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Emerging and well-characterized chlamydial infections detected in a wide range of wild Australian birds

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Abstract

Birds can act as successful long-distance vectors and reservoirs for numerous zoonotic bacterial, parasitic and viral pathogens, which can be a concern given the interconnectedness of animal, human and environmental health. Examples of such avian pathogens are members of the genus *Chlamydia*. Presently, there is a lack of research investigating chlamydial infections in Australian wild and captive birds and the subsequent risks to humans and other animals. In our current study, we investigated the prevalence and genetic diversity of chlamydial organisms infecting wild birds from Queensland and the rate of co-infections with beak and feather disease virus (BFDV). We screened 1114 samples collected from 564 different birds from 16 orders admitted to the Australia Zoo Wildlife Hospital from May 2019 to February 2021 for *Chlamydia* and BFDV. Utilizing species-specific quantitative polymerase chain reaction (qPCR) assays, we revealed an overall *Chlamydiaceae* prevalence of 29.26% (165/564; 95% confidence interval (CI) 25.65–33.14), including 3.19% (18/564; 95% CI 2.03–4.99%) prevalence of the zoonotic *Chlamydia psittaci*. *Chlamydiaceae* co-infection with BFDV was detected in 9.75% (55/564; 95% CI 7.57–12.48%) of the birds. Molecular characterization of the chlamydial *16S* rRNA and *omp*A genes identified *C. psittaci*, in addition to novel and other genetically diverse *Chlamydia* species: avian *Chlamydia abortus*, *Ca. Chlamydia ibidis* and *Chlamydia pneumoniae*, all detected for the first time in Australia within a novel avian host range (crows, figbirds, herons, kookaburras, lapwings and shearwaters). This study shows that *C. psittaci* and other emerging *Chlamydia* species are prevalent in a wider range of avian hosts than previously anticipated, potentially increasing the risk of spill-over to Australian wildlife, livestock and humans. Going forward, we need to further characterize *C. psittaci* and other emerging *Chlamydia* species to determine their exact genetic identity, potential reservoirs, and factors influencing infection spill-over.

KEYWORDS
beak and feather disease virus, *Chlamydia*, *Chlamydia psittaci*, genetic diversity, infections, wild birds

1 | INTRODUCTION

Birds can act as successful long-distance vectors and reservoirs for numerous zoonotic bacterial, parasitic and viral pathogens, with co-infection of these also being very common (Levison, 2015; Tsiodras et al., 2008). Such avian pathogens are often actively shed into the environment, potentially infecting humans and other animals through direct or indirect methods, such as environmental contamination and transmission via arthropod vectors (Wille & Holmes, 2020). A key example of such an avian pathogen is *Chlamydia psittaci*, one of the most studied and widely recognized chlamydial species, currently detected in over 460 avian species globally (Stokes et al., 2021), including parrots, pigeons, poultry, seabirds, songbirds and waterfowl (Harkinezhad et al., 2009; Zaręba-Marchewka et al., 2020). Avian *C. psittaci* infections may vary from subclinical, with persistent organism shedding, to severe acute disease (also referred to as ornithosis in non-psittacine birds, or psittacosis/parrot fever in psittacine species) (Knittler & Sachse, 2015). The same pathogen can also cause diseases in livestock (Jenkins et al., 2018), and most importantly, a zoonotic event can cause severe respiratory disease in humans (Knittler & Sachse, 2015).

In the past decade, an expansion in the genus *Chlamydia* came from emerging avian chlamydial species such as *Chlamydia avium, Chlamydia buteonis*, *Chlamydia gallinaceae* (Zaręba-Marchewka et al., 2020) and *Ca. Chlamydia ibidis* (Vorimore et al., 2013). These species are comparatively understudied, with either a suspected or unknown pathogenicity, zoonotic potential and a limited known host range. Perhaps one of the most recent surprising findings is the increasing emergence of avian *Chlamydia abortus* strains, a phylogenetic intermediary between the livestock *C. abortus* and *C. psittaci* (Longbottom et al., 2021). Traditionally, *C. abortus* is an economically significant ruminant pathogen with zoonotic potential causing ovine enzootic abortions (Borel et al., 2018). Despite being characterized as primarily an ovine pathogen, *C. abortus* has also been detected in chickens and various wildfowl in Poland (Szymańska-Czerwińska, Mitura, Niemczuk, et al., 2017; Szymańska-Czerwińska, Mitura, Zaręba, et al., 2017), and parrots from Argentina (Origlia et al., 2019). Furthermore, recent molecular analyses have revealed *C. psittaci* strain 84/2334, isolated from an imported, yellow-crowned Amazon parrot (*Amazona ochrocephala*) from Germany, and Polish wildfowl *C. abortus* strains, are phylogenetically related. Therefore, a proposition exists to expand the *C. abortus* clade to include classical livestock and avian isolates, previously referred to as *C. abortus/C. psittaci* intermediaries (Longbottom et al., 2021; Szymańska-Czerwińskaet, Mitura, Niemczuk, al., 2017; Zaręba-Marchewka et al., 2019).

Contrary to global studies, there is a lack of research investigating *C. psittaci* and other chlamydial species in Australian wild and captive birds. Australia and its offshore islands host a diverse range of over 850 avian species (Dolby & Clarke, 2014), with 45% endemic to Australia. Queensland is Australia's second-largest and most naturally diverse state (Queensland Government, 2021), hosting over three-quarters of the avian species documented in Australia (Chesser, 2009; Lepage, 2020). *C. psittaci* infections in Australian birds are certainly not new, being described in various psittacine species to date, including scaly-breasted and rainbow lorikeets (*Trichoglossus chlorolepidotus*, *Trichoglossus haematodus*), Australian king-parrots (*Alisterus scapularis*), little corellas (*Cacatua sanguinea*), crimson rosellas (*Platycercus elegans*), cockatiels (*Nymphicus hollandicus*), budgerigars (*Melopsittacus undulatus*) and sulphur-crested cockatoos (*Cacatua galerita*) (Stokes et al., 2021). Infections have also been detected in non-psittacine species, mainly in single hosts, including a superb lyrebird (*Menura novaehollandiae*), Australian white ibis (*Threskiornis molucca*) and introduced species such as a domestic pigeon (*Columba livia domestica*) and a spotted dove (*Spilopelia chinensis*). These findings indicate that the Australian avian host range of *C. psittaci* is grossly underestimated (Anstey et al., 2021; Stokes et al., 2021, 2020; Sutherland et al., 2019). Furthermore, these infections were only described in a handful of surveillance studies conducted in one distinct Australian region, in the state of Victoria (Amery-Gale et al., 2020; Stokes et al., 2019, 2020; Sutherland et al., 2019) or as a discrete part of genotyping or equine/human infection studies (Anstey et al., 2021; Branley et al., 2016; Jelocnik et al., 2017; Jenkins et al., 2018). Regarding other avian chlamydial species in Australia, to date, only a single report describes *C. gallinaceae* in free-range chickens and a single wild galah (Stokes et al., 2019), starkly contrasting global avian chlamydial species and host diversity.

In addition to *C. psittaci*, a recent study investigating disease in Australian wild cacatuids revealed that co-infection could occur with multiple agents, including beak and feather disease virus (BFDV) (Sutherland et al., 2019). Beak and feather disease is caused by a highly infectious circovirus and is the most significant viral disease affecting native psittacine (Martens et al., 2020; Sutherland et al., 2019) and non-psittacine avian species in Australia, causing acute or chronic disease often fatal to both adult and juvenile birds (Amery-Gale et al., 2017; Rahaus et al., 2008).

Considering the role of birds in chlamydial zoonoses and the pathogenic potential of avian species, we investigated the prevalence, host range, co-infection with BFDV and genetic identity of species from the genus *Chlamydia* infecting wild birds located in South East Queensland (SEQ), Australia. To gain further insights into the genetic diversity of infecting *C. psittaci* and other emerging avian species in Australia, we applied a molecular typing scheme (*omp*A genotyping) on a range of positive samples. In doing so, we identified genetically diverse traditional and other emerging chlamydial organisms infecting an expanded avian host range.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This study collected 1114 samples from 564 wild birds of 107 species belonging to 17 orders admitted to the Australia Zoo Wildlife Hospital (AZWH, Beerwah, Queensland) between May 2019 and mid-December 2020. Samples consisted of pooled ocular and choanal ($n = 558$), cloacal ($n = 553$) and lung/liver ($n = 3$) dry swabs. All dry swabs were obtained from euthanized birds admitted to the AZWH. The attending veterinarians decided on the euthanizing and admission cause of the birds. Various metadata, including the date, admission cause, location and clinical manifestations were recorded for each sampled bird. Clinical manifestations were noted and recorded by the veterinarians (Supporting Information 1). The birds were collected from 136 different locations across SEQ.

2.2 | Sample processing and DNA extraction

The stepwise methodology used in this study is outlined in Figure S1. All swabs were processed in a Biosafety Cabinet by vortexing and heat lysis on 90°C for 10 min, followed by DNA extraction using the QiaAMP DNA mini kit according to the manufacturer's instructions (Qiagen, Australia). The extracted DNA was labelled with a 'C, E, Li' or 'Lu' at the end of the accession sequence representing a cloacal, eye/choana, liver or lung sample, respectively, and stored in a −20°C freezer until further analyses. This study's ethical approval was granted by the University of the Sunshine Coast Animal Research Ethics Committee (ANE1940, ANE2057).

