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Research Article

Contrasting dynamics of two incursions of low-pathogenicity avian influenza virus into Australia

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Abstract

The current panzootic of high pathogenicity avian influenza virus H5N1 demonstrates how viral incursions can have major ramifications for wildlife and domestic animals. Herein, we describe the recent incursion into Australia of two low pathogenicity avian influenza virus subtypes, H4 and H10, that exhibited contrasting evolutionary dynamics. Viruses detected from national surveillance and disease investigations between 2020 and 2022 revealed 27 genomes, 24 of which have at least one segment more closely related to Eurasian or North American avian influenza lineages than those already circulating in Australia. Phylogenetic analysis revealed that H4 viruses circulating in shorebirds represent a recent incursion from Asia that is distinct from those circulating concurrently in Australian waterfowl. Analysis of the internal segments further demonstrates exclusive, persistent circulation in shorebirds. This contrasts with H10, where a novel lineage has emerged in wild waterfowl, poultry, and captive birds across Australia and has likely replaced previously circulating H10 lineages through competitive exclusion. Elucidating different dynamics for avian influenza incursions supports effective disease risk identification and communication that better informs disease preparedness and response.

Keywords: avian influenza; bird migration; H4; H10; influenza A virus; low-pathogenicity avian influenza; LPAI; viral incursion; evolution

Introduction

High pathogenicity avian influenza virus (HPAIV) H5N1 is causing a major disease burden on the poultry industry and in wild birds and marine mammals (Wille et al. 2022). The current panzootic encompasses Europe, Asia, Africa, North America, South America, and Antarctica, with Oceania being the only continent still free from HPAIV H5N1 (Wille et al. 2024). Despite the negative consequences for wild bird populations, migratory avian hosts facilitate long-distance virus dispersal of HPAIV H5N1, a role that has increased over the years with the dramatic expansion of HPAIV H5Nx clade 2.3.4.4 in 2014 and 2.3.4.4b in 2021 (Global Consortium for H5N8 and Related Influenza Viruses 2016, Wille et al. 2022, Adlhoch et al. 2023, Klaassen and Wille 2023). Improved understanding of avian-borne viral movement and incursions

has therefore become increasingly important to improve disease preparedness and in mounting appropriate responses.

Beyond HPAIV H5N1, wild birds are the natural reservoirs for low pathogenicity avian influenza viruses (LPAIVs). Wild birds, particularly members of the orders *Anserifomes* (waterfowl, including ducks, geese, and swans) and *Charadriiformes* (shorebirds and gulls), are principal reservoirs for LPAIV, with 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes identified to date (Olsen et al. 2006). These host orders play differing roles in LPAIV ecology, with contrasting patterns of prevalence (Wille et al. 2023), seasonal ecology (Maxted et al. 2016), and roles in long-distance dispersal between continents (Rimondi et al. 2018, Wille et al. 2022), which are clearly apparent in the Australian context (e.g. Hansbro et al. 2010, Hoque et al. 2015, Hoye et al. 2021, Wille et al. 2022). In addition, there is relatively little transfer of viral lineages between these host groups although it does occur (Hicks et al. 2022). In contrast to waterfowl from other continents, Australian wild waterfowl are endemic to the Australo-Papuan region and do not connect Australia with Eurasia and North America through migration (McCallum et al. 2008). Rather, long-distance migratory shorebirds annually migrate between Australia and breeding areas in Eastern Siberia and Alaska, with key stop-over sites across Asia (Tracey et al. 2004, McCallum et al. 2008). As such, viral introductions to Australia are likely facilitated by long-distance migratory shorebirds rather than waterfowl, with intercontinental reassortment being a feature of some viral genomes from shorebirds sampled in Australia (Hurt et al. 2006, Hoye et al. 2021, Wille et al. 2022). Following these infrequent introductions, the persistent circulation of LPAIV lineages has been observed within the continent, presumably by nomadic waterfowl. As such, Australia can generally be regarded as a sink for virus diversity (Wille et al. 2022).

