

Opportunistic sampling of wild native and invasive birds reveals a rich diversity of adenoviruses in Australia

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Abstract

Little is known about the diversity of adenoviruses in wild birds and how they have evolved and are maintained in complex ecosystems. In this study, 409 samples were collected from woodland birds caught for banding (droppings), birds submitted to a wildlife hospital (droppings and tissues), silver gulls (droppings or tissues), and feral pigeons (*Columbia livia*; oral, cloacal swabs, or tissues) from the Greater Sydney area in NSW, Australia. Additional samples were from native pigeons and doves (swabs) presented to the Healesville Sanctuary, VIC, Australia. Samples were screened for adenovirus DNA using degenerate primers and polymerase chain reaction. Adenovirus sequences were detected in eighty-three samples representing thirty-five novel amino acid sequences. Fourteen novel sequences were atadenoviruses, seven were aviadenoviruses, twelve were siadenoviruses, and one was a mastadenovirus. Sequences from passerine birds were predominately found to form a single lineage within the atadenoviruses, a second lineage in the siadenoviruses, and a third smaller aviadenovirus lineage. These viruses appeared to have co-evolved with a diverse group of woodland birds that share similar habitat. Evidence for host/virus co-evolution in some viruses and a wide host range in others was observed. A high prevalence of adenovirus infection was found in rainbow lorikeets (*Trichoglossus haematodus*), galahs (*Eolophus roseicapilla*), and sulphur-crested cockatoos (*Cacatua galerita*). Sequences were either identical to or mapped to already established lineages in the *Aviadenovirus*, *Siadenovirus*, and *Atadenovirus* genera, suggesting a possible origin of the psittacine adenoviruses in ancestral Australian psittacine birds. The sequences of passerine and psittacine origin provided insight into diversity and structure of the *Atadenovirus* genus and demonstrated for the first-time viruses of passerine origin in the *Aviadenovirus* genus. Four unrelated adenovirus sequences were found in silver gull samples (*Chroicocephalus novaehollandiae*), including one of pigeon origin, suggesting environmental virus exposure. Three pigeon adenovirus types were detected in feral pigeons and infection

prevalence was high. Evidence for host switching between invasive species and native species and native species and invasive species was documented. A variant of a murine adenovirus was detected in kidney tissue from two bird species suggesting mouse to bird transmission.

Key words: adenovirus; Australia; bird; diversity; evolution.

1. Introduction

The family *Adenoviridae* contains five genera, the *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, *Ichtadenovirus* and one proposed genus (*Testadenovirus*) (Harrach et al. 2012). *Siadenoviruses* infect a frog (Clark et al. 1973; Davison, Wright, and Harrach 2000), a tortoise (Rivera et al. 2009), and multiple species of domestic, wild, and captive birds (Zsivanovits et al. 2006; Beach et al. 2009; Katoh et al. 2009; Wellehan et al. 2009; Kovács and Benkő 2011; Park et al. 2012; Lee et al. 2016; Phalen et al. 2019). *Atadenoviruses* are thought to predominantly infect squamates (snakes and lizards) (Benkő et al. 2002; Wellehan et al. 2004; Farkas, Harrach, and Benkő 2008; Szilovicza et al. 2016; Prado-Irwin et al. 2018) but have also been detected in ruminants (cattle, sheep, goats, and deer; *Odocoileus* spp.) (Harrach et al. 1997; Dán et al. 1998; Both 2004), two species of marsupial, the brushtailed possum (*Trichosurus vulpecula*) (Thomson, Meers, and Harrach 2002) and the kowari (*Dasyuroides byrnei*) (Gál et al. 2017), and in bird species, the Southern mealy Amazon parrot (*Amazona farinosa*) (To et al. 2014) and most recently in a long-tailed finch (*Poephila acuticauda*) (Phalen et al. 2019) and in pooled faeces from a Brazilian parrot (*Psittacara leucophthalmus*) and three species of mammals (Duarte et al. 2019). It was thought that aviadenoviruses exclusively infected birds, however, a recent study detected an aviadenovirus sequence in tissues from a pine marten (*Martes martes*) (Walker et al. 2017).

The evolution of adenoviruses and their relationship to their hosts appears to reflect a combination of processes including host–virus co-divergence and host switching. Virus–host co-divergence may explain, on a broad scale, the predominance of mammalian adenoviruses in *Mastadenovirus* genus, avian adenoviruses in the *Aviadenoviridae* and *Siadenoviridae* genera, squamate adenoviruses in the *Atadenoviridae* genus, and the exclusive presence of turtles and tortoises in the proposed *Testadenovirus* genus. On a smaller scale, host–virus co-divergence has also been identified in closely related species (Papp et al. 2009; Prado-Irwin et al. 2018; Diffo et al. 2019). Host–virus co-divergence, however, cannot fully explain the phylogenetic relationships of adenoviruses to their hosts within genera. For instance, how a pine martin can be infected with an aviadenovirus (Walker et al. 2017) and ruminants (Harrach et al. 1997; Both 2004) and birds (Davison, Benkő, and Harrach 2003; Phalen et al. 2019) can be infected with an atadenovirus is not known. These apparent anomalies, instead, appear to be the result of host switching events (Benkő and Harrach, 2003). In fact, it has been suggested that host–virus co-divergence was an uncommon phenomenon in adenoviruses, but host switching occurred frequently (Geoghegan, Duchêne, and Holmes 2017). The recent discovery of multiple recombination events between distantly related adenoviruses, also suggests that host switching between unrelated host species may be occurring resulting in adenovirus co-infections providing an opportunity for crossing over events (Das et al. 2017).

The epizootiology and diversity of adenoviruses in wild vertebrates are largely unknown. Studies in domestic animals

(Teske et al. 2017; Schachner et al. 2018), wild birds, and wild birds kept as pets or in breeding collections (Oaks et al. 2005; Zadavec et al. 2011; Phalen et al. 2019; Yang et al. 2019) suggest that many adenoviruses are highly infectious, but infections are predominately sub-clinical and persistent resulting in lifelong infection and continuous or intermittent shedding. Some of these studies also suggest that maintaining the ability to infect a range of hosts may also be a strategy that allows some adenoviruses to persist within bird populations (Oaks et al. 2005; Zadavec et al. 2011; Teske et al. 2017; Benge et al. 2019; Phalen et al. 2019).

