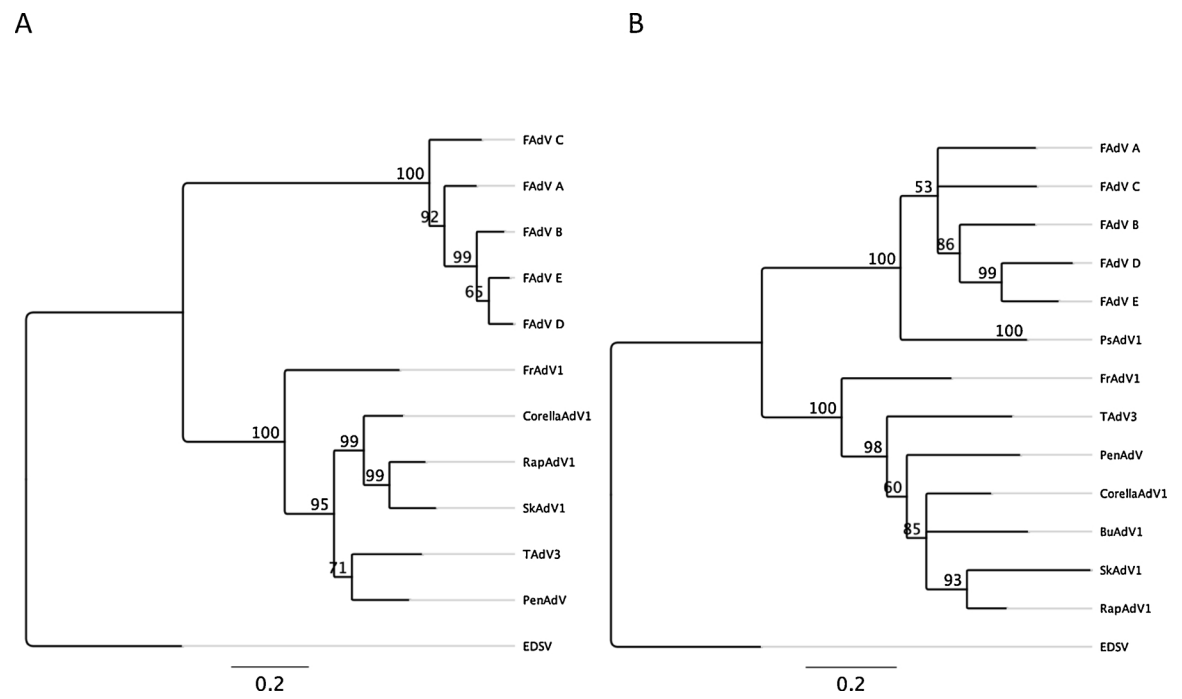


**Fig. 2.** Phylogenetic tree illustrating the relationship of a novel psittacid adenovirus detected in a little corella (*Cacatua sanguinea*) to other representative adenoviruses. A. Longer Hexon predicted protein sequence. B. Truncated sequence allowing the inclusion of BuAdV1 and PsAdV1 in the analysis. The proposed name for the novel virus is Corella adenovirus-1 (CorAdV-1). Accession numbers available in Supplementary Material.

bird that tested positive for multiple infectious agents (bird #46), the coding-complete genome was amplified using the primers and PCR conditions described by Sarker et al. (2013a, 2013b, 2014a, 2014b). The virus detected (GenBank accession no. MK163676) had a 98% match to previously reported sequences of BFDV, KF385463.1 and KF385406.1 that have been identified in Australian galahs and Major Mitchell cockatoos (*Lophochroa leadbeateri*), respectively.

### 3.4. *Chlamydia psittaci* was detected by next generation sequencing

All qPCRs were negative for *C. psittaci*, however, a single bird (bird #46), an adult little corella, was found to be positive for *C. psittaci* (Table 1) using NGS, giving an overall prevalence of 1.81% (95% CI 0.3–9.6) in the study population. In total, 47, 757 paired reads mapped to the 6BC reference genome, providing a maximum depth of coverage of 52, and a median depth of coverage of 4. Multilocus sequence typing (MLST) determined that this sample contained *C. psittaci* sequence type (ST) 24 strain, belonging to the highly virulent *C. psittaci* 6BC lineage. This classification was supported by the phylogenetic tree produced from the RealPhy output (848,653 bp in length), which placed the *C. psittaci* amongst the clade containing the 6BC reference strain (Fig. 3).

