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Liver virome of a Little Corella (*Cacatua sanguinea*) reveals coinfection with a novel parvovirus and two beak and feather disease viruses

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Emerging diseases are acknowledged as a growing threat to wildlife, with the continued identification of pathogenic and potentially pathogenic viruses in avian species resulting from ongoing advances in molecular diagnostic techniques. Parvoviruses under the genus *Chaphamaparvovirus* (subfamily *Hamaparvovirinae*) are highly divergent. The detection and characterisation of parvoviruses in psittacine birds is limited. This study reports a novel parvovirus, tentatively named psittaciform chaphamaparvovirus 3 (PsChV-3) under the genus *Chaphamaparvovirus*, identified in an Australian free-ranging little corella (*Cacatua sanguinea*). The PsChV-3 genome is 4277 bp in length and encompasses four predicted open-reading frames, including two major genes, a nonstructural replicase gene (NS1), and a structural capsid gene (VP1). The NS1 and VP1 genes showed the closest amino acid identities of 78.8% and 69.7%, respectively, with a recently sequenced psittaciform chaphamaparvovirus 2 from Australian *Neophema* species grass parrots. In addition, the presence of two complete novel beak and feather disease (BFDV) genomes, 1993 and 1868 nt in length, respectively, were detected from the same bird. Both these BFDV genomes contained two bidirectional ORFs encoding the putative Rep and Cap proteins. Phylogenetic analysis showed that the sequenced novel BFDV genomes clustered in a distinct subclade with other BFDVs isolated from Australian cockatoos. This study contributes to the characterisation chaphamaparvoviruses and BFDV in Australian parrots and supports the need for ongoing monitoring and molecular studies into the avian virome in native Australian psittacine bird species.

Keywords Avian; beak and feather disease virus; circovirus; co-infection; next-generation sequencing; parvovirus

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Viral pathogens may pose a significant concern at an individual animal, species, and ecosystem level. The diversity of viruses in birds is not well understood, and much of our current knowledge relates to zoonotic viruses (e.g. avian influenza virus), viruses of economic concern for poultry flocks (e.g. Newcastle disease virus [avian paramyxovirus-1]), or viruses that result in notable wildlife mortality (e.g. beak and feather disease virus; BFDV).[1]

Parvoviruses are small (4-6 kb in length), nonenveloped, linear, single-stranded DNA viruses that encode two gene cassettes: a nonstructural replicase gene (NS1) encoding viral replication enzymes, and a capsid (VP1) gene that encodes structural proteins.[2] The family *Parvoviridae* comprises three subfamilies: *Parvovirinae* (10 genera), *Desnovirinae* (11 genera), and *Hamaparvovirinae* (five genera).[2,3] Parvoviruses are frequently associated with gastrointestinal disease in a wide range of animals.

Birds have been identified as reservoirs of several parvoviruses from the subfamily *Parvovirinae (Dependoparvovirus* and *Aveparvovirus* genera), and the subfamily *Hamaparvovirinae* (*Chaphamaparvovirus*). From the genus *Dependoparvovirus*, these include *Anseriform dependoparvovirus 1* (causative agent of the goose parvovirus [GPV; Derzy's disease] and the Muscovy duck parvovirus [MDPV] that cause a fatal disease characterised by diarrhoea, lethargy, anorexia, and prostration in ducklings [MDPV] and goslings [GPV]), and short beak and dwarfism syndrome (SBDS) in mule ducks, caused by a variant of GPV that may be assisted by the presence of duck hepatitis virus.[4,5] These diseases cause significant threats to domestic waterfowl production and industry, as well as wild waterfowl populations, and are capable of independent replication in rapidly growing tissues.[6] The avian adenovirus-associated viruses (AAAV) are helper-dependent, non-pathogenic members of the *Avian dependovirus 1* family that require coinfection with a helper virus (herpesvirus or adenovirus) for productive infection.[2,6] Evolutionarily, MDPV and GPV and the AAAV share a common ancestor.[7] In the genus *Aveparvovirus*, parvoviruses affecting poultry include chicken parvovirus and turkey parvovirus. These also cause enteric disease in affected birds.[7] Recently, several galliform chaphamaparvoviruses (GaChPVs) have been identified as likely aetiological agents of spotty liver disease in association with *Campylobacter hepaticus* in Australia. These GaChPVs belong to the genus *Chaphamaparvovirus*, family *Parvoviridae*, subfamily *Hamaparvovirinae*.[8] Peafowl have also been documented as reservoirs of parvoviruses, with clinical signs including enteritis and pneumonia.[9] Pigeon parvovirus is closely related to turkey and chicken parvoviruses that reside in the genus *Aviparvovirus*, which are thought to circulate widely amongst both wild and domestic avian species.[10] In psittacine birds, novel chaphamaparvoviruses have been detected in asymptomatic rainbow lorikeets[11] and *Neophema* spp grass