It is likely that the diversity of adenoviruses in wildlife far exceeds that which is currently known and that surveys of wildlife are likely to reveal one or more novel adenoviruses in most studied species. As examples, two novel adenoviruses were discovered in pine martens in the UK along with a novel adenovirus in the Eurasian otter (*Lutra lutra*) (Walker et al. 2017), three novel adenoviruses were found in three species of wild lizards (Iberian worm lizard [*Blanus cinereus*], Carpetane rock lizard [*Iberolacerta cyreni*], Iberian green lizard [*Lacerta schreiberi*]) in Spain (Szilovicza et al. 2016), four novel adenoviruses were found in a single Caribbean reptile (the brown anole [*Anolis sagrei*]) (Prado-Irwin et al. 2018), and adenoviruses have been identified in wild gulls (*Larus* spp.) (Bodewes et al. 2013), South Polar skuas (*Stercorarius maccormicki*) (Park et al. 2012), chinstrap penguins (*Pygoscelis antarcticus*) (Lee et al. 2016) multiple species of raptors (Oaks et al. 2005; Kovács and Benkő, 2011) and a single wild passerine species, the great tit (*Parus major*) (Kovács et al. 2010). Perhaps the most revealing study on the potential for adenovirus diversity was a survey of wild rodents and shrews in Cameroon that found fourteen different mastadenovirus genotypes in relatively few species of mammals (Diffo et al. 2019).

The diversity of adenoviruses in wildlife may also be impacted by the intentional and unintentional movement and release of invasive species. Recently, helodermatid adenovirus 2, originally identified in captive Mexican bearded lizards (*Heloderma horridum*) and wild gila monsters (*Heloderma suspectum*) in the USA (Papp et al. 2009), was detected in a captive central bearded dragon (*Pogona vitticeps*) in the USA, and a nearly identical sequence was identified in a captive death adder (*Acanthophis antarcticus*) in Australia (Benge et al. 2019). How this virus moved between continents is not known, but it was speculated that movement of animals for the pet trade may have been responsible. Millions of birds have been moved worldwide as the result of the international trade in wild-caught and captive-raised pet birds and the international sale of racing pigeons (*Columbia livia*), so it is likely that many known and unknown avian adenoviruses have been distributed globally as well (Bergman 2009; Gilbert et al. 2012).

The purpose of this study is to test the hypotheses that a previously unrecognised diversity of adenoviruses is cryptically circulating in wild birds in Australia and that wild Australian psittacine birds are the likely source of many adenoviruses found globally in pet psittacine birds. Having confirmed these hypotheses, we then explore the possible role that maintaining a wide host range may play in the epizootiology of these

adenoviruses. We also screen invasive bird species to determine what adenoviruses they may have introduced to Australia and to determine if these viruses have disseminated to native species. Lastly, we investigate the possibility that invasive bird species might become infected with adenoviruses of native birds.

2. Methods

2.1 Animal ethics and permits

Gull samples were collected under the NSW OEH NP Scientific License No. SL100109 and sample collection was approved by the University of Wollongong Animal Ethics Committee Animal Research Authority AE17/20. The research on Five Islands National Park was approved by NPWS Highlands Illawarra Area Manager. Sample collection in the Australian Botanic Garden was performed under Australian Bird and Bat Banding Scheme (ABBBS) license No. A 656, New South Wales National Parks and Wildlife Service permit to trap, band, and release protected fauna (license No. SL 100621) and Animal Research Authority No. 141209/04. For Windsor Downs Nature Reserve and Wianamatta Nature Reserve sample collection, the license numbers are ABBBS Banding Authority No. 1893, ABBBS project approval—cooperative project 8529, New South Wales National Parks and Wildlife Service—Scientific License No. SL101929. For the site at Ungarie, the permit numbers are ABBBS project approval—project 1893-2, New South Wales National Parks and Wildlife Service—Scientific License No. SL101918. For sample collection at the Weddin Mountains National Park, the license numbers are Weddins Cooperative Banding Site Project 8515/1, ABBBS Banding Authority 1277, NSW NPWS Scientific License SL100990. Feral pigeons were supplied by an accredited and licensed pigeon control company.

2.2 Sample collection from wild birds

Samples were collected opportunistically in seven different locations in NSW, Australia (Fig. 1). Droppings from live birds or kidney samples from birds that died or were euthanized were collected from injured or diseased birds brought to the Avian, Reptile, and Exotic Pet Hospital of the University of Sydney, Camden Campus (34°0'10.61"S, 150°37'27.84"E), between 1 December 2018 and 30 April 2019 by members of the public (Supplementary Table S1). Droppings were collected using sterile swabs. Birds that died or were euthanized were necropsied and kidney samples were collected using cleaned and bleached instruments. Kidney, spleen, and liver were also collected from eleven feral pigeons killed as a part of pest control programme in Sydney, NSW, Australia.

Droppings ($n = 242$) were also collected from holding bags from thirty-nine bird species caught in mist nets as part of fauna surveys in the Australian Botanic Gardens Mount Annan (−34.065686, 150.769603), Windsor Downs Nature Reserve (−33.644051, 150.803406), Wianamatta Nature Reserve (−33.693320, 150.730140), Weddin Mountains National Park (−34.012656, 148.057075), and the city of Ungarie (−33.732826, 146.857419) (Supplementary Table S2). Swab samples were collected from ten clean holding bags to serve as negative controls.

Fresh droppings were collected off the ground from sixteen silver gulls (*Larus novaehollandiae*) on their breeding grounds on Five Islands (−34.492062, 150.927735) off the coast of Port Kembla, NSW, Australia (Fig. 1). An additional four dead silver gulls (1-day-old chick, one fledgling, and two adults) were presented for necropsy as a part of a disease outbreak investigation

that was occurring on the Five Islands between October 2018 and December 2018. Kidney, spleen, and liver were collected from these birds. All samples were stored at −20 °C until analysis.

Archived DNA samples (combined cloacal and choanal tissue) from four feral pigeons collected from the greater Sydney area, and oral swabs obtained from two feral spotted turtle-doves (*Spilopelia chinensis*), and seventeen wild native pigeons submitted to the Healesville Sanctuary, Healesville, VIC, Australia (−37.682510, 145.530900) were also included in the study. The native pigeons and doves examined included nine white-headed pigeons (*Columba leucomela*), three common bronze-wings (*Phaps chalcoptera*), two wonga pigeons (*Leucosarcia melanoleuca*), two brush bronzewings (*Phaps elegans*), and one brown cuckoo-dove (*Macropygia phasianella*) (Phalen et al. 2017). Archived oral swab samples from feral pigeons collected from the greater Sydney area (Phalen et al. 2017) were tested to determine if this kind of sample would be efficient in the adenovirus detection on the native pigeon samples.

2.3 Nested-PCR for adenovirus detection and sequencing

DNA was extracted from all samples using a commercial kit (Purelink™ Genomic DNA Mini Kit, Invitrogen, CA, USA) according to the manufacturer's instructions. Extracted DNA was screened for the presence of adenovirus DNA using a panadenovirus nested-PCR (polymerase chain reaction) (Wellehan et al. 2004) modified by Yang et al. (2019). Amplicons of the appropriate mass were purified using a commercial kit (Amicon Ultra-0.5 Centrifugal Filter Unit, Millipore) according to the manufacturer's instructions and sequenced using the forward primer (Australian Genome Research Facility, Westmead, NSW, Australia). Sequencing with reverse primers of ten random amplicons and six amplicons for which less than 200 bp of sequence was obtained was also undertaken.