## 4. Discussion

Wildlife health is recognised as an important part of the 'One Health' paradigm as it is interconnected with humans, domestic animals and their ecosystems, and affects biodiversity (Whiteley, 2013). Avian hosts are frequently involved in the epidemiology of infectious diseases, and have been implicated as spillover hosts in outbreaks of zoonotic diseases, including a recent epizootic of *C. psittaci* in New South Wales (NSW), Australia, most likely originating in parrots (Whiteley, 2013; Branley et al., 2016; Jelocnik et al., 2017; Jenkins et al., 2018).

Beak and Feather Disease Virus, a circovirus, is well known for causing a chronic and ultimately fatal disease in psittacine birds, although non-psittacine birds may also be affected (Amery-Gale et al., 2017). Acute forms of the disease may affect juvenile birds, while in adult birds, a chronic, debilitating feather dystrophy develops progressively with each moult, owing to the long viral incubation period (Pass and Perry, 1985; Ritchie et al., 1989; Raidal et al., 1993; Schoemaker et al., 2000). The disease is associated with a continuous high level of excretion of viral particles. Within the order Psittaciformes, the virus exhibits host-generalism with a wide species susceptibility (Sarker et al., 2013a, 2013b). BFDV is considered a significant threat to the conservation of native parrots in Australia, where it is endemic (Raidal et al., 2015), as well as being a problem where birds are kept in captivity worldwide (Bassami et al., 2001).

The overall prevalence of BFDV across all birds (n = 46) tested by PCR was 69.6%, which is comparable to a recent study that documented a BFDV prevalence of 67.7% (95% C.I. 50.3–82.1%) in wild cacatuids (n = 31) presenting to a wildlife hospital in Victoria (Amery-Gale et al., 2017), and work done by Raidal et al. (1993) that documented BFDV seroprevalences ranging between 41 and 94% in different wild cockatoo flocks in NSW, Australia. All three of the species tested in this study had birds that tested PCR positive for BFDV, with a prevalence of 92.3% (95% C.I. 66.7–98.6%) in tested sulphur-crested cockatoos and 56.7% (95% C.I. 39.2–72.6%) in tested long-billed corellas. All three little corellas tested in the present study were positive for BFDV. The estimated prevalence of BFDV in sulphur-crested cockatoos in our study was consistent with earlier work (Amery-Gale et al., 2017). Amery-Gale et al. (2017) also documented that the single little corella PCR tested for the presence of BFDV was positive, and did not record any long-billed corellas testing PCR positive for BFDV (n = 1). Our data further support the presence of BFDV as an endemic virus in wild Australian birds.

A selected sample from a little corella that showed evidence of having a BFDV and an avian herpesvirus by PCR was further investigated using high-throughput NGS. This study demonstrates evidence of concurrent infection with multiple infectious agents in a little corella including BFDV, an avian adenovirus, a herpesvirus and *C. psittaci*. The BFDV genome sequenced in this study was 1902 bp in length, and using a BLASTN search, the complete BFDV genome sequence demonstrated a 98% nucleotide match to BFDV genotypes identified in Australian galahs (KF385436.1), and Major Mitchell cockatoos (KF385406.1). BFDV is well known for having a wide host