In this study, we analysed recently detected LPAIV genomes associated with two recent viral introductions to better understand incursions to and dispersal within Australia. Through time-scaled phylogenetic analysis, we estimated the dates and probable origins of these viral introductions and illustrated the contrasting patterns of viral maintenance in wild birds following these events.

Materials and Methods

Ethics statement

Capture, banding, and sampling of shorebirds and ducks were conducted under Australian Bird and Bat Banding Scheme authorities 2915 and 8001. Animal ethics permits were provided by Deakin University Animal Ethics permit number B39-2019 and Philip Island Nature Parks Animal Ethics permit number SPFL20082. Faecal environmental surveillance samples collected by the Department of Primary Industries, Parks, Water and Environment, Tasmania; Primary Industries and Regions South Australia; Department of Primary Industries and Regional Development Western Australia (WA); and New South Wales (NSW) Department of Primary Industries did not require permits. Samples collected for disease investigations, similarly, did not require permits.

Sample collection and screening

Sample collection through the National Avian Influenza in Wild Birds (NAIWB) programme was undertaken as per Grillo et al. (2015). Between 2020 and 2022 samples from wild caught birds, hunted birds, and fresh faeces from the environment were collected. In addition, we included passive surveillance samples from affected domestic or captive birds as part of diagnostic investigations (Supplementary Table S1). Viral screening and sequencing were undertaken as per Wille et al. (2022). Briefly, RNA was extracted from swab samples in virus transport media and screened by real-time RT-PCR using primers and probes targeted against the influenza A virus matrix gene (Spackman et al. 2002, Heine et al. 2015) using established and accredited approaches. Influenza A virus genome segments were amplified using the SuperScript III one-step RT-PCR system with high-fidelity Platinum Taq DNA polymerase (Thermo Fisher Scientific) and universal influenza A virus gene primers as previously described (Zhou et al. 2009). Either original samples (with Ct <30) or viral isolates or both were sequenced on an Illumina MiSeq, with up to 24 samples pooled per sequencing run by use of dual-index library preparation, the Nextera XT DNA Library Preparation kit, and 300-cycle MiSeq Reagent v2 kit (Illumina). Sequence reads were trimmed for quality and mapped to respective reference sequence for each influenza A virus gene segment using Geneious Prime software (www.geneious.com) (Biomatters, Auckland, NZ) (Wille et al. 2022). Only segments with >10× coverage were included; genome coverage for viruses generated in this study is available in Supplementary Table S2.

Sequences generated in this study have been deposited in GenBank (accession numbers are available in Supplementary Table S1).

Phylogenetic analysis

All full-length H4, H10, N1, N2, N6, N7, N8 sequences were downloaded from the Bacterial and Viral Bioinformatics Resource Centre Influenza portal (www.bv-brc.org) for the construction of global maximum likelihood trees. As H10 genome sequences from Oceania reported in (Vijaykrishna et al. 2013) are not in GenBank, we queried them from the Global Initiative on Sharing All Influenza Data database (https://gisaid.org/). The resulting HA and NA phylogenies vary in the total number of sequences owing to the differing number of sequences available for these subtypes in GenBank. For the internal segments, we used phylogenetic tree backbones from Wille et al. (2022) and supplemented these with more recent sequences from BLAST results of sequences generated in this study. Internal segment trees comprised ∼2500 sequences per segment. The temporal, geographic, and HA subtype distributions of these phylogenies are reported in Supplementary Table S3. Sequences were aligned using MUSCLE v3.8.425 (Edgar 2022) integrated within Geneious Prime. Maximum likelihood trees incorporating the best-fit model of nucleotide substitution were estimated using Smart Model Selection (Lefort et al. 2017), and aBayes (Anisimova et al. 2011) node support were estimated using PhyML v3.0 (Guindon et al. 2010).

We selected ∼100 sequences for construction of time-structured phylogenetic trees. For all trees, except H10, we selected sequences from the Eurasian lineage. For the H10 tree, we selected sequences from the North American lineage. We ensured that trees comprised all sequences from Oceania, which fell into the same clade. The 10 to 20 most closely related sequences identified by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were incorporated, followed by removal of identical genomes with the same collection date and location. Generally, sequences were from samples collected between 2010 an 2022, although we did include a number of older sequences to ensure clock-like structure. The temporal, geographic, and HA subtype distributions of the HA and NA phylogenies are reported in Supplementary Table S3, and all XML files are available on GitHub (https://github.com/michellewille2/H4_H10).