2.4 Phylogenetic analysis

Complete and partial sequences of the DNA polymerase gene (DNA pol) from available adenoviruses were downloaded from the NCBI database to represent all five adenovirus genera (Table 1). A total of sixty-eight publicly available sequences were used in the phylogeny and were from mammals ($n = 16$), birds ($n = 26$), and reptiles ($n = 26$). All sequences were translated into proteins using Mega (Kumar et al. 2018), trimmed and aligned with the newly generated sequences using the programme Muscle (Edgar 2004) and Mega-X (Kumar et al. 2018). A bootstrap network was generated using SpiltsTree4 version 4.15.1. using default parameters (Huson and Bryant 2006).

3. Results

3.1 Detection prevalence, species of origin, and spatial distribution of adenovirus sequences

A total of 409 samples were collected and screened for the presence of adenovirus DNA. Of these, 111 samples produced an amplicon of expected mass and eighty-three of these sequences were confirmed to be those of an adenovirus by sequencing. The distribution and prevalence of positive samples found, as a function of species in which they were found, is shown (Table 2). Amplicons that could not be confirmed to be adenoviruses appeared to contain many overlapping reads. Whether these represented multiple adenovirus sequences or multiple overlapping non-specific sequences is not known. Adenovirus



Figure 1. Map of Southeast Australia showing sites where wild birds were caught in mist nets and sampled and location of the University of Sydney Avian Reptile and Exotic Pet Hospital.

DNA was not detected in the samples from native doves and pigeons or the two Eurasian collared doves. The ten swabs from the clean bags (negative controls) were negative for adenovirus DNA. Sequencing with reverse primers of sixteen amplicons resulted in additional sequence data for samples from a long-billed corella and a bowerbird.

Thirty-six novel adenovirus DNA sequences were identified which represented thirty-five novel amino acid sequences. Of the novel amino acid sequences fifteen were atadenoviruses, seven were aviadenoviruses, twelve were siadenoviruses, and one was a mastadenovirus, their GenBank accession number, abbreviations used in Fig. 2, and species of origin are shown (Table 3). Of these thirty-five novel amino acid sequences, the DNA coding sequences for five of them were less than 200bp long and therefore could not be submitted to GenBank. These sequences are also shown (Supplementary Table S3). Five adenovirus sequences had a high per cent identity (>95%) to previously sequenced viruses (Table 3). These included a strain of psittacine adenovirus 1 detected in rainbow lorikeet kidneys, pigeon adenoviruses 1 and 2, and pigeon adenovirus 4 in pigeon cloacal swabs and pigeon adenovirus 2 in pigeon kidneys. In addition, a DNA sequence with 97.6 per cent similarity to murine adenovirus 2 was detected in droppings of two tawny frogmouths and two galahs, and in the kidney of one southern boobook and one sulphur-crested cockatoo. One sample from a silver gull, referred to subsequently as pigeon adenovirus 2 variant 1, had 97.6 per cent identity with the pigeon adenovirus 2.

Seventeen adenovirus sequences were found in more than one sample (range: 2–7 samples) (Table 3). Seven adenovirus sequences were detected in more than one bird species (Table 3). More than one adenovirus sequence was detected in fifteen species of birds although simultaneous infection with more than one adenovirus in a single bird was not detected. Samples from rainbow lorikeets and silver gulls had the most sequences ($n=4$ for each). The novel siadenovirus sequence detected in four superb fairy-wrens was found in samples collected in the Mount Annan Botanical Garden, the Wianamatta Nature Reserve and the Weddin Mountains National Park. The novel aviadenovirus sequence detected in three eastern yellow robins and one crested shrike-tit was detected in samples

collected at the Mount Annan Botanical Garden and the Windsor Nature Reserve. The novel siadenovirus sequence found in three noisy miners, one New Holland honeyeater, and one Indian mynah was detected in samples collected at the Wianamatta Nature reserve, at the Avian Reptile and Exotic Pet Hospital, and Weddin Mountains National Park. All other repeated sequences were detected in birds sampled at one site.

3.2 Bootstrap network analysis

The network analysis grouped previously described and genetically well-characterised adenoviruses into their appropriate adenovirus genera (Fig. 2). The terminal branches of the network were well supported with most branches occurring in greater than 90 per cent of the bootstrap iterations. Basally, multiple possible evolutionary pathways were identified and were poorly supported.

By combining the novel adenoviruses from this study with known atadenoviruses, an evolutionary relationship between viruses in the *Atadenovirus* genus appears to be unfolding. Four lineages with Lineage 3 containing sub-lineages 3A and 3B of viruses, which may represent five different clades, were identified in the *Atadenovirus* genus. Lineage 1 contained six virus sequences from Australian honeyeaters (family *Meliphagidae*) and two additional virus sequences found in four other species of Australian passerine. One sequence was found in a red-browed finch, silvereye, and superb fairy-wren, all species that use similar habitats. A virus, amniota adenovirus, whose only known sequence was detected in pooled psittacine bird and native mammal faeces from Brazil (Duarte et al. 2019), also occurred in this lineage. Lineage 2 contained the atadenovirus sequences from ruminants. Three virus sequences, lacertid adenoviruses 1 and 2 and virus sequence from noisy miner (Miner 4) mapped in between Lineages 1 and 2. Lineage 3 contained a diverse array of virus sequences from squamates (lizards and snakes). All of the viruses in Lineage 3B are from reptiles; however, Lineage 3A contained sequences from three reptile viruses, one detected in Spain in an Iberian worm lizard, and two from Australian species, the blue-tongued skink and central bearded dragon, and three avian virus sequences, one from a native Australian species, the satin bowerbird (source faeces), and one from an

Table 1. DNA polymerase sequences included in the phylogenetic analysis, their GenBank numbers, and the abbreviations used in the phylogram.