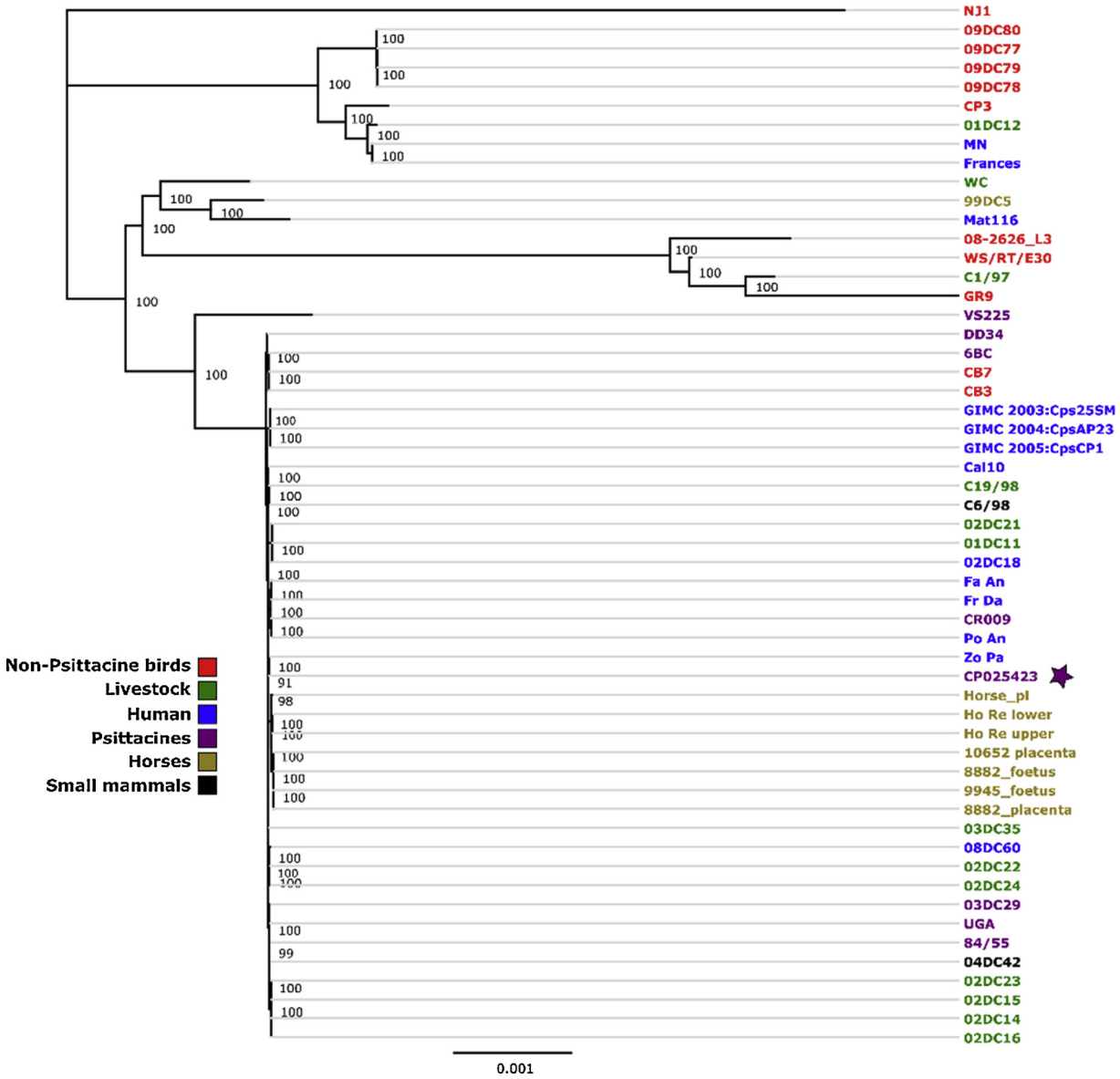


**Fig. 3.** Phylogenetic tree illustrating the relationship of the *Chlamydia psittaci* DNA reads (indicated with a star) to *C. psittaci* genomes from the NCBI RefSeq database, colour coded by host. An alignment of 848,653 bp produced with RealPhy was used in conjunction with MrBayes with the following parameters: 4 chains, 1,000,000 chain length, subsampled every 1000 iterations, 10% burn in, with GTR + I+G substitution model. Accession numbers available in Supplementary Material.

range and the capacity for flexible host switching and cross-species transmission (Sarker et al., 2014a, 2014b; Das et al., 2015), with evidence suggesting that all species in the order Psittaciformes may be susceptible to infection with all genotypes of BFDV (Khalesi et al., 2005; Raidal et al., 2015). In addition, sulphur-crested cockatoos and galahs often form mixed species flocks (Raidal et al., 1998), and so it is not unexpected that transmission of BFDV strains would be transmitted between these species.