2.3 | *Chlamydiaceae*-specific quantitative polymerase chain reaction detection

The *Chlamydiaceae* DNA detection was performed on all samples ($n = 1114$) using the *Chlamydiaceae* family-specific probe-based qPCR targeting the 110 bp fragment of the chlamydial 23S ribosomal RNA gene (Ehricht et al., 2006) (Table S1). The quantitative polymerase chain reaction (qPCR) assays were carried out in a total volume of 20 μl, consisting of 10 μl iTaq Universal Probe Supermix (Bio-Rad, Australia), 0.4 μl of the 10 μM probe (Sigma Aldrich, Australia), 5.6 μl PCR grade water, 0.5 μl of each 10 μM forward and reverse primer and 3 μl DNA template. All samples were run in duplicate, and positive (cultured isolates *C. muridarum* and *C. trachomatis* genomic DNA) and negative (MilliQ H_2O) controls were included in each assay. The qPCR conditions were as follows: 95°C for 3 min; 40 cycles of 95°C for 5 s and 60°C for 30 s. In this study, an animal was considered positive for *Chlamydia* spp. if chlamydial DNA was detected in duplicate from any single anatomical site and had a *Cq* value ≤36. The *Cq* cut-off value and the detection limit were determined using a 10-fold serial dilution from 10^6 to 10° copies of quantified *C. pecorum* and *C. psittaci* genomic DNA in triplicate.

2.4 | *C. psittaci*, *C. pecorum* and *Chlamydia pneumoniae* species-specific qPCR assays

In order to confirm the presence of *C. psittaci*, *C. pecorum* and *C. pneumoniae* DNA, all *Chlamydiaceae*-positive samples were subsequently screened with species-specific Sybr Green-based qPCR assays targeting 263 bp, 209 bp and 168 bp of the conserved Cps_0RF_0607, CpecG_0573 (Jelocnik, Laurence, et al., 2019) and *C. pneumoniae ompA* genes, respectively (Table S1). The qPCR assays were carried out in a total volume of 15 μl, consisting of 7.5 μl iTaq Universal SYBR Green Supermix (Bio-Rad), 3.5 μl PCR grade water, 0.5 μl of each 10 μM forward and reverse primer and 3 μl DNA template. All samples were run in duplicate, and positive (cultured isolates *C. pecorum*, *C. psittaci* and *C. pneumoniae* genomic DNA, respectively) and negative (MilliQ H_2O) controls were included in each assay. The qPCR conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 15 s, 57°C for *C. psittaci* and *C. pecorum* and 63°C for *C. pneumoniae* for 25 s, 72°C for 30 s and final extension on 72°C for 7 min, followed by high-resolution melt analyses by a melt of 77.5, 78.0 and 79 ± 0.5°C for *C. pecorum*, *C. psittaci* and *C. pneumoniae*, respectively. In this study, an animal was considered positive for the three *Chlamydia* species if their DNA was detected in duplicate from a single anatomical site and had a *Cq* value ≤33 and high resolution melts (HRMs) of 77.5, 78.0 and 79 ± 0.5°C. The *Cq* cut-off values were determined using a 10-fold serial dilution from 10^6 to 10° copies of quantified *C. pecorum*, *C. psittaci* and *C. pneumoniae* genomic DNA in triplicate.

2.5 | BFDV qPCR detection

Due to the high prevalence of clinical BFDV manifestation in birds admitted to the AZWH, all DNA samples were screened for BFDV utilizing a qPCR assay targeting a 495 bp fragment of the ORF C1 capsid protein (Table S1). The qPCR assay was carried out in a total volume of 15 μl, consisting of 7.5 μl iTaq Universal SYBR Green Supermix (Bio-Rad), 3.5 μl PCR grade water, 0.5 μl of each 10 μM forward and reverse primer and 3 μl DNA template. All samples were run in duplicate, and positive BFDV (positive sulphur-crested cockatoo DNA sample) and negative (MilliQ H2O) controls were included in each assay. The qPCR conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 15 s, 63°C for 25 s, 72°C for 30 s and final extension on 72°C for 7 min, followed by high-resolution melt analyses. In this study, an animal was considered positive for BFDV if viral DNA was detected in duplicate from a single anatomical site and had a *Cq* value ≤33 and an HRM 80 ± 0.5°C for BFDV. The *Cq* cut-off values were determined using a 10-fold serial dilution from 10^6 to 10° copies of purified and quantified 495 bp ORF C1 fragment in triplicate.

For all qPCR assays in this study (chlamydial family and species, and BFDV), samples with discordant results (such as those with only one replicate amplifying) or suspected inhibited amplification were retested.

2.6 | Initial characterization of infecting species using signature *Chlamydiales 16S* rRNA gene sequence

2.6.1 | Conventional *16S* rRNA PCR assays

To further characterize the infecting chlamydial species, we amplified 806 and 476 bp fragments of the signature *Chlamydiales 16S* ribosomal RNA gene (Everett et al., 1999; Jelocnik, Taylor-Brown, et al., 2019) using all 242 *Chlamydiaceae*-positive qPCR samples (Table S1; Figure S1). The conventional PCR reactions were performed in 35 μl volume, consisting of 17.5 μl Amplitaq Gold mix (ThermoFisher, Australia), 12.5 μl PCR grade water, 1 μl of each 10 μM forward and reverse primer and 3 μl DNA template. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 58°C for 45 s for 806 bp *16S* rRNA or 60°C for 35 s for 476 bp *16S* rRNA, 72°C for 45 s and a final extension at 72°C for 7 min. Positive (*C. muridarum* or *C. trachomatis* DNA) and negative (MilliQ water) controls were included in each assay. PCR products were electrophoresed on a 1.5% agarose gel, followed by visual confirmation under a UV transilluminator. Based on band intensity and DNA concentration, amplicons were bidirectionally Sanger sequenced at Macrogen, South Korea (Macrogen). A total of 65 of the 806 amplicons and 476 bp amplicons (from *Chlamydiaceae*-positive samples) were chosen for sequencing.

2.6.2 | Sequence and phylogenetic analysis

The resulting chromatograms for each sequenced amplicon were assessed for quality using their Phred quality scores (>30) and minimum sequence length for both forward and reverse chromatograms in Geneious Prime 2020.2.2 (https://www.geneious.com). Based on the criteria that both chromatograms were of high quality across at least 370 and 700 nt per sequence, we successfully resolved a total of 31 longer (806 bp) and 11 shorter (476 bp) *16S* sequences, respectively. Paired (806 and 476 bp) amplicons were sequenced from eight samples as quality control. The remaining sequences did not fit into the specified criteria, or the sequencing failed, potentially due to low copy numbers or mixed infections in the sample and were excluded for downstream analyses. The newly determined 41 sequences were chosen for further analyses. Preliminary sequence identity was determined using the online NCBI tool, BLAST (Altschul et al., 1990), using blastn against the nr/nt and 16S/ITS databases. The sequence length, top BLAST hit and percent (%) sequence identity were recorded (Supporting Information 1). Sequences were deposited in GenBank under accession numbers MZ823638–MZ823678.

Both shorter and longer newly determined *16S* sequences were used for the phylogenetic analyses. A set of 41 shorter sequences (≤476 bp) and 31 longer sequences (≤806 bp) were aligned to 41 publicly available *16S* rRNA gene sequences from the order *Chlamydiales* using Clustal Omega, as implemented in Geneious Prime, and trimmed to 377 and 757 nt, respectively. Prior to the tree construction, we estimated the best fit model for nucleotide substitution using both 377 and 738 nt *16S* rRNA gene alignments with jModelTest2 (Darriba et al., 2012). The predicted best-fit model for all alignments according to AIC was the GTR+I+G for the 377 the 738 nt alignments. To evaluate preliminary phylogenetic relationships, confirm family-level clustering and determine an appropriate outgroup, maximum likelihood phylogenetic trees were constructed using IQ-Tree v1.6.12 (Nguyen et al., 2015), utilizing the 377 and 757 nt Clustal Omega alignments of the *Chlamydiales 16S* rRNA gene sequences (Figure S2).

Next, we employed both Bayesian and Maximum Likelihood methods to further evaluate the phylogenetic relationship of the *16S* rRNA gene sequences from this study within the family *Chlamydiaceae*. Two separate Bayesian phylogenetic trees were constructed utilizing MrBayes 3.2.6 (Ronquist et al., 2012) (as implemented in Geneious Prime) from the alignment of 41 short (377 bp) and 31 longer (738 bp) *16S* rRNA gene sequences from this study with 19 representative *16S* rRNA gene sequences from 18 species from the family *Chlamydiaceae* (Figure 4). The Bayesian trees parameters included: GTR+I+G nucleotide model for both alignments, with four MCMC chains with million generations, sampled every 1000 generations and with the first 100,000 trees discarded as burn-in. The *16S* rRNA sequence from *Ca. Amphibiichlamydia salamandrae* was used as an outgroup, as inferred above. We additionally constructed maximum likelihood phylogenetic trees with IQ-Tree v1.6.12 (Figure S3) also using GTR+I+G nucleotide model to further confirm the phylogenetic relationships of the same alignments and compare them to that of Bayesian analyses.

2.7 | Evaluating strain diversity using chlamydial major outer membrane protein A (*ompA*) genotyping

2.7.1 | Conventional *ompA* PCR assays

Chlamydial *ompA* genotyping is commonly used to evaluate the genetic diversity of infecting strains (Robbins et al., 2020; Sachse et al., 2008). Following species characterization, we utilized both full length (>1000 nt) and partial (500–720 nt) *ompA* gene sequences for *C. psittaci*, avian *C. abortus*, *C. pecorum* and *C. pneumoniae* strain typing (Table S1). The *ompA* PCR reactions were also performed in 35 μl volume, consisting of 17.5 μl Amplitaq Gold mix (ThermoFisher), 12.5 μl PCR grade water, 1 μl of each 10 μM forward and reverse primer and 3 μl DNA template. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 58°C for 30 s for partial, and/or 55–61°C for 60 s for full length *ompA*, 72°C for 45 s and a final extension at 72°C for 7 min (Table S1). Positive (targeted species DNA) and negative (MilliQ water) controls were included in each assay. PCR products were electrophoresed on a 1.5% agarose gel, followed by visual confirmation under a UV transilluminator. Based on band intensity and DNA concentration, at least 35 amplicons were bidirectionally Sanger sequenced at Macrogen, South Korea.