Adenovirus name	Abbreviation used in the phylogram	Accession number
Agamid adenovirus 1	agamid	FJ196812.1
Amniota adenovirus	amniota	MN025530
Amphisbaenian adenovirus 1	amphisbaenian	KT950887.1
<i>Anolis sagrei</i> adenovirus 1	anolis 1	MH558562.1
<i>Anolis sagrei</i> adenovirus 2	anolis 2	MH558564.1
<i>Anolis sagrei</i> adenovirus 3	anolis 3	MH558565.1
<i>Anolis sagrei</i> adenovirus 4	anolis 4	MH558563.1
<i>Anolis sagrei</i> adenovirus 5	anolis 5	MH558569.1
<i>Anolis sagrei</i> adenovirus 6	anolis 6	MH558568.1
<i>Anolis sagrei</i> adenovirus 7	anolis 7	MH558567.1
<i>Anolis sagrei</i> adenovirus 8	anolis 8	MH558566.1
Bat adenovirus TMJ	bat	GU226970.2
Bovine adenovirus 4	bovine 4	AF036092.3
Bovine adenovirus 1	bovine 1	NC_044934
California sea lion adenovirus 1	sea lion	KJ563221.1
Chameleon adenovirus 1	chameleon	AY576679.1
Canine adenovirus type 1	canine 1	U55001.1
Duck atadenovirus A	duck A	AC_000001
Estrildidae adenovirus 1	estrildidae 1	MK413652.1
Estrildidae adenovirus 2	estrildidae 2	MK413651.1
Eublepharid adenovirus 1	eublepharid	AY576677
Fowl aviadenovirus A	fowl A	AC_000014.1
Fowl adenovirus 5	fowl 5	KC493646.1
Fowl aviadenovirus C	fowl C	GU188428.1
Fowl aviadenovirus 2	fowl 2	HM853995.1
Fowl adenovirus 6	fowl 6	KT862808.1
Frog adenovirus 1	frog	NC_002501.1
Gekkonid adenovirus 1	gekkonid	AY576681.1
Goose adenovirus 4	goose 4	JF510462.1
Gouldian finch adenovirus 1	g. finch	KF031569.1
Great tit siadenovirus A	great tit	FJ849795.1
Gull adenovirus	gull	KC309438.1
Helodermatid adenovirus 1	helodermatid	AY576680.1
Human mastadenovirus A	human A	AC_000005.1
Kowari adenovirus	kowari	KT696557.1
Lacertid adenovirus 1	lacertid 1	KT950888.1
Lacertid adenovirus 2	lacertid 2	KT950885.1
Little corella adenovirus	l. corella	MK227353.1
Lizard adenovirus 2	lizard 2	KJ156523.1
Marten adenovirus	marten	KY705358.1
Meyer's parrot adenovirus 1	Meyer's parrot	AY644731.1
Murine adenovirus 1	murine 1	AC_000012.1
Murine adenovirus 2	murine 2	HM049560.1
Murine adenovirus 3	murine 3	EU835513.1
Odocoileus adenovirus A	deer A	NC_035619.1
Ovine adenovirus 7	ovine 7	U40839.3
Ovine adenovirus A	ovine A	AC_000001.1
Pancake tortoise adenovirus	pancake tortoise	JN632575.1
Penguin siadenovirus A	penguin	KP144329.1
Pigeon adenovirus 1	pigeon 1	FN824512.1
Pigeon adenovirus 2	pigeon 2	KX121164.1
Pigeon adenovirus 4	pigeon 4	KX555531.1
Pigeon adenovirus 5	pigeon 5	KX555532.1
Psittacine adenovirus 3	psittacine 3	KJ675568.1
Psittacine 3 type 1	psittacine 3 type 1	MN025529.1
Psittacine adenovirus 2	psittacine 2	EU056825.1

Table 1. (continued)

Adenovirus name	Abbreviation used in the phylogram	Accession number
Psittacine aviadenovirus B	psittacine B	KX577802.1
Psittacine adenovirus 1	psittacine 1	MH580295.1
Psittacine siadenovirus D (proposed name)	psittacine D	MN687905
Raptor adenovirus 1	raptor 1	NC_015455.1
Red-eared slider adenovirus	red-eared slider	JN632580.1
Scincid adenovirus 1	scincid 1	AY576682.1
Siadenovirus IDL-3602	pacific parrotlet	MK695679
Simian adenovirus 3	simian 3	AY598782.1
Skunk adenovirus PB1	skunk	KP238322.1
South polar skua adenovirus-1	skua 1	HM585353.1
Snake adenovirus 2	snake 2	FJ012163.1
Snake adenovirus 3	snake 3	FJ012164.1
Southern Psittacara leucophthalmus aviadenovirus 1	white-eyed parrot	MN153802.1
Turkey adenovirus 1	turkey 1	GU936707.2
Turkey siadenovirus A	turkey A	AC_000016.1
Western bearded dragon Adenovirus 1	bearded Dragon	HQ005514.1
Yellow-bellied slider adenovirus	yellow-bellied slider	JN632578.1

invasive species, the common starling (source kidney), and a sequence from the well-characterised duck atadenovirus 1. Lineage 4 contains virus sequences from birds and possibly a mammal. Five sequences were derived from psittacine birds, two closely related ones from South American parrots, and two closely related sequences, from a sulphur-crested cockatoo and a long-billed corella, and a more distantly related one in a rainbow lorikeet. Lineage 4 also contained two sequences from an Australian ibis and a silver gull that appear to be monophyletic. The only marsupial adenovirus for which there is a DNA polymerase sequence, the kowari adenovirus, appears to map to Lineage 4, based on this analysis. Also, the relationship of *Anolis sagrei* adenoviruses 7 and 8 to the known adenovirus genera could not be determined, but they appeared to fall outside of the *Atadenovirus* genus or may be sister species to the *Atadenovirus* genus (Fig. 2).

The G + C content of partial and complete DNA polymerase genes varied markedly (Fig. 2), but distinct trends in G + C content were observed with similar G + C content being found in clusters of closely related virus sequences. The lowest G + C content (<40% G + C) was found in Lineage 2 (ruminant viruses), a cluster of viruses in of Lineage 1 containing viruses from two passerine species and a virus sequence from Brazil and the sequences lacertid adenoviruses 1 and 2 and a sequence from a noisy miner (Miner 4). The sequences containing the highest G + C content (51.0–71.2%) were found in a cluster of sequences of passerine origin within Lineage 1, the *Anolis sagrei* viruses 3, 4, 5, 6 and a virus from a chameleon in Lineage 3B, two reptile and bird sequences in Lineage 3B, and four of six viruses in Lineage 4. The kowari adenovirus 1 which may represent a member of Lineage 4 also had a high G + C content (65.6%). The remaining atadenoviruses had G + C contents between 40 and 50 per cent.

Two distinct lineages of virus sequences were demonstrated in the *Siadenovirus* genus. Lineage 1 contained the Sulawesi

Table 2. Positive adenovirus samples from wild Australian birds and their sample site of origin.

Species	Sample	N	Confirmed adenovirus sequences	Percentage of positive birds
Rainbow lorikeet	Droppings/kidney	6/24	3/8	37
Feral pigeons	CS/KLS	4/11	4/5	60
Silvereye	Droppings	52	5	9.6
Noisy miner	Droppings	18	7	39
Silver gull	Droppings/KLS	16/4	5/1	30
Superb fairy-wren	Droppings	20	5	30
Eastern yellow robin	Droppings	29	3	10
Australian magpie	Droppings	4/1	3/0	60
Grey fantail	Droppings	8	2	25
Galah	Droppings/K	6/6	2/1	25
White-plumed honeyeater	Droppings	7	4	57
New Holland honeyeater	Droppings	6	3	50
Tawny frogmouth	Droppings/kidney	6/1	3/0	43
Golden whistler	Droppings	15	0	0
Red-browed finch	Droppings	16	2	12.5
Common barn-owl	Kidney	2	2	100
Little wattlebird	Droppings	2	2	100
Southern boobook	Kidney	4	2	50
Sulphur-crested cockatoo	Droppings/kidney	6/2	1/1	25
Australian ibis	Droppings/kidney	1/1	1/0	50
Australian raven	Droppings	1	1	100
Eastern shrike-tit	Droppings	1	1	100
Eastern spinebill	Droppings	4	1	25
Indian mynah	Kidney	2	1	50
Long-billed corella	Droppings	3	1	33.3
Rufous whistler	Droppings	6	0	0
Satin bowerbird	Droppings/kidney	2/1	1/0	33.3
Shearwater	Kidney	2	0	0
Common starling	Droppings	1	1	1
Spotted pardalote	Droppings	13	1	8
Yellow-faced honeyeater	Droppings	7	0	0
Total			83	