Prior to the estimation of time-scaled phylogenies, we evaluated the extent of molecular clock-like structure in the data by performing linear regressions of root-to-tip distances against the year of sampling using maximum likelihood trees using TempEst (Rambaut et al. 2016). As evidence for a molecular clock was obtained, time-scaled phylogenetic trees were estimated using BEAST v1.10.4 (Drummond et al. 2012), under the relaxed uncorrelated lognormal relaxed clock (Li and Drummond 2012) and SRD06 codon structured nucleotide substitution model (Shapiro et al. 2006), with the exception of M and NS segments for which we used the HKY+G model due to overlapping reading frames. The Bayesian skyline coalescent tree prior was used as this likely reflects the complex epidemiology dynamics of avian influenza viruses through time (Drummond et al. 2005). Hundred million generations were performed, and convergence was assessed using Tracer v1.8 (http://tree.bio.ed.ac.uk/software/tracer/). Maximum credibility lineage trees were generated using TreeAnnotator following the removal of 10% burnin, and trees were visualized using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). The most recent common ancestors (MRCAs) of clades of interest (i.e. the MRCA of Australian sequences and the MRCA of the node representing the divergence between Australian sequences and those from the closest relative in GenBank) were extracted from the maximum clade credibility trees using FigTree.

Mammalian adaptation

To understand whether there was any evidence of mammalian adaptation in the H10 viruses, we compared our genomes to a human H10 virus (Chen et al. 2014), as well as seal H10 viruses (Herfst et al. 2020). Viruses were also interrogated using FluServer (http://flusurver.bii.a-star.edu.sg).

Results

Virus detection

Both H4 and H10 viruses were recovered through the NAIWB-targeted surveillance programme and other *ad hoc* sampling between December 2020 and September 2022. Briefly, H4 viruses were recovered from hunter-shot ducks in Tasmania ($n=1$) and South Australia ($n=2$), faecal environmental samples in South Australia ($n=1$) a hunter-shot duck (*Anas gracilis*, $n=1$) in NSW, and live-captured shorebirds (*Calidris* spp, $n=6$) in Victoria. H10 viruses were recovered from faecal environmental samples collected in South Australia ($n=2$) and Western Australia ($n=1$) and from live-captured ducks (*Anas* sp) in Victoria ($n=10$) (Supplementary Table S1).

Additional H10 viruses were identified through passive surveillance events in domestic or captive birds by respective state government animal health laboratories in conjunction with the national reference laboratory at the Commonwealth Scientific and Industrial Research Organisation Australian Centre for Disease Preparedness. These occurred between June and August 2021 and included chickens (*Gallus domesticus*) from New South wales, farmed emu (*Dromaius novaehollandiae*) in Victoria, and captive Tawny Frogmouth (*Podargus strigoides*) in Western Australia (Supplementary Table S1). The detection of an H10N7 virus from a chicken in New South Wales involved a backyard chicken farm ~20 km from Canberra, Australian Capital Territory. The flock of 234 birds experienced a morbidity of 12% and a mortality of 6%. Movement restrictions were placed on birds from this farm with subsequent testing no longer able to detect avian influenza virus. In Victoria, an H10N3 virus was detected in farmed emu chicks aged 3–6 weeks with respiratory signs and low-level mortalities. Finally, in Western Australia, eight aviary-kept Tawny Frogmouths died suddenly at a Perth wildlife park, one of which was submitted for diagnostic laboratory investigation, resulting in the detection of an H10N7 virus.

Incursion and export events with a limited host range of H4 viruses from shorebirds

H4 viruses recovered from waterfowl and shorebirds had different evolutionary histories, with viruses recovered from waterfowl falling into an H4 lineage, which has persisted in Australia for four decades, compared to viruses from shorebirds, which fell into a different lineage, comprising a recent incursion event (Fig. 1, Supplementary Fig. S1).