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parrots,[12] but little is known about parvovirus in other psittacine bird species.

Beak and feather disease virus has become recognised as the dominant viral pathogen of psittacine birds worldwide.[13] The virus causes an often chronic and fatal disease in Psittaciformes, but can also affect birds from other avian orders, as it is a host-generalist that is capable of flexible host-switching.[14–17] It induces an immunosuppressive condition that affects multiple organs but particularly causes feather dystrophy and loss and keratin deformities of the beak and claws. Clinically, the virus has acute, peracute and chronic forms, and in many cases, is ultimately fatal.[18] A member of the family Circoviridae, BFDV possesses a small, circular, single-stranded DNA genome (approximately 2.0 kb in length) that is encapsidated into a non-enveloped, spherical icosahedral virion.[13,19] In order to replicate its genome, BFDV needs to invade the host nucleus to access its transcriptional machinery. Beak and feather disease virus is known to replicate in numerous tissues, including skin, liver, the gastrointestinal tract, and bursa of Fabricius; while the capsid antigen of BFDV is found in the spleen, thymus, thyroid, parathyroid and bone marrow.[20–22] Viral attachment and entry into host cells may not necessarily lead to viral replication; consequently, not all cells containing viral particles may contribute to the progression of disease. However, BFDV is thought to encode proteins that actively transport the viral genome into the nucleus in addition to factors that direct the precursor DNA to exit the cytoplasm, where it causes large globular intracytoplasmic paracrystalline arrays.[13,19]

Here, we characterise a novel parvovirus identified in tissue samples collected from a wild-caught little corella (*Cacatua sanguinea*), that additionally hosted two BFDV genomes.

Materials and methods

Sampling, ethical considerations and extraction of DNA

Liver tissue was obtained from a wild caught little corella that originated near Cranbourne, Victoria (38.105 S, 145.279 E) that was humanely euthanatized by a registered veterinarian due to multiple trauma-related untreatable injuries. At necropsy examination, fresh tissue samples were aseptically collected from the liver, kidney, and spleen, and stored frozen at −80°C. The samples were sent to La Trobe University for molecular diagnostic investigation. Virus particle enrichment was performed using the stated methods after the elimination of impurities (e.g. host cells, bacteria, and free nucleic acids) from the liver sample[1,23] with minor variations. Briefly, the liver sample was aseptically resuspended and vigorously homogenised in sterile phosphate-buffered saline (PBS) (1:10) and centrifuged at 2500 × *g* for 90 min at 4°C. The supernatant was filtered using a 0.80 µm syringe filter, and the filtrate was processed downstream. The sample was then ultracentrifuged at 178,000 × *g* and 30 psi for 1 h at 4°C using a Hitachi Ultracentrifuge CP100NX (Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant was discarded, and the pellet was suspended in 130 µL of sterile PBS. The filtrates were then nuclease-treated using 2 µL of benzonase nuclease (25–29 U/µL, purity > 90, Millipore; Merck KGaA, Darmstadt, Germany) and 1 µL of micrococcal nuclease (2,000,000 gel units/mL; New England Biolabs, Ipswich, MA, USA) and incubated at 37°C for 2 hours. The nuclease reaction was stopped by adding 3 µL of 500 mM ethylenediaminetetraacetic acid. The viral nucleic acids were extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) without carrier RNA, which allowed the simultaneous extraction of viral DNA and RNA. The quantity and quality of the isolated nucleic acids were determined using a Nanodrop and an Agilent Tape Station at the Genomic Platform, La Trobe University.