Avian herpesviruses and adenoviruses were detected with a low prevalence (1.81% [95% C.I. 0.3–9.0%]; and 1.81%, [95% C.I. 0.3–9.6%] respectively) in the study population. This is comparable to previous work that documented an absence of PsHV-1 in tested wild and captive birds in Australia (Raidal et al., 1998 [n = 411]; Amery-Gale et al., 2018 [n = 409]). The low prevalence of these viruses detected in our study, and the absence of them as detected by previous studies (despite their larger sample sizes) suggests that these viruses are circulating at low levels in wild cacatuid populations. However, differences in detection techniques (e.g. neutralising antibody titres Raidal et al., 1998 and PCR targeting the herpesvirus DNA polymerase gene in this study and Amery-Gale et al., 2018) and differences of sample types used for testing (choanal/cloacal swabs in this study and Amery-Gale et al., 2018; serum in Raidal et al., 1998); may also account for the differences in detection levels. Although PsHV-1 has not been reported in wild Australian avifauna by PCR testing, it has been reported in imported green-winged macaws on serological testing, and is suspected to be circulating with a low seroprevalence in some avicultural collections (Tomaszewski et al., 2006). In these conditions, latently infected birds may be potential sources of viral dissemination (Tomaszewski et al., 2006). However, it is important to note the differences in methodology between the PCR testing used to evaluate the presence of viral DNA in wild bird populations (the current study and Raidal et al., 1995; Amery-Gale et al., 2018), versus the serological testing used to determine the presence of viral antibodies in birds in avicultural collections (Tomaszewski et al., 2006), meaning it is not possible to make direct comparisons of PsHV-1 prevalence between wild and captive birds in Australia based on the information currently available. Herpesviruses, particularly related alphaherpesviruses, are often serologically indistinguishable, and the discovery of a novel avian

iltovirus that is most closely related to PsHV-1, suggests that serological studies are unlikely to have differentiated the two viral species.

In Psittaciformes, alphaherpesviruses of the genus *Iltovirus* have been documented to cause respiratory disease (Lazic et al., 2008; Shivaprasad and Phalen, 2012; Sutherland et al., 2017). Psittacid herpesvirus-1 (formerly known as Pacheco's Disease) causes an acute, highly contagious and frequently fatal respiratory disease in parrots. Particularly affecting cockatoos, macaws and Amazon parrots, the virus targets lymphocytes and hepatocytes, resulting in severe tissue necrosis and the formation of syncytial cells (Panigrahy and Grumbles, 1984; Thureen and Keeler, 2006). In addition, herpesviruses with a tropism for the respiratory tract have been reported in Bourke's parrots (*Neopsephotus bourkii*) and Indian ringnecks (*Psittacula krameri*) (Helfer et al., 1980; Tsai et al., 1993; Raidal et al., 1995; Lazic et al., 2008; Shivaprasad and Phalen, 2012; Sutherland et al., 2017).

Of the five genera of adenoviruses, avian adenoviruses are present in the genera *Atadenovirus*, *Aviadenovirus* and *Siadenovirus*. Lesions associated with adenovirus in psittacine birds include hepatitis (Scott et al., 1986; Pass, 1987; Gomez-Villamandos et al., 1992; Ramis et al., 1992; Droual et al., 1994), conjunctivitis (Jacobson et al., 1989), pneumonia (Ramis et al., 1992; Desmidt et al., 1991), enteritis (Droual et al., 1994; Mackie et al., 2003) and splenic lymphoid depletion (Gomez-Villamandos et al., 1995), although subclinical infections may occur (Droual et al., 1994). In Australia, avian adenoviruses have been associated with an acute fulminant disease characterised by multisystemic necrosis in red-bellied parrots (*Poicephalus rufiventris*) (Das et al., 2017); necrotising enteritis in lorikeets (*Trichoglossus* spp.) (Mackie et al., 2003); and necrotising hepatitis in various psittacine birds (Scott et al., 1986; Pass, 1987) and a tawny frogmouth (*Podargus strigoides*) (Reece et al., 1985).

While further work is required to understand the pathogenicity and clinical significance of the novel viruses detected in the present study, previous work has suggested that the presence of immunosuppressive diseases such as BFDV may increase the likelihood that subclinical infections with other viruses progress to clinical disease in birds infected with multiple agents (Latimer et al., 1993, 1996; Raidal et al., 1998). Beak and Feather Disease Virus infection is often associated with clinical evidence of acquired immunodeficiency, leading to a variety of secondary or opportunistic infections (Jacobson et al., 1986; Latimer et al., 1990, 1992, 1996). Additionally, impaired immune system function may contribute to the development of avian adenovirus infections (Scott et al., 1986), and a novel pathogenic PsAdV has been detected in Amazon parrots (*Amazona farinosa*) epidemiologically linked to a human psittacosis/avian chlamydiosis outbreak previously (To et al., 2014).