2.7.2 | The *omp*A sequence and phylogenetic analysis

Using the same sequence quality criteria as above, we successfully resolved a total of 28 *omp*A sequences. Of those, we generated 10 full-length *C. abortus* and *C. psittaci omp*A sequences, ranging from 965 to 1156 nt, in addition to seven shorter sequences ranging from 707 to 715 nt long. A further 10 *C. pecorum* and one *C. pneumoniae omp*A sequences were generated, of which two were full-length *C. pecorum omp*A sequences ranging from 958 to 1072 nt, and eight were shorter fragments ranging from 263 to 418 nt, in addition to a single 688 nt *C. pneumoniae omp*A fragment. The 10 *C. abortus* and *C. psittaci* full-length *omp*A sequences were aligned to 32 representative *C. psittaci*/*C. abortus omp*A genotypes using Clustal Omega (as implemented in Geneious Prime 2021.1.1) and trimmed to a final length of 962 nt. Additionally, the seven shorter sequences were also aligned as above (including the 10 longer sequences) and trimmed to a final length of 583 nt. Furthermore, two full-length *C. pecorum* and one 688 nt *C. pneumoniae omp*A sequence were aligned to 23 *C. pecorum* and 10 *C. pneumoniae* representative *omp*A sequences and trimmed to a final length of 960 nt for *C. pecorum* and 688 nt for *C. pneumoniae*, respectively. Finally, the remaining seven shorter *C. pecorum omp*A sequences were aligned as above (including the two full-length *C. pecorum*) and trimmed to a final length of 279 nt. The eight shortest *C. pecorum* 263 nt *omp*A fragment was omitted from this analysis. The *omp*A sequences from this study were deposited in GenBank under accession numbers MZ962159–MZ962186.

To assess the genetic diversity of *omp*A sequences obtained from this study to representative chlamydial *omp*A genotypes, both Maximum Likelihood and Bayesian phylogenetic trees were constructed. Prior to the tree construction, we estimated the best fit model for nucleotide substitution using both the 583 nt *C. abortus* and *C. psittaci omp*A alignment and the 279 nt *C. pecorum* and 688 nt *C. pneumoniae omp*A alignment with jModelTest2. The predicted best-fit model according to AIC was the GTR+I+G for all the *omp*A alignments.

Bayesian phylogenetic trees were constructed from the 583 nt *C. abortus* and *C. psittaci omp*A alignment, and the 279 nt *C. pecorum* and 688 nt *C. pneumoniae omp*A alignment, using MrBayes 3.2.6 and the following tree parameters: GTR+I+G nucleotide model, with four MCMC chains with million generations, sampled every 1000 generations and with the first 100,000 trees discarded as burn-in. *C. avium* PV4360_2 and *C. gallinaceae* JX-1 *omp*A sequences were used as outgroups (Figure 5). To further assess the phylogenetic relationships of the same alignments and compare it to that of Bayesian analyses, we also constructed maximum likelihood phylogenetic trees with IQ-Tree v1.6.12 (Figure S4). Additionally, two maximum likelihood phylogenetic trees were also constructed using IQ-Tree v1.6.12 for the 962 nt full-length *omp*A sequence alignments of the *C. abortus* and *C. psittaci*, and 960 nt *C. pecorum* and 688 nt *C. pneumoniae* sequences (Figure S5).

2.8 | Statistical analyses

Apparent prevalence and sample size calculations were performed using the Epitools online epidemiological calculators (Sergeant ESG, 2018), with the bird being the unit for calculations. The sample size required to estimate the prevalence of *Chlamydiaceae* within a 95% confidence interval (CI), a precision of ± 5%, and an assumed 10% chlamydial prevalence was determined using the sample size calculation tool (epitools.ausvet.com.au/oneproportion), as implemented in Epitools. Sample sizes to estimate apparent prevalence within a 95% CI from the two avian orders of interest (Columbiformes and Psittaciformes) were achieved. The estimated true and apparent prevalence of chlamydial infections from testing results using a test of known sensitivity (0.9) and specificity (0.95) within a 95% CI was determined using the estimating prevalence utility tool (epitools.ausvet.com.au/trueprevalence), as implemented in Epitools. Statistical software, including SPSS (IBM Corp. 2017) and R Studio (RStudio Team 2020), were utilized to perform chi-squared analysis, binomial logistic regression, estimate odds ratios and determine the presence, direction and magnitude of potential correlations between the detection of *Chlamydia* BFDV and various recorded metadata. *p*-Values ≤.05 were considered statistically significant. The results were obtained utilizing R version 3.0.1 using the ggcorrplot function from a ggplot2 extension package, GGally (Schloerke et al., 2018).

3 | RESULTS

3.1 | Sampled avian species located within SEQ and their admission causes

In this study, a total of 1114 (552 ocular/choanal, 560 cloacal, one lung and liver) swabs were taken from 564 birds, encompassing 107 species from 44 families within 16 avian orders were analyzed (Figure 1a). Due to the frequent detection of *Chlamydiaceae* in parrots (Psittaciformes) and pigeons (Columbiformes) globally, sampling continued until sufficient numbers were achieved to obtain the targeted statistical power calculations requirements in both avian orders. According to admission forms and clinical notes, birds from this study were admitted under three main causes: trauma (293/564; 51.95%), clinical signs/disease (134/564; 23.76%) and animal attacks (55/564; 9.75%). Birds were also admitted for other miscellaneous causes (11/564; 1.95%), including old age, land clearing, inability to fly and entrapment, in addition to unspecified reasons (71/564; 12.59%). Out of all specified admission causes, vehicle collisions (hit by a car) accounted for the largest amount of hospital admissions (81/564; 14.36%), whilst clinical manifestation of beak and feather disease was the second most common specified cause of admission (34/564; 6.02%) (Figure 1b).

In this study, the sampled birds were found from locations across SEQ as far North as Gladstone, down to suburbs situated near and within Brisbane, comprising a total sampling range of over 500 km.

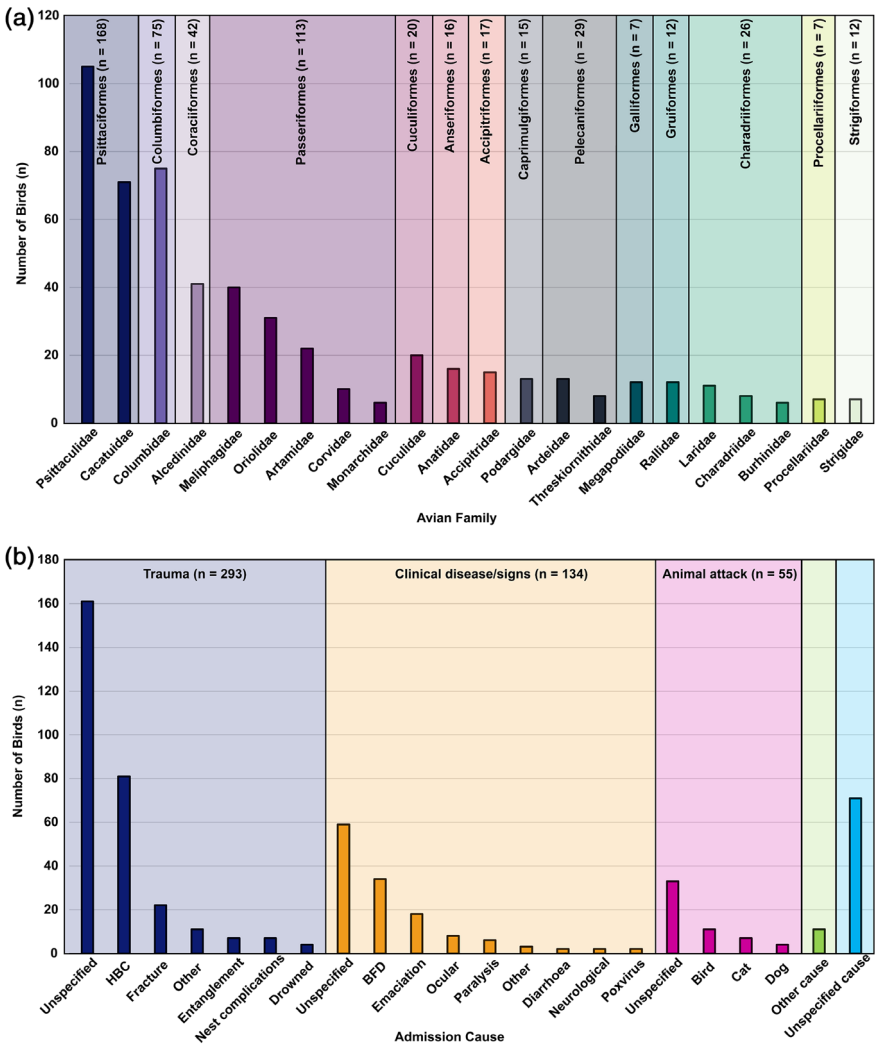

FIGURE 1 Sampled bird diversity and causes of admission. Histogram (a) shows the avian diversity ($n = 564$) and the number of avian families ($n = 44$) sampled in this study. Avian families (x-axis) are grouped by colour according to their respective order. Families containing a sample size $n \leq 5$ birds (y-axis) are excluded from this graph. Histogram (b) shows the various admission causes (x-axis) of sampled birds ($n = 564$; y-axis) from South East Queensland (SEQ) admitted to the Australia Zoo Wildlife Hospital (AZWH) between May 2019 and mid-December 2020. See Supporting Information 1 for the full list of individual avian species, their respective family, order and admission cause from this study. Within trauma, 'Other' includes eye trauma, plane and window collision, neurotrauma, fishing-related injuries and puncture wounds. Within clinical disease/signs, 'Other' includes lung disease, metabolic bone disease and botulism. 'Other cause' includes old age, land clearing, inability to fly and entrapment

The majority of birds in this study were collected from Caloundra/Mooloolah ($n = 104$) and Moreton Bay Regions ($n = 109$), whilst only a few birds were collected from locations including Toowoomba/Moonie ($n = 2$) and Noosa/Peregian Springs ($n = 6$). The geographical range of these birds (Figure 2) and the array of species within the sampled population (Figure 1a) provides a snapshot of the diversity of birds and avian chlamydial prevalence across SEQ.