KLS, pooled kidney, liver, and spleen; N, number of samples.

tortoise and frog adenoviruses. Based on analysis by others, these are likely to represent a clade of viruses that are basal to the rest of the viruses in the genus *Siadenovirus* (Davison, Wright, and Harrach 2000; Rivera et al. 2009). Lineage 2 contained sequences from a diverse array of birds, including psittacine species, Australian passerine species, a sequence from a penguin, skua, barn owl, turkey, hawk and a sequence from a pigeon. Specific evolutionary trends were not detected with the exception that most of the sequences from Australian passerine species formed a loose sub-lineage. One sequence in this sub-lineage was detected in two honeyeaters and the invasive Indian myna. The only known passerine siadenovirus from a non-Australian bird, the great tit adenovirus, also fell within this sub-lineage of sequences from Australian passerines. A single genetically distinct sequence that did not map to either lineage was found in two Australian magpies and a tawny frogmouth. Sequences from psittacine birds mapped to two distinct groups. Group 1 contained sequences from a virus detected in tissues from a plum-headed parrot and other psittacine adenovirus 2 strains. Group 2 contained sequences from a captive Australian budgerigar (proposed name psittacine siadenovirus D), a wild little corella from Australia, and a captive parrotlet (*Forpus coelestis*) from the USA. Two other virus sequences psittacine adenovirus 5, from a cockatiel in the USA, and budgerigar adenovirus from a budgerigar from Japan are also in this group

based on comparison of sequences of their hexon proteins (data not shown).

Four lineages of virus sequences were identified in the genus *Aviadenovirus*. Lineage 1 contained two pigeon viruses and a variant of pigeon adenovirus 2, which was detected in a sample from a silver gull, and a virus from tissues from a boobook owl. Lineage 2 contained the goose adenovirus sequence, as well as, an unrelated virus sequence from gull, tissues from a barn owl, a single virus sequence that was found in two species of Australian passerines, a sequence from a common marten (a European mammal), and a sequence from pooled parrot and mammal faeces from wild captive animals housed in a Brazilian veterinary hospital (Duarte et al. 2019). Lineage 3 contained the majority of the known chicken and turkey adenoviruses, the sequence of viruses found in nestling of two species of gulls (*Larus argentatus* and *Larus fuscus*) with adenovirus disease and unrelated sequences from tissues from a silver gull and tissues from a boobook owl. Lineage 4 contained three virus sequences from three psittacine birds all of which were detected in wild or captive birds in Australia and a fourth virus sequence from an African species that was held in a captive collection in the USA (Wellehan et al. 2005).

The only mastadenovirus sequence detected in this study was a variant of murine adenovirus 2. It was found in the droppings (galah) and kidney (sulphur-crested cockatoo) of these two species of ground feeding parrots and droppings (tawny

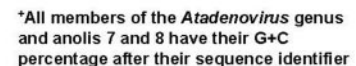


Figure 2. Bootstrap Network (1,000 replicates) generated using SplitsTree4.15.1 and partial DNA polymerase gene amino acid sequences from representative adenoviruses and adenovirus sequences obtained from the birds surveyed in this study. Bootstrap values that exceeded 70 per cent are shown. Murine adenovirus 2 variant 1 was found in the four species of birds in parenthesis; however, it is suspected to be of murine origin. Underlined virus sequences are adenoviruses of birds that have been documented in Australia. Virus sequences identified with an asterisk (*) where found in this study. Adenoviruses identified with a hashtag (#) were found in more than one species of bird, either in this study or were found in this study and/or one or more studies by others: Passerine 1: noisy miner, New Holland honeyeater, Indian mynah (all this study); Passerine 2: eastern shrike-tit, eastern yellow robin (all this study); Passerine 3: red-browed finch, silveryeye, superb fairy-wren (all this study); Psittacine 1 (psittacine adenovirus 1): Senegal parrot (Italy), rainbow lorikeet (this study); psittacine avia B (psittacine aviadenovirus B): red-bellied parrot, purple-crowned parrot (Australia); psittacine 2 (psittacine adenovirus 2): multiple species (USA and Europe) multiple captive species (Australia); psittacine D (proposed name psittacine siadenovirus D): Budgerigar (Australia), rainbow lorikeet and galah (both this study), variants of this virus sequences found in a budgerigar (Japan), and cockatiel (USA). Pigeon 1 (pigeon adenovirus 1), pigeon B (pigeon adenovirus 2), and pigeon 4 (pigeon adenovirus 4): Feral and domestic pigeons Europe and feral pigeons Australia (this study).

Table 3. Novel adenovirus DNA sequences detected in this study, their species of origin, and the abbreviation used in the phylogenetic tree.

GenBank accession number	Species or species of origin n = the number of times each sequence was detected	Reference name for this manuscript
MN238632	Common barn owl (n = 1)	barn owl 1
MN238633	Common barn owl (n = 1)	barn owl 2
MN238634	Southern boobook (n = 1)	boobook 1
MN238635	Southern boobook (n = 1)	murine 2 variant 2 (v2) ^a
MN238636	Sulphur-crested cockatoo (n = 1)	murine 2 variant 2 (v2) ^a
MN238637	Tawny frogmouth (n = 2)	murine 2 variant 2 (v2) ^a
MN238638	Galah (n = 2)	murine 2 variant 2 (v2) ^a
MN238639	Grey fantail (n = 2)	fantail
MN238640	Silver gull (n = 2)	silver gull 1
MN238641	Silver gull (n = 2)	silver gull 2
MN238642	Silver gull (n = 1)	pigeon 2 variant 2 (v2) ^b
MN238643	Little wattlebird (n = 1)	wattlebird 1
MN238644	Rainbow lorikeet (n = 1)	psittacine 1 variant 2 (v2) ^c
MN238645	Noisy miner (n = 3)	passerine 1 ^d
MN238646	New Holland honeyeater (n = 1)	passerine 1 ^d
MN238647	Indian mynah (n = 1)	passerine 1 ^d
MN238648	Noisy miner (n = 1)	noisy miner 1
MN238649	Noisy miner (n = 1)	noisy miner 2
MN238650	Eastern yellow robin (n = 3)	passerine 2 ^e
MN238651	Eastern shrike-tit (n = 1)	passerine 2 ^e
MN238652	Silvereye (n = 1)	silvereye 1
MN238653	Spotted pardalote (n = 1)	pardalote
MN238654	Superb fairy-wren (n = 4)	wren 1
MN238655	Sulphur-crested cockatoo (n = 1)	sulphur-crested cockatoo
MN238656	Long-billed corella (n = 1)	long-billed corella
MN238657	Eastern starling (n = 1)	starling
MN238658	Silver gull (n = 1)	silver gull 3
MN238659	Australian ibis (n = 1)	ibis
MN238660	Little wattlebird (n = 1)	wattlebird 2
MN238661	Rainbow lorikeet (n = 2)	lorikeet 1
MN238662	New Holland honeyeater (n = 2)	NH honeyeater 2
MN238663	Noisy miner (n = 1)	noisy miner 3
MN238664	Superb fairy-wren (n = 1)	passerine 3
MN238665	Silvereye (n = 4)	passerine 3
MN238666	Red-browed finch (n = 2)	passerine 3
MN238667	White-plumed honeyeater (n = 3)	wp honeyeater 1
MN238668	White-plumed honeyeater (n = 1)	wp honeyeater 2
MT079819	Satin bower bird (n = 1)	bowerbird
NA ^f	Eastern spinebill (n = 1)	spinebill
NA	Noisy miner (n = 1)	miner 3
NA	Australian magpie (n = 2)	magpie 1
	Also detected in Tawny frogmouth (n = 1)	
NA	Australian magpie (n = 1)	magpie 2
NA	Australian raven (n = 1)	raven