Viruses from waterfowl included H4N6 ($n=4$) and H4N1 ($n=1$), with six of the eight gene segments of these viruses falling into established clades circulating in Australia (Table 1; Fig. 1 and Supplementary Figs S2 and 3). The HA sequences of all five viruses fell into the same clade, despite being recovered from different Australian states (Fig. 1). The N6 sequences similarly all belonged to a single clade, and for both N1 and N6, the sequences were most similar to other viruses that have circulated in Australia since 2019 (Supplementary Fig. S2). The internal gene constellations were largely similar, with PB1, PA, M, and NP segments of all five H4 viruses belonging to phylogenetic clades previously detected in Australia. However, the two H4N6 viruses detected in South Australia had PB2 and NS segments most closely related to sequences from Asia, and these viruses are likely the result of a reassortment event with undetected, recently introduced viruses of Eurasian lineage (Table 1; Fig. 1 and Supplementary Figs S2 and 3).

The five H4N8 viruses isolated from Red-necked Stints (*Calidris ruficollis*) ($n=5$) in 2020 and one from a Sharp-tailed Sandpiper (*Calidris acuminata*) ($n=1$) in 2021 comprised segments from both recent introduction events (i.e. were novel incursions) and established lineages circulating in Australia. As all five viruses from Red-necked Stints were recovered from birds captured at the same sampling event, and thus it is unsurprising that the viruses had the same genome constellation (Table 1; Fig. 1 and Supplementary Fig. S4). Notably, the virus recovered from the Sharp-tailed Sandpiper, captured approximately a year later, had all eight segments fall into the same clade as those viruses from the Red-necked Stints, and the sequences for all segments were consistently ≥99.9% similar to each other. Both the HA and NA segments comprised a novel incursion into Australia from Eurasia, with the HA and NA diverging from the most closely related LPAIV sequences in databases with mean dates of August 2018 [95% highest posterior density (HPD) October 2017 to April 2019] and September 2018 (95% HPD September 2017 to June 2019), respectively (Fig. 1, Supplementary Table S4). There was a time lag of ~1 year between the mean date of divergence from these reference sequences and the mean MRCA of the clade comprising the six shorebird sequences recovered in Australia. In contrast, the mean MRCA for the six virus glycoprotein gene sequences, particularly the N8 (April 2020, 95% HPD November 2018 to October 2020), was very close to the sample collection date (December 2020), suggesting likely proliferation through the population following a single introduction into Australia (Fig. 1 and Supplementary Table S4). For both the HA and NA segments, the most closely related LPAIV publicly available sequences were viruses sampled from South Korean wild birds although a general lack of publicly available sequence data prevented any firm conclusions being drawn with regard to the geographical source of the viral incursion from Asia.

Table 1. Viruses sequenced in this study and lineage delineations for all genome segments.