Next-generation sequencing

Before library construction, extracted nucleic acids were subjected to cDNA synthesis, and amplification was carried out using the Whole Transcriptome Amplification Kit (WTA2, Sigma-Aldrich, Darmstadt, Germany) as per manufacturer instructions. Amplified polymerase chain reaction (PCR) products were then purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega, Madison, WI, USA). The quantity and quality of the purified products was checked using a Qubit dsDNA high sensitivity assay kit with Qubit Fluorometer v4.0 (Thermo Fisher Scientific, Waltham, MA, USA). The library construction was performed using the Illumina DNA Prep (Illumina, San Diego, CA, USA) as per kit instructions, starting with 250 ng of DNA as measured by a Qubit Fluorometer v4.0 (Thermo Fisher Scientific, USA). The quality and quantity of the prepared library was assessed by the Australian Genome Research Facility, Melbourne, Australia. Cluster generation and sequencing of the library was performed with 150 bp paired-end reads on the Illumina® NovaSeq chemistry, according to the manufacturer's instructions.

Bioinformatic analyses

The resulting raw sequencing reads were analysed as per the established pipeline[24–27] using Geneious Prime® (version 2022.1.1, Biomatters, New Zealand) and CLC Genomics Workbench (version 9.0.1). Briefly, preliminary quality evaluation for all raw reads was generated and pre-processed to remove ambiguous base calls and poor-quality reads and trimmed to remove the Illumina adapter sequences. Trimmed sequence reads were mapped against the chicken genome *Gallus* (GenBank accession number NC_006088.5) to remove likely host DNA contamination. In addition, reads were further mapped to the *Escherichia coli* bacterial genomic sequence (GenBank accession no. U00096) to remove possible bacterial contamination. Cleaned and unmapped reads were used as input data for de novo assembly using a SPAdes assembler (version 3.10.1)[28] under the "careful" parameter in the LIMS-HPC system (a High-Performance Computer specialised for genomics research at La Trobe University). The resulting contigs were compared against the nonredundant nucleotide and protein databases on GenBank using BLASTN and BLASTX,[29] respectively, with an E-value threshold of 1×10^{-5} to remove potential false positives. Contigs that had significant BLAST hits with bacteria, eukaryotes or fungi were filtered out to remove non-viral reads. Virus contigs of interest greater than 300 nucleotides (nt) were imported into Geneious Prime (version 2022.1.1) for further functional analysis. The detected viruses were annotated using Geneious Prime (version 2022.1.1), where genus specific published viruses were used as a reference guideline.

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Comparative genomics and phylogenetic analyses

Genomic features of the newly sequenced viral genomes were analysed using Geneious Prime (version 2022.1.1). Sequence similarity percentages between representative viruses were determined using tools available in Geneious Prime (version 2022.1.1).

For phylogenetic analyses, representative viral genome or gene sequences were downloaded from GenBank, and virus-specific trees were constructed using CLC Genomics Workbench (version 9.0.1) and Geneious Prime (version 2022.1.1, Biomatters, New Zealand). Amino acid sequences of protein-coding genes and nucleotide sequences of the selected partial genes were aligned using the MAFTT L-INS-I algorithm implemented in Geneious (version 7.388)[30] implemented in Geneious Prime® (version 2022.1.1). To determine the best-fit model to construct phylogenetic analyses, a model test was performed using CLC Genomics Workbench (version 9.0.1) using default parameters, favouring a general-time-reversible model with gamma distribution rate variation and a proportion of invariable sites (GTR + G + I). Phylogenetic analyses for nucleotide and protein sequences were performed using the GTR and WAG substitution model, respectively, with 1000 bootstrap support in CLC Genomics Workbench (version 9.5.4).

Results

Detection of a novel psittaciform chaphamaparvovirus 3 (PsChPV-3)

The assembled genome of psittaciform chaphamaparvovirus 3 (PsChPV-3) was a linear single-stranded DNA (ssDNA) molecule of 4277 nucleotides (nt) in length and had a similar basic organization as previously described members of the family *Parvoviridae*. The PsChPV-3 genome was shown to contain 33.8% A, 19.8% G, 23.2% T and 23.2% C, with an A + T content of 57.0% and a C + G content of 43.0%. The overall genomic organization of PsChPV-3 is similar to other parvoviruses, with two major predicted open reading frames (ORFs) that typically contained a replication initiator protein, called NS1, and a viral capsid (VP1).