^aAlthough each has unique DNA sequence; all have the same amino acid sequence and this sequence is highly similar (98% homology) to murine adenovirus 2 making it a variant of murine adenovirus 2.

^bAlthough this has a unique amino acid sequence, its sequence is 95.24 per cent identical to as pigeon adenovirus 2, making it a variant of pigeon adenovirus 2.

^cAlthough this has a unique amino acid sequence; it has a 96.2 per cent homology with the amino acid sequence as psittacine adenovirus 1 making it a variant of this virus.

^dEach of these three viruses has identical DNA sequences.

^eBoth viruses have the same sequences.

^fNot applicable: The nucleotide sequences are less than 200 base pairs so they could not be submitted to GenBank. These sequences are provided in Supplementary Table S3.

frogmouth) and kidney (southern boobook) of two species that would be expected to eat mice.

4. Discussion

To test the hypothesis that wild birds harbour a previously unrecognised diversity of adenoviruses, we screened birds from the Greater Sydney area and adjacent inland regions of NSW, Australia. Additional samples from native and culled feral pigeons and three feral species were screened for adenoviruses to investigate the potential impact that the introduction of a feral species might have on adenovirus diversity in native wildlife. Thirty-five novel adenovirus sequences and six previously described adenovirus sequences were detected in this study. Given they were found in only thirty-seven species of birds and over 900 species of birds are found in Australia (Dolby and Clarke 2014), it is likely that more will be found in the species that have not been studied. The novel DNA sequences identified were from four adenovirus genera (*Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Mastadenovirus*) and their discovery has important implications for the understanding of adenovirus evolution.

The most significant evolutionary implications from the discovery of these virus sequences are for the genus *Atadenovirus*. Based on the emerging picture of the structure of the *Atadenovirus* genus it appears that the four lineages of the *Atadenovirus* genus recognised so far have arisen from ancient adenoviruses circulating in and between primitive birds and squamates that then co-evolved with a smaller host range. Lineages 1 and 4 co-evolved with birds, although the two lineages may not be able to infect the same species. Lineages 3A and 3B have co-evolved in squamates, with Lineage 3B possibly being exclusive to squamates. Lineage 3A also appears to be a squamate lineage. However, it contains three sequences from birds, one from the droppings of a satin bowerbird, one from the kidney of a European starling, and a sequence from the well-characterised pathogen of domestic ducks, duck atadenovirus 1. The sequence from the bowerbird clusters with the duck atadenovirus 1 sequence suggesting it is an avian adapted virus. The starling sequence was most closely related to one found in a blue-tongued lizard and could represent a spill-over from a reptilian species, particularly as starlings feed predominately on the ground. Lineage 2 appears to represent viruses that originated from a host switching event, either from a reptile (Lineage 3) or avian (Lineage 1) virus; the current analysis suggests that it was from the avian Lineage 1. Given that viruses in this lineage have been found in both deer species (Cervidae) and cattle, sheep, and goats (Bovidae) the host switching event would have occurred on or before the time that the Bovidae and Cervidae separated from other artiodactyls approximately 20 million years ago (Chen et al. 2019). In this analysis, the kowari adenovirus (Gál et al. 2017), a virus of a marsupial (*D. byrnei*), also appears to be in the *Atadenovirus* genus but it is unclear as to whether it is a member of Lineage 4 or represents a separate lineage.

Based on this analysis, the relationship of three adenovirus sequences, Lacertids 1 and 2 and miner 4, to other atadenovirus lineages could not be determined. They mapped between Lineages 1 and 2 and shared a low or relatively low G+C content with the viruses in Lineage 2 and the most closely related viruses in Lineage 1 suggesting a close evolutionary relationship to these other virus sequences. However, additional sequencing of these viruses will be required to confirm their phylogenetic relationship to other atadenovirus sequences.

The *Atadenovirus* genus was named for the low G+C percentage (A+T bias) observed in the bird, reptile, and ruminant viruses that were originally sequenced (Benkő and Harrach 1998). Various hypotheses have been suggested for their low G+C content, including their possible origin from a poikilotherm (Harrach 2000; Wellehan et al. 2004), or virus adaption following a host switch to avoid the hosts immune system (Benkő and Harrach 2003). Based on currently available data, low G+C content is not a consistent feature of the *Atadenovirus* genus and G+C percentage can vary from as low as 31 per cent to as high as 67 per cent. Nor is the G+C percentage predictive of whether the virus sequence will come from a bird, reptile, or mammal. In fact, virus sequences at the extremes in G+C content can be found in similar species, such as the passerines, and even within the same species such as *Anolis sagrei*. Instead, the G+C percentage is a feature of clusters of closely related viruses where divergence in G+C content (>10%) is uncommon to rare (Fig. 2). This feature is so striking that it can be used to distinguish between relatively closely related sequences that originate from similar species of birds. An example of this is two virus clusters originating from passerine birds in *Atadenovirus* Lineage 1, where one cluster of viruses has a G+C sequence percentage ranging from 46 to 60 per cent and the other has a G+C sequence percentage ranging from 37 to 39 per cent.

The *Siadenovirus* genus has been previously shown (Clark et al. 1973; Davison, Wright, and Harrach 2000; Rivera et al. 2009) to contain a virus from a Sulawesi tortoise and a virus from a frog (Lineage 1) that are basal to the remaining viruses, all of bird origin (Lineage 2), present in this genus. The data from this study also show this and greatly expands the diversity of adenovirus sequences found in this genus. The new data suggest that some degree of virus-host co-evolution has occurred in this genus as the bulk of the sequences originating from passerines, including one from a European species, map to a single cluster of viruses. Host-virus co-evolution may also be occurring in parrots, although it appears that the two parrot sub-lineages have evolved independently. The presence of virus sequences from passerines, a barn owl, other raptors, a pigeon, and two Antarctic species, the south polar skua and a penguin in a loose cluster of distantly related viruses is more consistent with virus switching between hosts by these viruses rather than co-evolution. Additional sequences from different species of birds will be required to verify this hypothesis.