3.2 | Overall *Chlamydiaceae* prevalence in SEQ avian hosts

Chlamydiaceae DNA was detected in all sampled bird orders (16/16; 100.00%) within 31 families (31/44; 70.45%) and from 65 avian species (65/106; 61.32%) across almost all SEQ locations (Figure 2), resulting in a total positivity rate of 29.26% (165/564; 95% CI 25.65–33.14%) (Table 1, and Supporting Information 2). In our targeted orders, *Chlamydiaceae* DNA was detected in a total of 54/168 (32.14%; 95% CI 25.55–39.54%) parrots (Psittaciformes), with similar detection rates of 33.80% (24/71; 95% CI 23.88–45.38%) and 30.92% (30/97; 95% CI 22.60–40.70%) in the Cacatuidae and Psittaculidae families, respectively (Supporting Information 2). Among Columbiformes, *Chlamydiaceae* DNA was detected in 15/75 (20.00%; 95% CI 12.51–30.41%). There was no significant difference in *Chlamydiaceae* DNA detection between these two orders (p-value = .053). Avian orders with the highest *Chlamydiaceae* DNA detection included seabirds (Procellariiformes, 6/7; 85.71%; 95% CI 48.69–97.43%), raptors (Accipitriformes, 8/17; 47.05%; 95% CI 26.17–69.04%) and shorebirds (Charadriiformes, 11/26; 42.31%; 95% CI 25.54–61.05%). Although, the lowest positivity (in families with $n \geq 5$ birds) was detected in landfowl (Galliformes) (1/7; 14.29%; 95% CI 2.57–51.31%) (Supporting Information 2). No significant difference exists between *Chlamydiaceae* detection and any other avian orders in this study (p-value > .05).

3.3 | Chlamydial and BFDV DNA co-detection

Due to observations recorded by veterinarians of birds with clinical signs consistent with BFDV infection, we also screened for the presence of BFDV DNA. Overall, BFDV DNA was detected in 34.75% (196/564; 95% CI 30.94–38.77%) of the sampled birds in this study

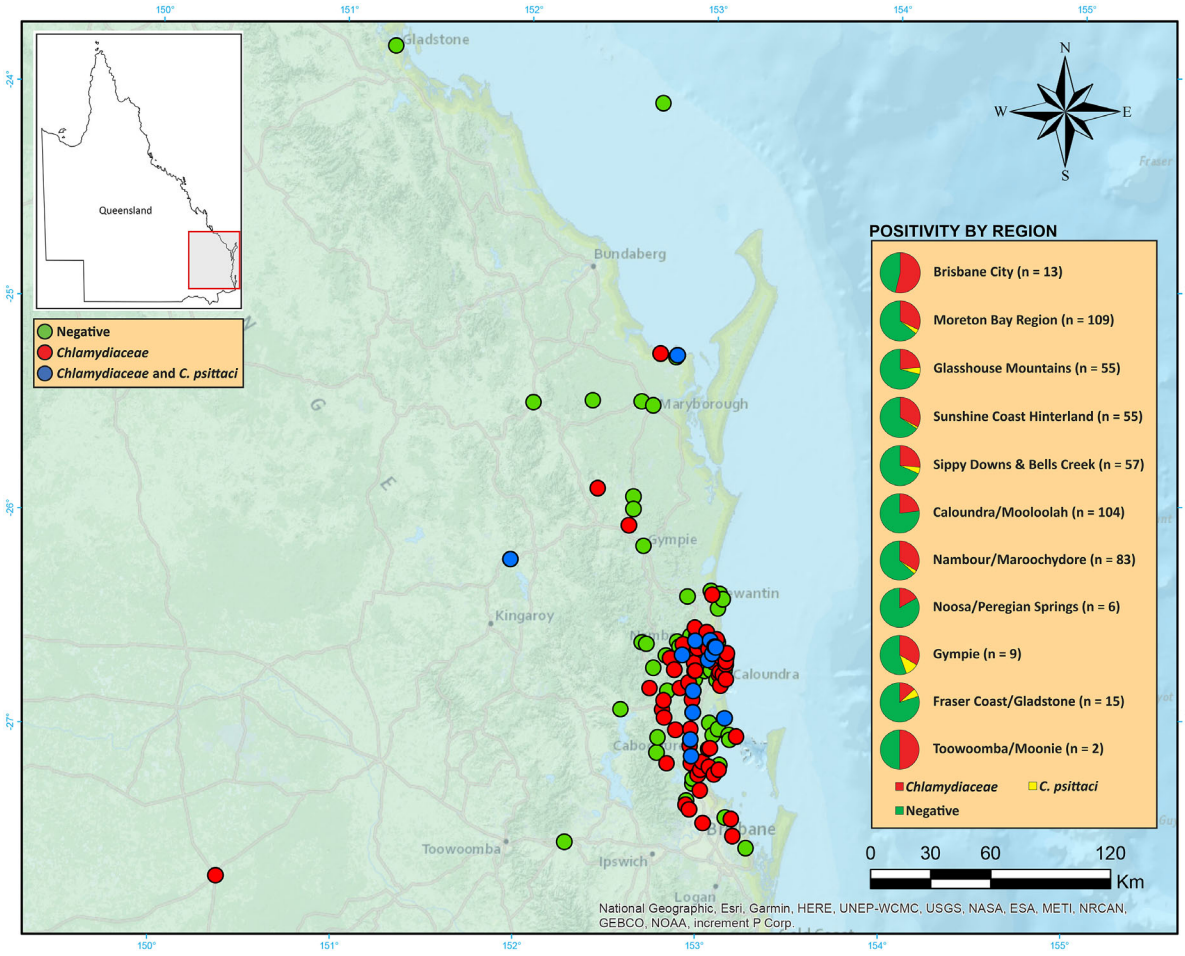

FIGURE 2 A map of South East Queensland (SEQ) showing the various locations birds were sourced prior to the Australia Zoo Wildlife Hospital (AZWH) admission. Each location is marked with a coloured circle dependant on chlamydial prevalence. Green indicates no detection of *Chlamydiaceae*, red indicates *Chlamydiaceae* detection and blue indicate that *Chlamydiaceae* and *C. psittaci* were both detected in that location. Locations were further divided into 11 regions within SEQ, with pie charts showing the percentage of chlamydial prevalence within each region, respectively. For full details on regional prevalence, see Table S2

TABLE 1 Detection rates of *Chlamydiaceae* and beak and feather disease virus (BFDV) DNA in birds and swab samples from this study

	Total number positive	Apparent prevalence[a] (%)	True prevalence[b] (%)
Birds *Chlamydiaceae* positive	165/564	29.26 (CI 25.65–33.14)	28.54 (CI 24.30–33.11)
Swabs *Chlamydiaceae* positive	242/1114	21.72 (CI 19.40–24.24)	19.67 (16.94–22.64)
Birds BFDV positive	196/564	34.75 (CI 30.94–38.77)	35 (CI 30.51–39.73)
Swabs BFDV positive	316/1114	28.37 (CI 25.80–31.08)	30.75 (CI 24.47–30.69)
Birds *Chlamydiaceae*/BFDV positive	55/165	33.33 (CI 26.59–40.83)	25.40 (CI 25.40–42.16)
Swabs *Chlamydiaceae*/BFDV positive	75/242	30.99 (CI 25.50–37.08)	30.58 (CI 24.12–37.74)

Abbreviations: BFDV, beak and feather disease virus; CI, confidence interval.
[a]Wilson CI.
[b]Blaker CI testing results using a test of known test sensitivity 90%, test specificity 95%.

(Table 1). In *Chlamydiaceae*-positive birds, BFDV DNA was detected in 33.33% (55/165, 95% CI 26.59–40.83%) of birds (Table 1). *Chlamydiaceae* and BDFV DNA were co-detected in all orders except for falcons (Falconiformes), landfowl (Galliformes), bustards (Otidiformes) and owls (Strigiformes), with the highest detection rates observed in parrots (Psittaciformes) (17/55; 30.90%) (Supporting Information 2). No significant relationships exist between *Chlamydiaceae* and BFDV coinfection (p-value = .947).