Comparative analysis of the protein sequences encoded by the predicted ORFs, using BLASTX and BLASTP, identified significant protein sequence similarity (E value $\leq 10^{-5}$) for all four ORFs, according to the BLAST database. The 5′ ORF1 is 639 nt long, and a BLAST search using the putative amino acid sequence revealed a 59.3% protein similar to the hypothetical protein of Phoenicopteridae parvo-like hybrid virus (GenBank accession no. QTE03742.1). The PsChPV-3 non-structural (NS) protein 1 (NS1) ORF was 2016 nt long and shown to the highest amino acid identities to the recently identified PsChPV-2 (protein similarity 78.8%, GenBank accession no. OK482703.1), followed by Ara ararauna chaphamaparvovirus (protein similarity 57.1%, GenBank accession no. QTE04010.1) and PsChPV-1 (protein similarity 55.5%, GenBank accession no. QKX49056.1). Similar to other paroviruses, the complete NS1 gene of PsChPV-3 was 671 amino acids in length and encodes the helicase, including the conserved ATP- or GTP-binding Walker A loop (GPxNTGKT/S; $_{317}$**GPSNTGKS**$_{324}$), Walker B (xxxWEE; $_{355}$IGV**WEE**$_{360}$) Walker B′ (KQxxEGxxxxxPxK; $_{372}$**KQ**VL**EG**MQTSI**PIK**$_{385}$) and Walker C (PxxxTxN; $_{396}$**P**III**TTN**$_{402}$) aa motifs. In addition, the NS1 protein contains two conserved replication initiator (endonuclease) motifs, xxHuHxxxx (IF$_{108}$**H**V**H**$_{110}$AMLQ) and YxxK ($_{166}$**Y**LM**K**$_{169}$) (conserved amino acids are indicated in bold letters, and "u" indicates a hydrophobic residue).

The major 3′ ORF was 1713 nt long and encoded a protein similar to the *Parvoviridae* capsid protein VP1. At the amino acid level, the PsChPV-3 VP1 protein exhibited the greatest similarities to PsChPV-2 (protein similarity 69.7%, GenBank accession no. MZ364297.1), followed by Ara ararauna chaphamaparvovirus (protein similarity 49.0%, GenBank accession no. QTE04010.1) and PsChPV-1 (protein similarity 47.7%, GenBank accession no. QKX49056.1). The PsChPV-3 genome also contained a 555 nt long ORF, homologous to the NS2 protein of PsChPV-2 (protein similarity 89.7%, GenBank accession no. MZ364297.1), followed by turkey parvovirus TP1-2012/HUN (protein similarity 50.8%, GenBank accession no. AHF54688.1) and peafowl parvovirus 2 (protein similarity 50.8%, GenBank accession no. QGJ83205.1).

Phylogenetic analyses of the parvovirus subfamilies based on the amino acid sequence of the viral replication initiator protein have supported their familial organisation by viral host range (invertebrate or vertebrate).[3] A phylogenetic analysis based on the parvoviral replication initiator protein (NS1) sequences clearly support the addition of the newly sequenced PsChPV-3 to the genus *Chaphamaparvovirus*. The maximum likelihood (ML) tree shows that the sequenced PsChPV-3 clustered in a distinct subclade with other parvoviruses including psittaciform chaphamaparvovirus 1 and 2 (PsChPV-1 and PsChPV-2), galliform chaphamaparvovirus 1 and 2, and peafowl parvovirus (Figure 1). Using the same set of NS1 protein sequences, we found that the maximum interlineage sequence identity values between the novel PsChPV-3 and other parvoviruses was 57%–60%.

Evidence of two beak and feather disease viruses

Beak and feather disease virus (BFDV) is a member of the *Circoviridae* family and has a relatively simple but compact circular, ambisense single-stranded DNA (ssDNA) genome of approximately 2.0 kb encoding a replicase (Rep) and a single capsid protein (Cap) which facilitates whole-genome viral epidemiological analysis.[19,31–33] Two complete novel genomes of BFDV (1993 and 1868 nt in length, average coverages of 89,380× and 90,035×, respectively) were detected in this study. The nucleotide sequence identity between the two BFDV genomes sequenced in this study was 90.5%, and therefore, we have designated them as two different BFDV genotypes. Both the genomes of BFDV (GenBank accession no. OK482701 and OK482702) contained two bidirectional ORFs encoding the putative Rep and Cap proteins. Phylogenetic analysis based on selected completed genome sequences of BFDV showed that the sequenced novel BFDV genomes in this study clustered in a distinct subclade with other BFDVs isolated from Australian cockatoos (Figure 2), suggesting that the BFDVs we sequenced are likely to have originated from a common ancestor.