The virus sequences now available from the *Aviadenovirus* genus show a similar pattern of lineages seen in the genus *Siadenovirus* representing virus-host co-evolution and a lineage composed of viruses originating from diverse and unrelated bird species as was demonstrated in the *Siadenovirus* genus. Lineages 1, 3, and 4 are composed of virus sequences that appear to have evolved predominately in pigeons (Lineage 1) and chickens and turkeys (Lineage 3) and exclusively in parrots (Lineage 4). Lineage 2, however, contains five virus sequences originating from unrelated species from Australia (multiple passerines species and a silver gull), Europe (pine marten and domestic goose) and South America (a psittacine bird). Given that Lineage 2 appears to be monophyletic it suggests that many other adenovirus sequences will be present in this lineage and will be found in many species of birds.

The genus *Mastadenovirus* has hitherto only been found to contain adenoviruses of mammal origin. Unexpectedly, a mastadenovirus sequence was detected in six bird samples. The sequence was a variant of murine adenovirus 2 and was found in droppings of two galahs and two tawny frogmouths. These viruses could have represented ingested viruses passing through

the digestive tract, as galahs feed on the ground and could ingest food items contaminated with mouse faeces or urine and tawny frogmouths feed on mice. The finding, however, of this same sequence in the kidney of a sulphur-crested cockatoo and a boobook owl suggests that this mastadenovirus was replicating in these birds and they were likely infected by contaminated food while ground feeding (sulphur-crested cockatoo) and ingested mice (boobook owl). Whether any of these species can support mastadenovirus dissemination requires additional investigation.

In a previous study by Prado-Irwin et al. (2018), two sequences (*Anolis sagrei* adenoviruses 7 and 8) identified in a brown anole (*Anolis sagrei*) were found to map basally to the viruses in the *Mastadenovirus* genus and were proposed to be a sister clade to the *Mastadenovirus* genus. In this study, they mapped to the *Atadenovirus* genus but with relatively little support. These have also a low G+C content which would be more consistent with a relationship with an adenovirus lineage. Additional sequencing of these viruses will be required to resolve their phylogeny.

Passerines species made up the largest cohort of birds surveyed in this study and the most common source of novel adenovirus sequences ($n=22$). The relationship between passerine adenoviruses and their hosts is complex and appears to represent a combination of host adaption in some of the viruses and the ability to infect a range of diverse species of passerine birds in others. Both the passerine siadenoviruses and atadenoviruses occur in lineages that are not monophyletic, suggesting that they originated with a host switching event that was followed by virus/species co-evolution in cohabitating, but diverse, woodland avian species, particularly honeyeaters (family Meliphagidae), finches (family Fringillidae), fairy-wrens (family Maluridae), the common silvereye (family Zosteropidae), and the Eastern yellow robin (family Eopsaltria). Three virus sequences were found in only one species (superb fairy-wren and noisy miner) or two species (Eastern yellow robin and crested shrike-tit) but at multiple and relatively distant geographic locations suggesting that these viruses prefer these species and are being maintained in them, possibly across their range. In contrast, three virus sequences were found in multiple passerine species and one was found in a passerine (Australian magpie) and a Caprimulgiformes (tawny frogmouth) demonstrating that some, perhaps many of the passerine adenoviruses maintain the ability to infect a wide range of passerine species and possibly some species that are not passerines. There may be limitations in the host range of the adenovirus found in passerines, however, as there was no evidence of recent or past cross species infections between passerine and psittacine birds, even though rainbow lorikeets share the same environment as many nectar feeding passerine birds included in this study.

Psittacine birds were the second most common source of adenovirus sequences in this study. These findings provide new insights into how adenoviruses are being maintained in Australian psittacine birds in the wild, the origin of adenoviruses in psittacine birds in general, and how the pet trade has resulted in the global dissemination of at least some of them. Prior to this study, only a single adenovirus sequence had been detected in a wild psittacine bird (little corella) in Australia (Sutherland et al. 2019). The new findings of this study show that sub-clinical adenovirus infections occur commonly and are likely maintained in wild rainbow lorikeets, galahs, and sulphur-crested cockatoos. The potential importance of lorikeets as reservoirs of psittacine adenoviruses is also supported by a previous study of a psittacine aviadenovirus B-associated

mortality event in red-bellied parrots (*Poicephalus rufiventris*) held in an Australian aviary (Das et al. 2017). In this study, while the captive red-bellied parrots, an African species, died following infection, purple-crowned lorikeets (*Glossopsitta porphyrocephala*), a native Australian species, were found to be subclinically infected.

The adenovirus sequences detected in the little corella and in other wild Australian psittacine birds in this study map to all but one (*Siadenovirus* genus psittacine Lineage 1) of the psittacine virus lineages previous found in captive-raised or wild-caught psittacine birds in other countries suggesting that these lineages originated in Australian species. Although not detected in wild Australian psittacine birds, the *Siadenovirus* genus Lineage 1 may also be of Australian psittacine bird origin as psittacine adenovirus 2, a member of this lineage, is widespread in captive parrots in Australia. It is enzootic in all captive breeding populations of the critically endangered orange-bellied parrot (*Neophema chrysogaster*) (Yang et al. 2019) and is apparently widespread in avicultural collections of the native Bourke (*Neopsephotus bourkii*) and scarlet-chested parrots (*Neophema splendida*) (Phalen et al. 2019). Further surveys of wild populations of lorikeets, *Neophema* spp., and *Neopsephotus* spp. will be required to determine if these viruses are also of Australian parrot origin.

If the adenoviruses of psittacine birds did first evolve in ancestral Australian psittacine species it is likely that they evolved in the regions of Gondwanaland that subsequently became Australia and New Zealand (Urantowska et al. 2018). How they then became global could either have been the result of co-evolution with parrots as they dispersed out of Australia or be the result of more recent dissemination through the trade in wild-caught and captive-raised parrots or both. Deciphering the evolutionary origins of these viruses is challenging as virus sequences identified outside of Australia were predominately identified in captive-raised or wild-caught birds that had been exposed to psittacine birds from all geographic origins and have been identified in many species of diverse geographic origin. In addition, some of these viruses were first detected in psittacine birds that died with adenovirus infection and associated disease (Wellehan et al. 2005; Mefenyana 2007; Milani et al. 2018) suggesting that species in which they were originally detected were not the co-evolved species of origin.