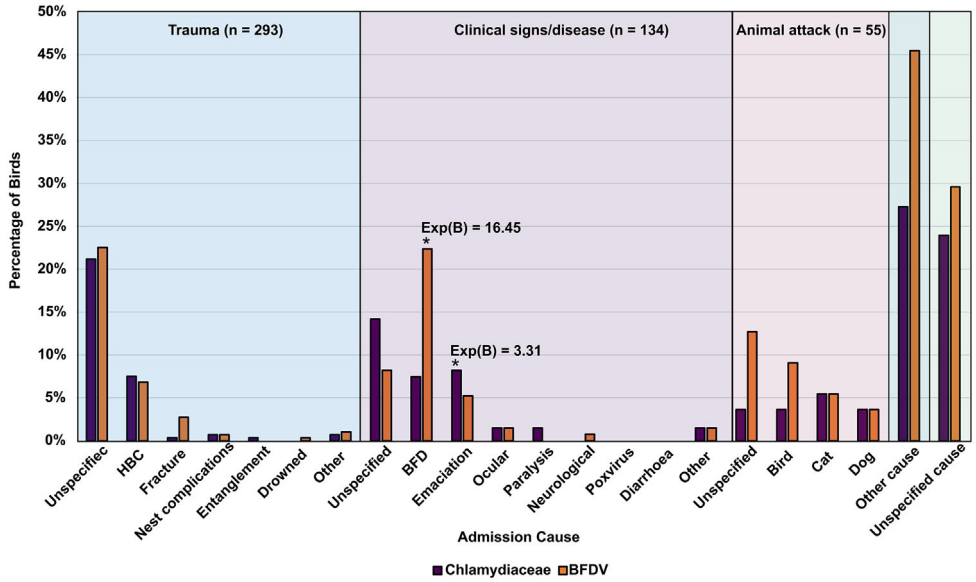

FIGURE 3 Chlamydial and beak and feather disease virus (BFDV) detection percentages within each admission cause category. The histogram displays the percentage of birds within each category (*y*-axis) detected with *Chlamydiaceae* and those co-detected with BFDV, separated based on their respective admission causes (*x*-axis). All significant relationships (*p*-value ≤.5) are denoted with an asterisk in addition to their respective odds ratio (exp(*B*))

3.4 | Relationships between chlamydial and BFDV DNA detection and admission causes

Chlamydiaceae DNA was detected in birds admitted under all admission causes. Within clinical signs/disease, a significant positive relationship exists between *Chlamydiaceae* detection and emaciation, as *Chlamydiaceae* detection rates were the highest in emaciated birds (11/18; 61.11%; *p*-value = .032). Furthermore, emaciated birds were three times more likely to be infected with *Chlamydiaceae* than other admission causes (exp(*B*) = 3.31). Additionally, a significant positive relationship exists between clinical signs of BFDV and DNA detection of BFDV (*p*-value < .005; exp(*B*) = 16.45), with 88.23% (30/34) of birds manifesting signs of BFDV also testing positive by BFDV-specific qPCR (Figure 3). Despite detecting viral DNA in all sampled avian orders, the clinical manifestation of BFDV was only observed in parrots (Psittaciformes).

3.5 | Sequence analyses reveal genetically diverse novel and traditional chlamydial taxa in an expanded avian host range

3.5.1 | *16S* phylogenetic analyses

BLAST analyses of the amplified sequences from this study revealed hits to multiple members within the *Chlamydiaceae* family, ranging from a 95.96%–100% nucleotide identity for the top BLAST hit (Supporting Information 1). This was further confirmed with the phylogenetic analyses using *16S* rRNA sequences of representative members of the *Chlamydiales* order (Figure S2). The phylogenetic analyses using both shorter (377 nt; Figure 4a) and longer (757 nt; Figure 4b) sequences from this study further resolved the relationships between taxa detected in birds from this study and representative taxa of the *Chlamydiaceae* family. Using the 377 nt *16S* rRNA sequence alignment, a total of 16 sequences detected from eight parrots, an Australian bustard (*Ardeotis australis*), sooty shearwater (*Ardenna grisea*), sacred kingfisher (*Todiramphus sanctus*), two Australasian figbirds (*Sphecotheres vieilloti*) and masked lapwing (*Vanellus miles*) were identical to and clustered in a well-supported clade with *C. psittaci* (Figure 4a). Sequences detected from three Torresian crows (*Corvus orru*), a laughing kookaburra (*Dacelo novaeguineae*), and a sulphur-crested cockatoo, in addition to a set of sequences from two short-tailed shearwaters (*Ardenna tenuirostris*), a white-faced heron (*Egretta novaehollandiae*), magpie-lark (*Grallina cyanoleuca*) and pied oystercatcher (*Haematopus longirostris*) formed two well-supported, genetically distinct clades that did not cluster with any known reference sequences, potentially indicative of new chlamydial species (Figure 4a).

Sequences from an eastern koel (*Eudynamys orientalis*), galah (*Eolophus roseicapilla*), rainbow (*Trichoglossus haematodus*) and scaly-breasted lorikeets (*Trichoglossus chlorolepidotus*) were identical to and clustered with *C. pecorum*. Additionally, a sequence from a laughing kookaburra was identical to and clustered with *C. pneumoniae* (Figure 4a). To the best of our knowledge, this is the first time that both *C. pecorum* and *C. pneumoniae* have been detected in birds in Australia. Lastly, the sequence detected in a white-faced heron (*E. novaehollandiae*) was 99.57% identical to and clustered with *Ca. Chlamydia ibidis* representative strain 10–1398/6, although it formed a separate lineage (Figure 4a). Using the 757 nt sequence alignment, we could further resolve and characterize chlamydial taxa detected in birds from this study (Figure 4b). Sequences clustering together in Figure 4a

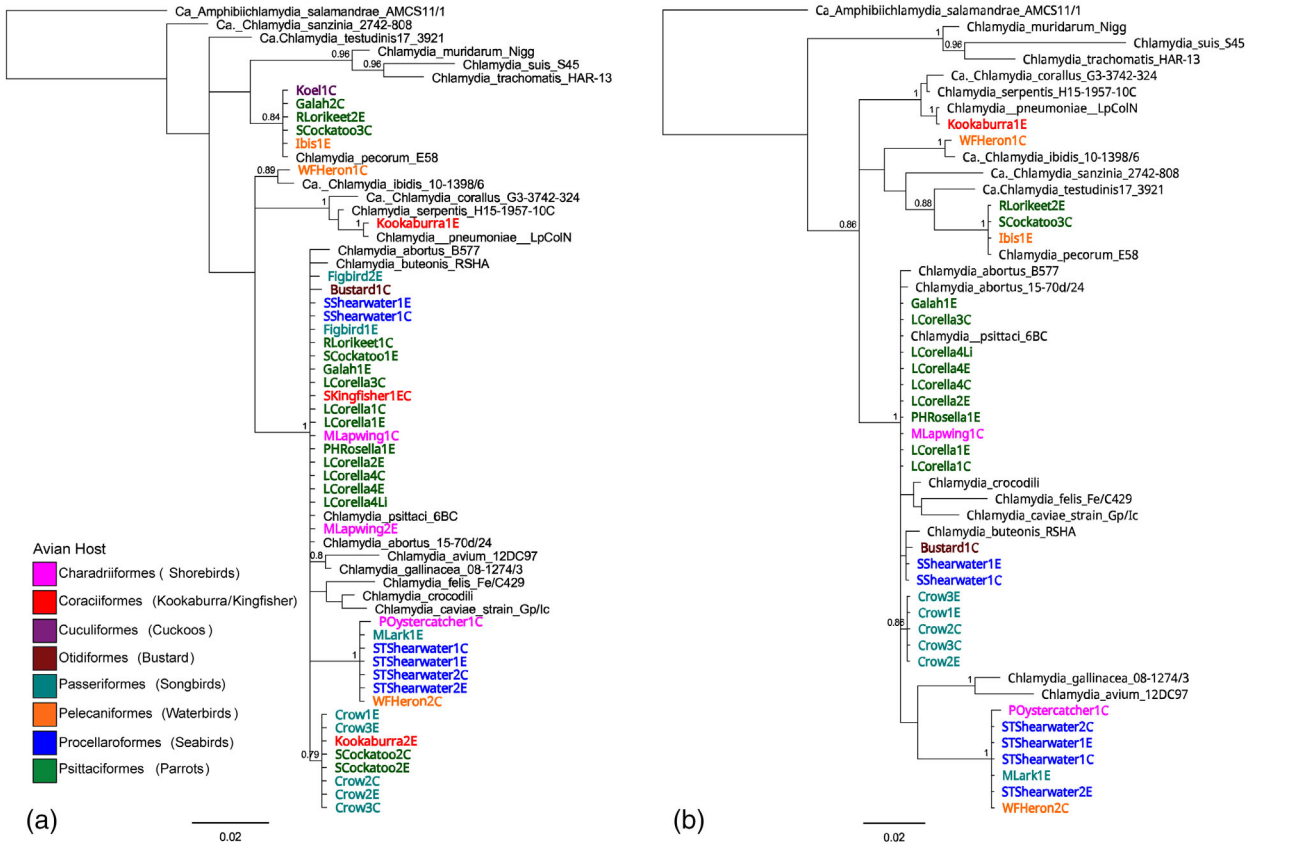

FIGURE 4 Phylogenetic relationships between chlamydial taxa detected in Australian birds from this study and related chlamydial *16S* rRNA sequences. Bayesian phylogenetic trees were constructed using MrBayes 3.2.6 (as implemented in Geneious) for both (a) 377 nt and (b) 757 nt alignments of *Chlamydiaceae 16S* rRNA sequences, using *Ca. Amphibiichlamydia salamandrae* as an outgroup. Representative species of the genus *Chlamydia* are in black, while samples from this study are in bold and coloured according to their avian order, as outlined in the host figure legend. The scale bar indicates the number of nucleotide substitutions per site. Support values ≥0.75 are shown

predominantly clustered the same as presented in Figure 4b. However, two sequences detected from a sooty shearwater and one from an Australian bustard (initially clustering with *C. psittaci* in Figure 4a) clustered together with *C. buteonis* as the closest related representative species in Figure 4b.