Discussion

Advances in molecular diagnostic tools have led to the recent discovery of many novel avian virus species, although it is likely that known viruses represent only a fraction of the overall avian viral diversity. In this study we characterised a novel parvovirus from the

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genus *Chaphamaparvovirus* in a wild-caught little corella, and additionally detected two novel BFDV genomes in the same bird.

Metagenomic sequencing has enabled the identification of several new parvoviruses with some of them identified in avian species.[8,9,12,34,35] Most of these new parvoviruses, including the one identified in this study, have been classified in the recently established *Chaphamaparvovirus* genus, within the *Hamaparvovirinae* subfamily, which includes parvoviruses that infect both vertebrate and invertebrate hosts, and have an amino acid sequence identity for the NS1 protein above 30%.[36] In the present study, a novel parvovirus—PsChPV-3—was detected by NGS in liver samples from a wild-caught little corella. The parvovirus we identified has the closest phylogenetic identity to PsChPV-2, with 57%–60% genome identity and 69.7% amino acid sequence identity. PsChPV-2 was recently detected in faecal samples from apparently healthy *Neophema* spp grass parrots in Australia.[12]

The clinical significance of PsChPV-3 is unknown, as causation between the virus and a disease process that may have led to the susceptibility of the bird in our study to the initial trauma was not established. Histopathological and electron microscopic investigations may have given further insight into its pathogenicity but were not performed as part of our work. Further research into novel avian *Chaphamaparvovirus* species in both wild and captive birds is warranted to determine the evolution and pathogenicity of these viruses.

Beak and feather disease virus is a highly prevalent and widely disseminated pathogen with a host range of over 370 avian species.[37] It is recognised for its high genetic diversity and flexible host switching within the order Psittaciformes and also distantly related non-psittacine species.[15,16,33,37] Multiple variant BFDV infections have been previously described in individual Australian birds. In a