This study provides evidence that the parrot trade is responsible for the globalisation of at least some of the adenoviruses infecting psittacine birds. In this study, an amino acid sequence in a rainbow lorikeet was detected that was identical to the amino acid sequence of psittacine adenovirus 1 found in captive parrots in Europe (Lüschow et al. 2007; Milani et al. 2018) and another amino acid sequence was detected in a wild rainbow lorikeet that only varied by 4 per cent to the psittacine adenovirus 1 sequence. Likewise, as much or more intra lineage variation between sequences obtained from Australian species was found (e.g. 70.7% identity between lorikeet 1 as compared to the sequence from a sulphur-crested cockatoo) as was found in the same lineage between sequences obtained from wild Australian birds as compared to a captive parrot from another geographic location, for example psittacine adenovirus A detected in a captive South American parrot (mealy Amazon parrot), had 70.1 and 74 per cent identity to lorikeet 1 and sulphur-crested cockatoo sequences, respectively.

The second sets of viruses that also appear to have disseminated from Australia as the result of the parrot trade are the viruses in *Siadenovirus* genus Lineage 2. This lineage is present in both wild and captive Australian psittacine species. The first

sequence in this lineage was found in wild little corella (Sutherland et al. 2019). Subsequently, another sequence in this lineage (psittacine siadenovirus D) was found in a captive budgerigar held in an outdoor enclosure in VIC, Australia (MN687905). In this study, partial DNA polymerase gene sequences of psittacine siadenovirus D were also detected in a wild galah and a rainbow lorikeet. Globally, a nearly identical DNA polymerase sequence (QER78597.1) to that found in psittacine siadenovirus D was found in a captive South American psittacine bird (*F. coelestis*) in the USA and based on hexon gene sequences (DNA polymerase gene sequences for these two viruses are not available), close variants of this virus have been found in captive budgerigars in Japan (Kato et al. 2009) and in another Australian species, the cockatiel, in the USA (Cassman et al. 2019).

In addition to the murine adenovirus 2 variant found in a boobook owl, three dissimilar sequences were found in the tissues of two barn owls and a second boobook owl. They were widely distributed within the siadenoviruses and aviadenoviruses and their sequences were sufficiently different from the most closely related sequences that they may each represent novel adenovirus species. These findings are consistent with those seen in psittacine and passerine birds where adenoviruses from multiple genera have evolved independently in the same or similar species. Another species for which multiple (four) dissimilar virus sequences were detected was the silver gull. Only one of these was from tissue (silver gull 1) and therefore suggests it came from an infected bird. The other three were from faeces and so the infection status of these birds is uncertain. The silver gull 1 sequence was an aviadenovirus as is the original gull adenovirus detected in the gull species (*L. argentatus* and *L. fuscus*) in the Netherlands (Bodewes et al. 2013). However, because of their phylogenetic distance, they do not share a recent evolutionary history and are likely to have evolved in family Laridae independently. The silver gull 2 sequence mapped to a cluster of sequences from three unrelated species. These sequences originated from samples collected in Brazil and Europe and Australia suggesting an ancient origin of this cluster of viruses and the likelihood of additional unrecognised diversity in it. Silver gull sequence 3 appears to be a variant of pigeon adenovirus 2. The latter may be explained given that both species scavenge for food in urban environments. The remaining adenovirus sequence was an atadenovirus and was a sister sequence to one detected in a white ibis, however, they only had 64 per cent amino acid sequence homology. Together these findings suggest that gulls may prove to be useful sentinel species that can be used to detect adenovirus diversity in their environment.

The last two objectives of this study were to determine if feral species of birds had introduced adenoviruses to Australia and whether feral species of birds might be the source of adenoviruses for native species or that they might be susceptible to enzootic adenoviruses. Four invasive species were sampled, the feral pigeon, Eurasian collared dove, common starling, and Indian mynah. Three of five known adenovirus sequences (pigeon adenoviruses 1, 2, and 4) of pigeons (Marek et al. 2014; Ballmann and Harrach 2016) were detected in the feral pigeons demonstrating that these viruses have been introduced to Australia and should now be considered enzootic. This adds to the documentation of columbid herpesvirus (Phalen et al. 2017), pigeon circovirus (Phalen and Walker 2008), pigeon paramyxovirus (Cowan, Monks, and Raidal 2014), and pigeon rotavirus (McCowan et al. 2018) in domestic and feral pigeons, highlighting the ability for this species to disseminate viruses globally.

Fortunately, no evidence of adenovirus transfer from feral pigeons to native Australian pigeons and doves was detected, however, the sample size was small and some of the samples may not have been the optimum ones to screen. An adenovirus sequence was detected in renal tissue from both the invasive European starling and the Indian myna. The sequence from the Indian myna was identical to those found in other native Australian species proving that at least this invasive species is susceptible to infection with an enzootic adenovirus. The sequence from the European starling was only distantly related to sequences found in native Australian birds and it could not be determined if it was a virus of the European starling or of Australian origin.

In this study, we used PCR and degenerate primer sets that were developed by Wellehan et al. (2004) to detect novel and known adenovirus DNA polymerase sequences. This method has proved to be a very powerful tool and has resulted in the discovery of many adenoviruses (Wellehan et al. 2005, 2009; Diffo et al. 2019; Phalen et al. 2019). The limitation of this methodology, however, is that only a relatively short sequence of amino acids is produced. As the numbers of short adenovirus sequences grow, the ability to produce an accurate phylogeny of these viruses' decreases and marked changes in the phylograms occur with the addition or removal of one or a few sequences. Using our sequences, the four of the five accepted adenovirus genera (species in the *Ichtadenovirus* genus were not included in the phylogeny) and the one proposed adenovirus genus were clearly defined. In addition, structure within each adenovirus genus was similar to what has been shown previously and clearly defined patterns of virus evolution were identified. However, in many cases, nodal support was poor, particularly in the deeper nodes. Therefore, it will require that much longer sequences of the DNA polymerase or other genes be generated before a more precise relationship between the novel adenovirus sequences reported here can be determined.

In conclusion, this study shows that a previously unrecognised diversity of adenoviruses has evolved in wild birds in Australia and, based on these findings, investigation into adenovirus diversity in birds in other complex ecosystems in Australia and on other continents is warranted. The results of this study also predict that viruses from multiple adenovirus genera can be expected to have independently evolved in the same or closely related bird species and that the *Atadenovirus* genus is likely to contain an equal diversity of avian adenoviruses as it does adenoviruses of reptile origin. The finding of similar or identical adenovirus sequences in multiple species of often distantly related birds and even in mammals suggests that maintaining a wide host range may be a key survival strategy for many adenoviruses. Viruses similar or identical to known psittacine adenoviruses have now been shown to be circulating in captive or wild Australian species in Australia suggesting that at least some of them may have originated in Australian psittacine birds. Lastly, this study showed that the feral pigeon is an important vector for the globalisation of pigeon adenoviruses and that at least one of these viruses may be able to infect native Australian species and that at least one virus of an Australian passerine can infect an invasive passerine species.

Data availability

All data are available through GenBank via the accession numbers listed in this article. Smaller sequences are found in [Supplementary Table S3](#).

Supplementary data

[Supplementary data](#) are available at *Virus Evolution* online.

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