3.5.2 | *C. psittaci*, *C. pecorum* and *C. pneumoniae* detected in traditional and novel hosts

As *16S* rRNA analyses revealed detection of *C. psittaci*, *C. pecorum* and *C. pneumoniae*, we further investigated the prevalence and novel host range for these infections using species-specific assays. Out of the 165 *Chlamydiaceae*-positive birds, *C. psittaci* was detected in a total of 18 birds (10.91%; 95% CI 7.01–16.58%) (Table 2). Additionally, *C. psittaci* was detected in birds from seven different avian orders in this study, infecting an extended range of traditional and novel avian hosts. Looking at traditional hosts, the highest *C. psittaci* prevalence was found in parrots (Psittaciformes, 10/18; 55.56%; 95% CI 33.72–75.44%), whilst only a single brown-capped emerald dove (*Chalcophaps longirostris*) was positive for *C. psittaci* (Columbiformes, 1/18; 5.56%; 95% CI 0.99–25.76%). Previously undescribed novel hosts (in Australia and/or globally) for *C. psittaci* included two masked lapwings, two Australasian figbirds, a sacred kingfisher, Australian bustard and a sooty shearwater (Supporting Information 2). The majority of *C. psittaci*-positive birds were admitted due to trauma (8/18; 44.44%). However, other notable admission causes include clinical manifestation of BFDV (2/18; 11.11%), emaciation (1/18; 5.56%) and a cat attack (1/18; 5.56%). BFDV was co-detected in 38.89% (7/18; 95% CI 20.31–61.38%) of *C. psittaci*-positive birds, with 57.14% (4/7; 95% CI 25.05–84.18%) occurring in cockatoos (Cacatuidae). No significant relationship was evident for *C. psittaci* and BFDV co-detection (p-value = .708), nor between *C. psittaci* detection and any admission cause (p-value > .05).

Similarly, we also further investigated *C. pecorum* and *C. pneumoniae* infections with 10.91% (18/165; 95% CI 7.01–16.58%) and 1.21% (2/165; 95% CI 0.33–4.31%) of *Chlamydiaceae*-positive birds testing positive for *C. pecorum* and *C. pneumoniae* infections, respectively (Table 2). *C. pecorum* was detected from eight avian orders sampled in this study, which includes Australian-native species such as the Australian white ibis, laughing kookaburra and the tawny frogmouth, with the majority of *C. pecorum*-positive samples being detected from pigeons (Columbiformes, 6/18; 33.33%; 95% CI 16.28–56.25%) and parrots (Psittaciformes, 4/18; 22.22%; 95% CI 9.00–45.21%) (Supporting Information 2). *C. pneumoniae* was only detected in single samples

TABLE 2 Detection rates of *C. pecorum*, *C. pneumoniae* and *C. psittaci* in *Chlamydiaceae*-positive birds and swab samples from this study

	Total number positive	Apparent prevalence[a] (%)	True prevalence[b] (%)
Birds *C. pecorum* positive	18/165	10.91 (CI 7.01–16.58)	10.54 (CI 6.40–16.58)
Swabs *C. pecorum* positive	20/242	8.26 (CI 5.41–12.42)	7.73 (CI 4.70–12.15)
Birds *C. pneumoniae* positive	2/165	1.21 (CI 0.33–4.31)	0.2 (CI 0.00–3.52)
Swabs *C. pneumoniae* positive	2/242	0.83 (CI 0.23–2.96)	0.00 (CI 0.00–2.09)
Birds *C. psittaci* positive	18/165	10.91 (CI 7.01–16.58)	10.54 (CI 6.40–16.58)
Swabs *C. psittaci* positive	23/242	9.50 (CI 6.42–13.86)	9.05 (5.76–13.68)

Abbreviation: CI, confidence interval.
[a]Wilson CI.
[b]Blaker CI testing results using a test of known test sensitivity 95%, test specificity 99%.

from a native laughing kookaburra and a black kite (*Milvus migrans*), respectively (Supporting Information 2). Both birds were seemingly healthy but were admitted due to trauma.

Furthermore, 38.89% (7/18; 95% CI 20.31–61.38%) of birds detected with *C. pecorum* were also detected with BFDV, whilst no birds detected with *C. pneumoniae* had BFDV. No significant relationship exists between *C. pecorum* and BFDV co-detection (p-value = .708), nor between *C. pecorum*/*C. pneumoniae* detection and any admission cause (p-value > .05).

3.6 | Sequence analyses reveal genetically diverse chlamydial strains infect birds

3.6.1 | *C. abortus*/*C. psittaci ompA* phylogenetic analysis

To further assess intra-species genetic diversity, we performed *ompA* sequence typing on 28 samples that were successfully resolved via *16S* rRNA analyses (Figure 5; Supporting Information 1). A total of 11 *ompA* 583 nt sequences were obtained from various parrot species, including the little corella, galah, pale-headed rosella, sulphur-crested cockatoo and rainbow lorikeet, in addition to other non-parrot species, including a masked lapwing, sacred kingfisher, Australian bustard and sooty shearwater all had 100% identity to *C. psittaci ompA* genotype A, clustering in the same clade with other genotype A sequences detected in horses, humans and other avian hosts (Figure 5a). Additionally, the *ompA* sequences detected in a sooty shearwater had 99.74%–100% similarity to the *ompA* from the *C. psittaci* Ful127 strain identified in Fulmars (*Fulmarus glacialis*), also closely related to *C. psittaci* genotype A. The *ompA* sequence isolated from a Torresian crow had a 99.89% similarity to *ompA* from *C. psittaci* strain A113.6N, previously identified in a rook (*Corvus frugilegus*) from Korea and designated genotype 6N. The remaining two *ompA* sequences from two short-tailed shearwaters are genetically distinct, forming a separate lineage, with the closest *ompA* hits to *C. psittaci ompA* genotype D, identified in turkeys. However, these sequences only share a 77.97%–78.32% similarity to the reference *ompA* sequence, indicating a potentially novel species (Figure 5a).

3.6.2 | *C. pecorum* and *C. pneumoniae ompA* phylogenetic analysis

Out of the nine 279 nt *C. pecorum ompA* sequences, eight sequences belonging to various psittacine species, a brown cuckoo-dove (*Macropygia phasianella*), wonga pigeon (*Leucosarcia melanoleuca*), and short-tailed shearwater clustered together and were genetically identical to *C. pecorum ompA* genotypes denoted A, F and G commonly detected in Australian koalas. Additionally, a sequence from an Australian white ibis was 99.44% similar to an *ompA* sequence identified from Chinese cattle (strain F2137). Lastly, the 688 nt *C. pneumoniae ompA* sequence detected from a laughing kookaburra was 100% identical and clustered with *ompA* sequences detected in Australian marsupials; bandicoot B26 and koala LPCoLN strains (Figure 5b).

4 | DISCUSSION

4.1 | Genetically diverse *Chlamydiaceae* infections are common across birds from SEQ

This study sampled a wide taxonomic range of native and migratory avian species sourced from SEQ, a highly biodiverse region of Australia, and evaluated the prevalence and genetic identity of chlamydial organisms detected from these birds. We found that *Chlamydiaceae* were commonly detected across all 16 sampled avian orders and in nearly one-third of all sampled birds (29.26%; 165/564). Interestingly, our findings are somewhat contrasting compared to other global studies investigating *Chlamydiaceae* in wild birds. For example, our detection rate is markedly higher than a recent review and meta-analysis, which showed that avian chlamydial infections have been described on every continent in a wide range of birds, with a stable worldwide prevalence between 15% and 20% in the past decade (Sukon et al., 2021). Additionally, a study from Switzerland detected a low *Chlamydiaceae* prevalence of 0.9% from 42 avian families (n = 339) (Stalder, Marti, Borel, Mattmann, et al., 2020), and a study from Poland detected a *Chlamydiaceae* prevalence of only 14.8% in 33 different avian families (n = 894) (Szymańska-Czerwińskaet, Mitura, Niemczuk, al., 2017). However, no psittacine species were sampled in these studies, whereas