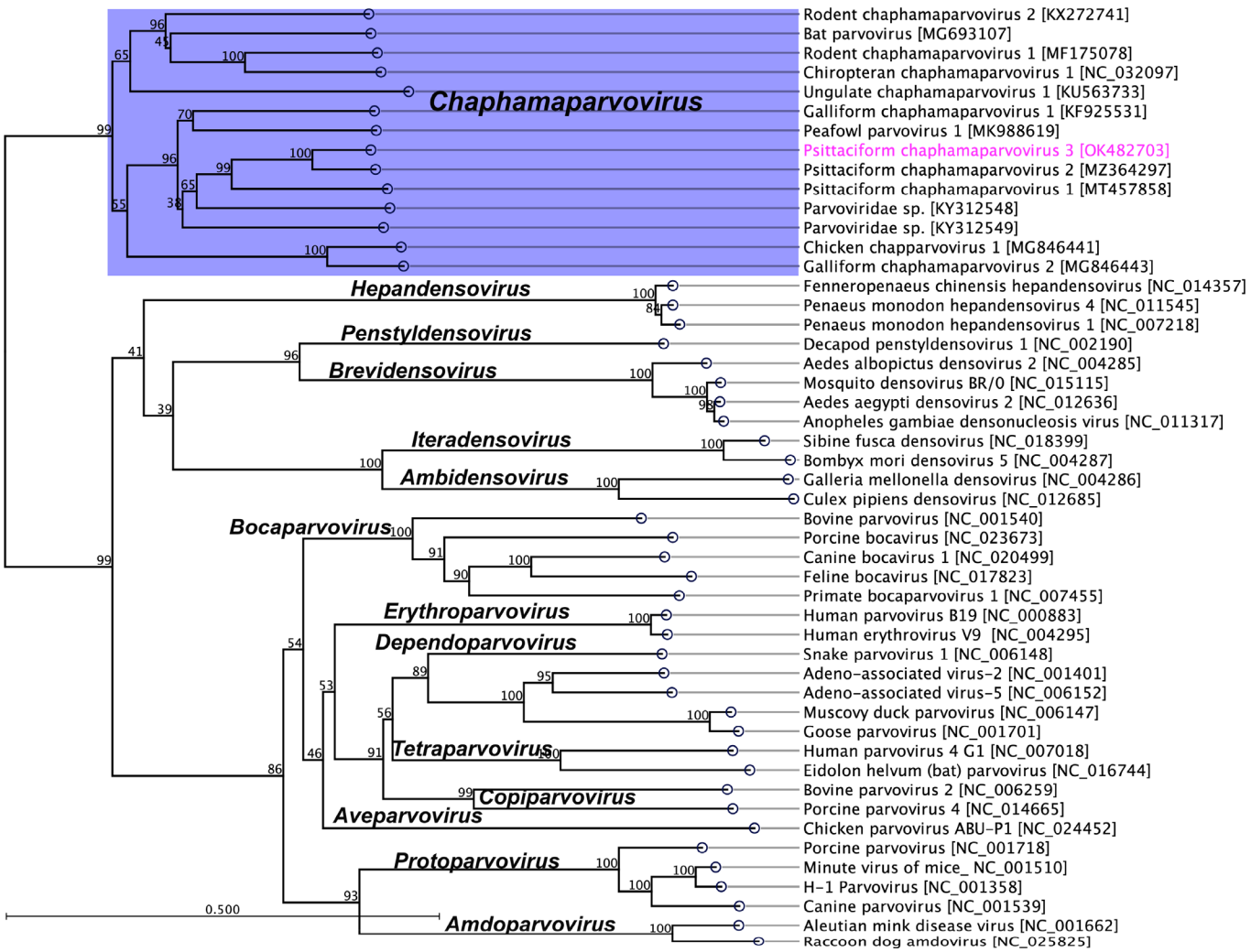

Figure 1. **Phylogenetic tree of the possible evolutionary relationship of psittaciform chaphamaparvovirus 3 with other selected parvoviruses. The maximum likelihood (ML) tree was constructed using the amino acid sequences of complete nonstructural protein (NS1). The numbers on the left show bootstrap values as percentages, and the labels at the branch tips refer to the original parvoviruses' species names, followed by their GenBank accession numbers in parentheses. The clade correspondence to the genus *Chaphamaparvovirus* has a purple background, and the psittaciform chaphamaparvovirus 3 sequenced in this study is shown in pink.**