*C. psittaci* is a bacterial pathogen that can cause avian chlamydiosis and human psittacosis. As well as being a primary pathogen that may cause multisystemic disease in birds and form latent infections, it has also been postulated that co-infection with multiple agents may increase the virulence of *C. psittaci* both between and within individual hosts (To et al., 2014). Investigations surrounding a 2014 outbreak of human psittacosis linked to avian chlamydiosis revealed a positive correlation between adenovirus load and the bacterial load of *C. psittaci* in the lungs of affected southern mealy Amazon parrots (*A. farinosa*) (To et al., 2014). Since adenoviruses are known to cause immunosuppression in avian hosts, these authors suggested that due to an impaired immune system caused by adenovirus infection, an increased number of individual birds were susceptible to infection by *C. psittaci*, as well as having a higher bacterial load, in turn leading to a greater chance of zoonotic transmission to humans. In Australia and elsewhere, human psittacosis is a notifiable disease. Molecular typing has suggested that Australian human *C. psittaci* isolates belong to a globally disseminated, highly virulent clonal 6BC clade, primarily acquired via contact with infected parrots (Branley et al., 2016; Jelocnik et al., 2017), and it was this *C. psittaci* lineage that was detected by MLST, and confirmed with 'RealPhy' analysis, in the little corella in the present study. The cause of the discrepancy between the qPCR and NGS results for *C. psittaci* may relate to the organism being present at a level too low to detect with the qPCR used in this study. The median depth of coverage when mapping the NGS reads to the 6BC reference was only 4, suggesting relatively few copies of the genomic DNA may have been present in the sample. Patterson et al. (2015) demonstrated that when using pure vector DNA, the limit of detection the 16SG qPCR when targeting *C. pecorum* and *C. pneumoniae* was 10 copies per reaction. However, it is unclear whether this sensitivity is reflected when detecting *C. psittaci* from choanal/cloacal swabs as collected in this instance. Although *C. psittaci* has long been considered endemic in Australian parrots (Burnet, 1935), spillover from wild and domesticated birds has recently been determined an important source of *C. psittaci* infection in humans, and has also been linked to equine reproductive loss in Australia (Jelocnik et al., 2017; Jenkins et al., 2018), highlighting its significance as an ongoing pathogen of concern.

For wild birds entering the pet trade, there is a significant risk that stress-induced shedding of viral and bacterial pathogens may occur as the birds enter captivity. Of particular note is that there were no clinically observed signs of illness in most of the live birds in this study (96.3%), despite a high detected prevalence of BFDV (69.3% [55.2–80.9%, 95% C.I.]).

Of the two birds that showed non-specific signs of illness (a fluffed appearance and closed eyes), one was PCR positive for BFDV and the other was concurrently infected with BFDV, a novel adenovirus, a novel herpesvirus and *C. psittaci*. It is therefore feasible that birds may enter the pet trade or present for veterinary treatment for reasons unrelated to their infectious disease status without showing clinical signs. Close contact between infected and susceptible birds during the quarantine or hospitalisation period, and for birds entering the pet trade during the sales process and as they enter their new homes facilitates pathogen transmission between hosts, particularly when they are taxonomically related (Amery-Gale et al., 2018). In addition, the risks for the transmission of zoonotic diseases during this period should not be underestimated.

The novel PsHV and PsAdV detected in a little corella are interesting additions to our knowledge of viruses in avian species, particularly the identification of a novel iltovirus. Genomic identification of previously undescribed avian viruses is essential for our understanding of their epidemiology, facilitating management of biosecurity aspects of the domestic and international bird trade, and conservation efforts of vulnerable species. The presence of multiple pathogens detected in a single bird presents a concerning example of the ease with which such infectious agents may enter the pet trade and how novel viruses circulating in wild populations have the potential for transmission into captive birds. While this may not be a representative example of the health of wild Victorian cacatuids since the affected bird presented to a veterinary hospital for treatment rather than being sampled as part of an apparently healthy wild flock, given the relatively small sample size of birds tested as part of this study, its importance should not be underestimated. Those involved in the pet bird trade, bird owners and veterinary staff should be mindful of instigating effective quarantine controls and the importance of testing new birds for potential pathogens to prevent the spread of potentially deadly diseases.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.012.

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