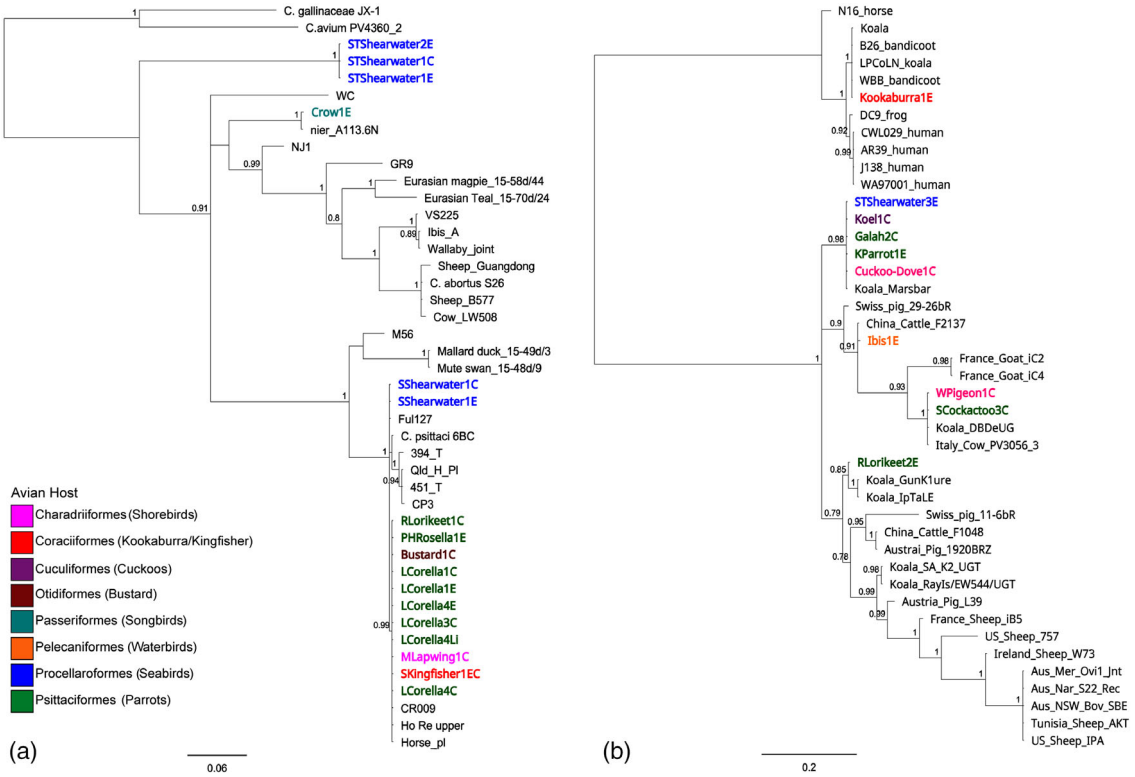

FIGURE 5 Phylogenetic relationships between chlamydial *omp*A sequences detected in Australian birds from this study and representative chlamydial *omp*A sequences. Bayesian phylogenetic trees were constructed using MrBayes 3.2.6 (as implemented in Geneious) for (A) 17 *C. psittaci* and avian *C. abortus omp*A, and (B) one *C. pneumoniae* and nine *C. pecorum omp*A alignments from this study. Representative *omp*A sequences of the genus *Chlamydia* are in black, while samples from this study are bold and coloured according to their avian order, as outlined in the host figure legend. The scale bar indicates the number of nucleotide substitutions per site. Support values ≥0.75 are shown. Tree A used *C. gallinaceae* strain JX-1 and *C. avium* strain PV4360_2 as an outgroup, whilst Tree B was mid-point rooted

multiple species of passerines were sampled in our study. Furthermore, most of the global findings are mainly driven by the ubiquitous reports of *C. psittaci* infections, with a paucity of studies describing other avian chlamydial species (Stokes et al., 2021; Sukon et al., 2021).

In our study, we detected and molecularly characterized *C. psittaci*, in addition to novel taxa highly similar to avian *C. abortus* and a potentially novel chlamydial species from various birds. We also detected *C. pecorum* genotypes highly similar to those found in koalas and livestock, in addition to the marsupial type-strain of *C. pneumoniae* and *Ca. C. ibidis* detected in birds for the first time in Australia. Despite *C. gallinaceae* previously reported in two Australian wild parrot species (Stokes et al., 2019), we did not detect this species in our study. We cannot exclusively say that *C. gallinaceae* or other avian species such as *C. avium* and *C. buteonis* were not present in our bird catalogue, as they may not have been characterized due to a low infection load. Additionally, Stokes et al. (2020) recently detected and molecularly described partial *16S* rRNA sequences similar to those of uncultured chlamydia-like organisms (CLOs) within the order *Chlamydiales*, such as *Parachlamydiaceae*, in crimson rosellas. It is possible that birds from this study could be infected with CLOs. However, this study focuses on *Chlamydiaceae* due to their recognized pathogenic potential.

In this study, we note a sampling bias as we sampled birds admitted to a wildlife hospital. Nearly one-quarter of all birds in this study were admitted due to clinical signs consistent with a variety of diseases (134/564; 23.76%). Of those, *Chlamydiaceae* were detected in 34.33% (46/134), with emaciated birds being 3.31× more likely to be infected with *Chlamydiaceae* than other admissions of clinical signs/diseases (Figure 3). These birds may have been shedding a higher chlamydial load, which contrasts naturally infected wild birds presenting with asymptomatic and low load infections (Amery-Gale et al., 2020; Borel et al., 2018; Konicek et al., 2016). Therefore, these results may not accurately reflect the true prevalence and genetic diversity of *Chlamydiaceae* in wild birds from SEQ but serve as a preliminary estimator of these parameters.

4.2 | Relationships between *Chlamydiaceae* and BFDV co-detection

Surprisingly, chlamydial infection was strongly independent of BFDV, and no significant relationship could be established between *Chlamydiaceae* and BFDV co-detection in this study (p-value = .947). Despite BFDV being highly prevalent and detected across 14/16 (87.50%) of the sampled avian orders, the clinical manifestation of the disease, such as the destruction of feather follicles and the necrosis of beak and claw matrices, was only evident in psittacine species. Our high BFDV

detection rate in clinically unaffected non-psittacine birds in our study aligns with a previous study from Victoria, Australia. This study also detected a high prevalence of BFDV in non-psittacine Australian birds, further indicating that many different Australian bird species could be considered potential reservoirs of the virus (Amery-Gale et al., 2017). Although BFDV is an immunosuppressive agent, attacking and replicating within the bursa of Fabricius and thymus, which are essential in the development and production of lymphocytes and healthy immune function of avian species (Raidal & Cross, 1995), it may not always cause immunosuppression and disease.

4.3 | Global emergence of avian *C. abortus* species

An additional unexpected finding in this study was that *16S* rRNA sequences from three Torresian crows (3/10; 30.00%) and a sulphur-crested cockatoo (1/24; 4.17%) displayed >99% sequence similarity to the avian and livestock *C. abortus 16S* rRNA reference sequences (Supporting Information 1). Novel sequences with 95%–97% similarity to avian and livestock *C. abortus* were also detected in an additional five birds, including an Australian pied oystercatcher (1/1; 100%), a magpie-lark (1/6; 16.67%), a white-faced heron (1/11; 9.09%) and two short-tailed shearwaters (2/4; 50.00%) (Supporting Information 1). Veterinarians noted that two-thirds of the Torresian crows, in addition to the magpie-lark and the Australian pied oystercatcher detected with these sequences, presented with clinical signs of disease—however, details regarding the extent of clinical disease were unspecified (Supporting Information 1). As *C. abortus* and *C. psittaci* are closely related species (Longbottom et al., 2021), these findings were confirmed by a *C. psittaci*-specific qPCR assay, with all samples testing negative. We could not further characterize the remaining sequences, apart from the additional *omp*A characterization from strains detected in a Torresian crow and two short-tailed shearwaters. The Torresian crow *omp*A sequence was 99.89% identical to the *omp*A from *C. psittaci* strain nier_A113, now denoted as an atypical avian *C. abortus*/*C. psittaci* intermediary (Stalder, Marti, Borel, Sachse, et al., 2020) (Figure 5a). This *omp*A genotype (genotype 6N) was first discovered in corvid species located in Russia (Yatsentyuk & Obukhov, 2007) and has since been identified in South Korean and Swiss corvid species (Jeong et al., 2017; Stalder, Marti, Borel, Sachse, et al., 2020). It is also interesting that corvid species from Australia, Asia and European countries share near-identical *omp*A sequences, particularly as Torresian crows are not a migratory species and have only been documented to travel across Australia, Papua New Guinea and other Indonesian Islands (Birdlife International, 2017). Corvid species, such as the Torresian crow, are common in urban environments and are found throughout SEQ (Clifton & Jones, 2017). Whilst predominantly feeding on invertebrates, corvid species are also notoriously known to scavenge and feed on carrion and may potentially ingest carcasses infected with various pathogens such as these novel avian *C. abortus* taxa (Clifton & Jones, 2017; Radomski et al., 2016). Furthermore, the characterized *omp*A sequences (together with the *16S* speciation) from the two short-tailed shearwaters indicate that the taxa infecting these birds could be potentially novel species in the expanding and diverse genus *Chlamydia*.

Out of the 17 *16S* rRNA sequences similar to the recently delineated atypical avian *C. abortus*/*C. psittaci* intermediary species, we were able to characterize four through *omp*A genotyping and showed that sequences from our study cluster with the above, rather than the traditional ovine abortigenic *C. abortus*. In Australia (and New Zealand), the traditional ovine abortigenic *C. abortus* is considered exotic (New South Wales Government 2021). Whether these detected emerging strains have any pathogenic potential or pose a zoonotic risk to livestock, humans and other mammals remains unclear. Therefore, studies investigating the prevalence, host range and genetic identities of these atypical avian *C. abortus*/*C. psittaci* intermediaries and novel taxa, particularly within Australia, may be noteworthy.

4.4 | Avian *C. pecorum* and *C. pneumoniae* infections extend the traditional host range for these pathogens

Our study detected the marsupial and livestock pathogen, *C. pecorum*, in a range of birds, alongside the marsupial-type strains of *C. pneumoniae* in a native laughing kookaburra and black kite. Globally, the detection of *C. pecorum* and *C. pneumoniae* infections in avian taxa are not common, with only a handful of reports describing *C. pecorum* infections in rock pigeons (*Columba livia*) from Germany and Japan (Sachse et al., 2012; Tanaka et al., 2005), and a variety of birds from Argentina (Frutos et al., 2015; Frutos et al., 2012). The latter study also described *C. pneumoniae* infections in various birds, including owls, falcons, and duck species, with *C. pecorum* co-infections detected in parrots, ratites, tanagers and thrushes (Frutos et al., 2015).