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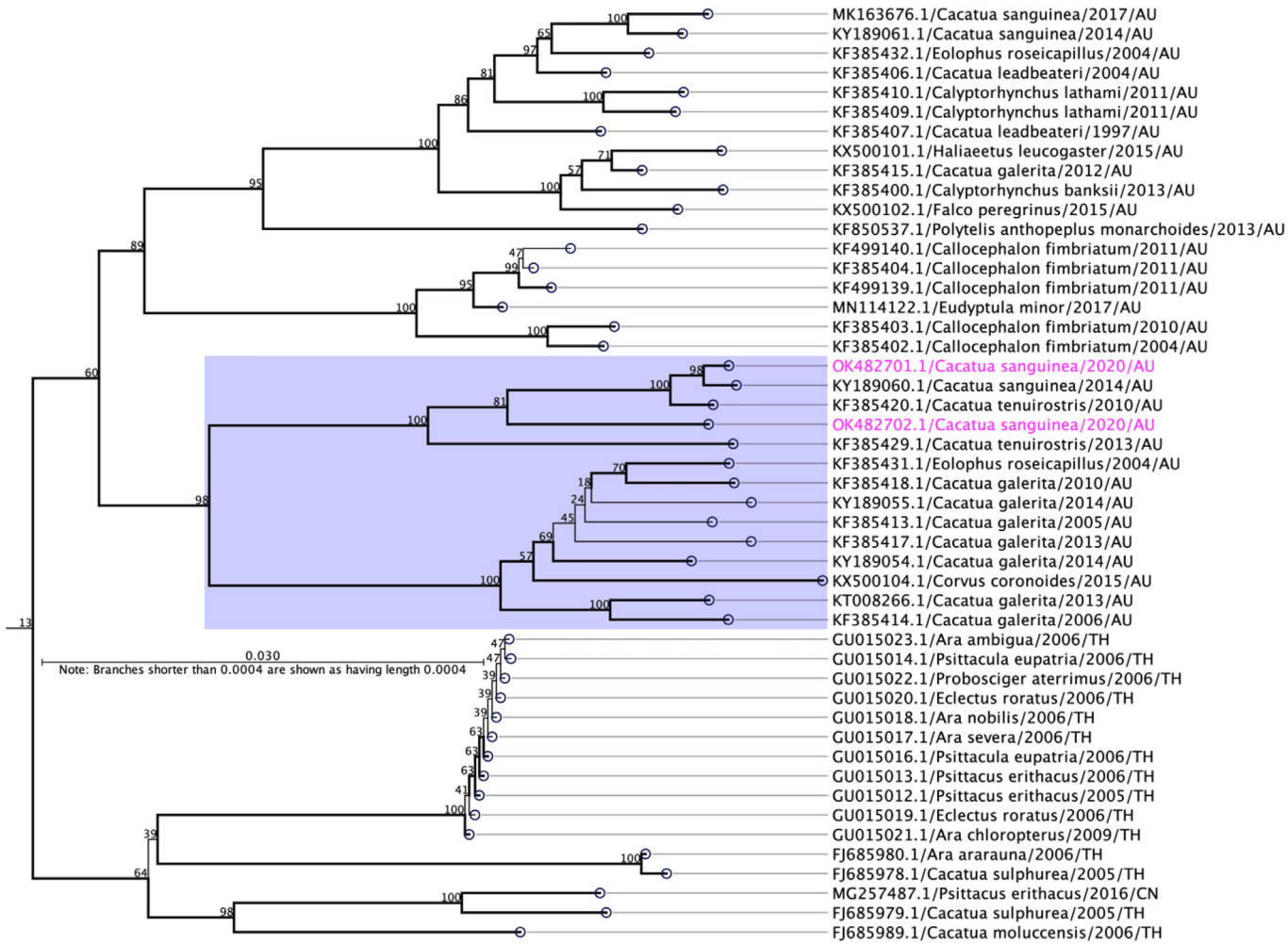

Figure 2. **Phylogenetic tree of the possible evolutionary relationships of the two novel BFDV genomes detected in a little corella (*Cacatua sanguinea*) with other selected BFDV genomes.**

2014 study, at least 30 genotypic variants of BFDV were identified in nine individual birds, with one bird containing up to seven BFDV variants.[33] The novel BFDV genomes we detected support the previously documented high mutation rate for BFDV (orders of magnitude in the range of 10^{-4} substitutions/site/year) and its recombination involving active cross-species transmission in avian subpopulations in Australia.[16,32,37] The flexible host-switching of BFDV is thought to be facilitated by horizontal transmission and its ability to persist for long periods in the environment.[33,38] The sequestration of BFDV genotypes within fomites such as nest hollows has been suggested as a factor extending the replication strategy of the virus, allowing the re-entry of ancestral BFDV genotypes into extant host populations and the emergence of new variants.[33]

Conclusion

Wild birds are major natural reservoirs and potential dispersers of a variety of infectious diseases.[11] Emerging viral pathogens are a significant concern, with potential consequences for human, animal and environmental health.[1,25,26] There are over 370 parrot species in Australia, including 85 critically endangered and vulnerable species, and 19 near risk of extinction.[39] Beak and feather disease virus is considered a key biosecurity threat due to the risks posed to threatened parrot conservation programs.[40] Hence, the identification of novel pathogenic variants and potential pathogens in free-ranging birds is of ongoing concern. Beak and feather disease virus is commonly detected in little corellas and other cacatuids, presenting a persistent threat since these species travel in large flocks, compete with other avian species for nesting sites, and shed the virus into local environments, enabling the transmission and maintenance of BFDV endemicity and facilitating the development of new viral variants.[37,41] Our findings highlight the need for ongoing surveillance and molecular studies for Chaphamaparvoviruses and BFDV, as their ecological impacts could be devastating to recovery programs for threatened native Australian birds.

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Conflicts of interest and sources of funding

The authors declare no potential conflict of interest associated with this publication.

Animal Ethics Approval

The Animal Ethics Committee at La Trobe University was informed that findings from the diagnostic materials were to be used in a publication, and a formar waiver of ethics approval was granted.

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