Our study is the first to detect genetically diverse *C. pecorum* in parrots, wild pigeon species and other birds, including the native Australian white ibis, laughing kookaburra and tawny frogmouth in Australia. These accounted for 10.91% of *Chlamydiaceae* infections in this study. Further characterization by *omp*A genotyping revealed the *C. pecorum* sequences found in parrots (Australian king-parrot, galah, sulphur-crested cockatoo and rainbow lorikeet) and other birds (short-tailed shearwater, eastern koel, brown cuckoo-dove and wonga pigeon) from this study were identical to *omp*A sequences from Australian koala strains. Interestingly, an *omp*A sequence detected from an Australian white ibis was closely related to *C. pecorum* strain F2137, detected in Chinese cattle. Despite the Australian white ibis not being a migratory species, the origins may be more complex and have possibly resulted from multi-species transmission pathways, including other migratory birds. To further answer this question, studies with a more targeted sampling towards migratory species would be necessary.

Genetically distinct animal strains of *C. pneumoniae* can infect and cause (respiratory) disease in a range of reptilian, amphibian, and mammalian animal hosts, including native Australian marsupials (Roulis et al., 2013). In our current study, *C. pneumoniae* detected from a laughing kookaburra resolved an *omp*A sequence 100% identical to that of *C. pneumoniae* marsupial (koala and bandicoot) strains. *C. pneumoniae* was

also detected in a black kite. However, no sequences could be resolved, potentially due to poor DNA quality or a low copy number present in the extracted DNA sample. In contrast to widespread and clinically severe *C. pecorum* infections in SEQ koalas (Quigley & Timms, 2020; Robbins et al., 2020), there has been no conclusive report about *C. pneumoniae* infections in koalas or other marsupials in the past decade, raising questions about the pathogenic potential of this species in marsupials (Jelocnik, 2019). Detection of *C. pecorum* and *C. pneumoniae* in wild Australian birds and determining what role do birds play in koala and livestock *C. pecorum* infection epidemiology certainly pose curious questions that will require further investigations.

4.5 | *C. psittaci* was detected in expanded avian host range

Although rates of occurrence varied across our study species, notable results included detecting *C. psittaci* in several novel hosts, described for the first time in Australia and to the best of our knowledge globally. These species include the Australasian figbird, masked lapwing, Australian bustard, sooty shearwater and sacred kingfisher. Aside from these novel detections, overall *C. psittaci* prevalence was relatively low at just 3.19% (18/564), with the majority of *C. psittaci* cases detected in parrots (10/168; 5.95%) (Supporting Information 1). These findings are comparable to other recent Australian studies, where *C. psittaci* prevalence ranged from 0.7% to 9.8% (Amery-Gale et al., 2020; Stokes et al., 2020). To date, there is a paucity of data on *C. psittaci* infections in Australian columbids, starkly contrasting studies from Europe and Asia, where pigeons are common hosts for *C. psittaci*. European studies report *C. psittaci* prevalence from 7.8% in German rock pigeons (Sachse et al., 2012) to 15.8% in wild pigeon populations across Switzerland (Mattmann et al., 2019). As recently reviewed, *C. psittaci* prevalence in feral pigeons across Asia ranges from 1% to 25% (Stokes et al., 2021). In Australia, recent studies only described *C. psittaci* in the introduced spotted dove and racing pigeon detected in New South Wales (Anstey et al., 2021), with this study being the first to evaluate *C. psittaci* infections in a larger sample size of wild columbids. However, we only detected one *C. psittaci* case in a single columbid species (1/75; 1.33%), the brown-capped emerald dove (1/2; 50.00%). Considering that Australian native doves and pigeons are phylogenetically distinct and can exhibit different behavioural characteristics to international examples such as European taxa, which flock in greater magnitudes, further studies across Australia are needed to determine a more exact prevalence and columbid host range for *C. psittaci*. Similarly, no *C. psittaci* was detected in any of the Anatid (duck) species in our study or Australia to date, despite wild and farmed ducks and geese being recognized hosts of this pathogen (Gedye et al., 2018; Soon et al., 2021; Vorimore et al., 2015).

C. psittaci is considered to have the broadest host range amongst all *Chlamydiaceae*, highlighting its effectiveness in infecting genetically diverse hosts, including humans and causing zoonotic disease (Borel et al., 2018; Knittler & Sachse, 2015; Radomski et al., 2016). Preliminary strain characterization using *omp*A genotyping from five parrots, an Australian bustard, masked lapwing and sacred kingfisher from this study revealed *omp*A genotype A, commonly detected in highly virulent, clonal, globally distributed psittacine, livestock and human ST24-type strains (Anstey et al., 2021; Vorimore et al., 2021) (Figure 5a). Australian and other global reports describe occupational, direct and indirect zoonotically acquisition of *C. psittaci* ST24/*omp*A genotype A-type strains (Anstey et al., 2021; Branley et al., 2016; Knittler & Sachse, 2015; Veletzky et al., 2021). These studies highlight the need for awareness and stringent biosecurity practices when handling avian species, posing a higher risk for wildlife hospital workers and veterinarians who frequently are in contact with birds (Chaber et al., 2021). Considering the above, we note that at least three parrots from this study infected with *C. psittaci omp*A genotype A were from densely populated urban regions in SEQ, potentially shedding this organism in the environment and posing an indirect risk to public health (Figure 2).

4.6 | Migratory seabirds are important reservoirs of *Chlamydia*

Australia is a host to many migratory seabirds, including the short-tailed and sooty shearwater. Our study showed a high number of shearwaters positive for *Chlamydiaceae* (6/7; 85.7%). These results are comparable to global studies, where traditional and unclassified *Chlamydiaceae* were detected in seabirds, including Charadriiformes, Pelecaniformes and Procellariiformes orders (Aaziz et al., 2015; Blomqvist et al., 2012; Franson & Pearson, 1995; Herrmann et al., 2006, 2000). Shearwaters are one of Australia's most abundant seabirds, as demonstrated by the annual commercial harvesting by recreational hunters (Tasmanian Government, 2021) and are known to migrate over 74,000 km to (and from) New Zealand from (and to) regions, including Alaska, California and Japan (Shaffer et al., 2006). Their ability to travel extended distances in large populations also results in numerous birds washing up on beaches and dying due to disease exhaustion, and extreme weather conditions, leaving serious concerns for zoonotic transmission of exotic diseases. A study published by Isaksson et al. (2015) denoted that due to migrations over large distances and often sharing close habitat with humans, seabirds might act as active transmission vehicles of *C. psittaci*, posing significant zoonotic risks to other wildlife and humans globally. An excellent case example is demonstrated in a previous study describing northern fulmars (*F. glacialis*) infected with *C. psittaci*, causing disease within the bird population and severe disease outbreaks in humans that have captured these birds for consumption (Herrmann et al., 2006; Wang et al., 2020). Fulmars also belong to the same avian order as shearwaters (Procellariiformes). Therefore, detecting identical *omp*A sequences from a sooty shearwater in this study to those in the pathogenic *C. psittaci* Ful127 strain is not surprising. Further investigations into seabirds from Australia are needed to evaluate the risks associated with human contact, particularly in Tasmanian populations who actively commercially harvest these species.

4.7 | Arrival of little corellas to coastal regions of SEQ: A concern for *C. psittaci* transmission?

Many protected Australian species, such as the little corella (*C. sanguinea*), are considered problematic agricultural pests in farming regions across Australia (Department of Environment & Heritage, 2007). In this study, we sampled 17 little corellas, of which five (29.41%) tested positive for *C. psittaci*, and two (11.76%) were co-detected with both *C. psittaci* and BFDV. These results make little corellas the avian species with the highest amount of *C. psittaci* (Supporting Information 2), contrasting a recent Australian study from Victoria where *C. psittaci* was detected in a single little corella, also co-infected with BFDV (1/55; 1.82%) (Sutherland et al., 2019). In the past, the little corellas predominantly inhabited inland Australia. However, periods of drought and the increase in the development and expansion of agricultural systems and peri-urban land use in coastal regions have altered ecological processes and facilitated the recent range extension of little corellas to the South-east Coastal Regions. Additional ecological factors further aided their population recruitment and led to increased local abundance in their now expanded habitat range (Blythman & Porter, 2020; Department of Environment & Heritage, 2007; Strang et al., 2014). Therefore, future epidemiological studies are recommended, particularly in SEQ, to evaluate the potential risk of zoonotic transmission of *C. psittaci* to humans posed by little corellas.

5 | CONCLUSION

Our study provides novel and important information about the prevalence, BFDV co-infection and genetic diversity of *Chlamydiaceae* in Australian free-range birds. We also show that avian chlamydial infections are common in many hosts and are more genetically diverse than expected. We further describe novel organisms such as avian *C. abortus* and traditional organisms such as the zoonotic *C. psittaci* and koala/livestock pathogen *C. pecorum* infecting novel hosts. Our findings also provide new insights into the avian chlamydial infection dynamics, raising new important questions about spill-over risks from birds to native marsupials, livestock and humans.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. This study's ethical approval was granted by the University of the Sunshine Coast Animal Research Ethics Committee (ANE1940, ANE2057). All handling and use of animals conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

AUTHOR CONTRIBUTIONS

Conceptualization: Vasilli Kasimov and Martina Jelocnik. *Methodology (including sampling, laboratory and bioinformatic work) and data analyses*: Vasilli Kasimov, Yalun Dong, Renfu Shao, Clancy Hall, Rosemary Booth, Gareth Chalmers, Susan I. Anstey, Aaron Brunton and Martina Jelocnik. *Writing—original draft preparation*: Vasilli Kasimov. *Funding acquisition*: Martina Jelocnik. *Writing—review and editing*: all authors. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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