Subtype	Designation	Sample date	PB2[a]	PB1[a]	PA[a]	HA[a]	NP[a]	NA[a]	M[a]	NS[a]
H4N1	A/Pacific Black Duck/Tasmania/22-1184-5/2022(H4N1)	5 March 2022	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (H4)	NAm—AUS	EUR-AUS (N1)	EUR-AUS (A)	A-EUR-AUS (A)
H4N6	A/Grey Teal/ NSW/14340X/2020(H4N6)	2 December 2020	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (H4)	NAm—AUS	EUR-AUS (N6)	EUR-AUS (A)	A-EUR-AUS (B)
H4N6	A/Grey Teal/South Australia/22-64233564-20/2022(H4N6)	21 May 2022	EUR—novel	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (H4)	NAm—AUS	EUR-AUS (N6)	EUR-AUS (A)	A-EUR—novel
H4N6	A/Grey Teal/South Australia/22-64233564-49/2022(H4N6)	21 May 2022	EUR—novel	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (H4)	NAm—AUS	EUR-AUS (N6)	EUR-AUS (A)	A-EUR—novel
H4N6	A/wild waterbird/South Australia/22-68204541-55/2022(H4N6)	16 August 2022	na[b]	na[b]	na[b]	EUR-AUS (H4)	NAm—AUS	EUR-AUS (N6)	EUR-AUS (A)	B-EUR-AUS
H4N8	A/Red-necked Stint/Victoria/15098/2020(H4N8)	9 December 2020	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H4N8	A/Red-necked Stint/Victoria/15109/2020(H4N8)	9 December 2020	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H4N8	A/Red-necked Stint/Victoria/15118(H4N8)	9 December 2020	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H4N8	A/Red-necked Stint/Victoria/15119/2020(H4N8)	9 December 2020	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H4N8	A/Red-necked Stint/Victoria/15159/2020(H4N8)	9 December 2020	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H4N8	A/Sharp-tailed Sandpiper/VIC/15469/2021(H4N8)	29 December 2021	EUR-AUS (B)	EUR-AUS (B)	EUR-AUS (B)	EUR—novel (H4)	EUR-AUS	EUR—novel (N8)	EUR-AUS (B)	A-EUR-AUS (C)
H10N2	A/Grey Teal/South Australia/22-64233564-37/2022(H10N2)	21 May 2022	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR—novel (N2)	EUR-AUS (A)	B-EUR-AUS
H10N3	A/emu/Victoria/21-03712/2021(H10N3)	19 August 2021	EUR—novel	EUR-AUS (A)	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N3)	EUR-AUS (A)	A-EUR-novel
H10N3	A/wild waterfowl/WA/AS-22-0673-0006/2022(H10N3)	16 February 2022	na[b]	na[b]	na[b]	NAm—novel (H10)	NAm—AUS	EUR-AUS (N3)	EUR-AUS (C)	A-EUR—novel
H10N7	A/chicken/ NSW/M21-10080-0006/2021(H10N7)	2 July 2021	EUR—novel	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	A-EUR—novel
H10N7	A/tawny frogmouth/WA/21-1823/2021(H10N7)	28 June 2021	EUR—novel	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR—novel (N7)	EUR-AUS (A)	A-EUR—novel
H10N7	A/Grey Teal/South Australia/22-64233564-17/2022(H10N7)	21 May 2022	EUR-AUS (A)	EUR-AUS (A)	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR-AUS (A)	A-EUR-AUS (A)
H10N7	A/Grey teal/Victoria/15848/2022(H10N7)	21 June 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/15974/2022(H10N7)	21 June 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS

Table 1. (Continued)

Subtype	Designation	Sample date	PB2[a]	PB1[a]	PA[a]	HA[a]	NP[a]	NA[a]	M[a]	NS[a]
H10N7	A/Chestnut teal/Victoria/16047/2022(H10N7)	28 June 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Chestnut teal/Victoria/16099/2022(H10N7)	28 June 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Chestnut teal/Victoria/16148/2022(H10N7)	5 July 2022	EUR—novel	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/16156/2022(H10N7)	5 July 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/16164/2022(H10N7)	5 July 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/16182/2022(H10N7)	5 July 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/16190/2022(H10N7)	5 July 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS
H10N7	A/Grey teal/Victoria/16195/2022(H10N7)	5 July 2022	EUR-AUS (A)	EUR—novel	EUR-AUS (A)	NAm—novel (H10)	NAm—AUS	EUR-AUS (N7)	EUR—novel	B-EUR-AUS

[a]Lineage information is as follows:
EUR-AUS: Established Australian clade in the Eurasian lineage.
EUR—novel: Novel incursion from the Eurasian lineage.
NAm—AUS: Established Australian clade in the North American lineage.
NAm—novel: Novel incursion from the North American lineage.
In cases were viruses fell into different North Australian lineages, we had appended an arbitrary letter in brackets (e.g. A, B, C) indicates that while viruses fell into Australian lineages, they fell into different lineages.
In the case of NS, whether the virus fell into the A or B allele is indicated prior to the lineage name, e.g. B-EUR-Aus.
Phylogenetic trees demonstrating sequence placement are presented in Figs 1 and 2, Supplementary Figs S1–3.
[b]PB2, PB1, and PA sequences were not recovered from A/wild waterfowl/WA/AS-22-0673-0006/2022(H10N3) nor A/wild waterbird/South Australia/22-68204541-55/2022(H4N6).

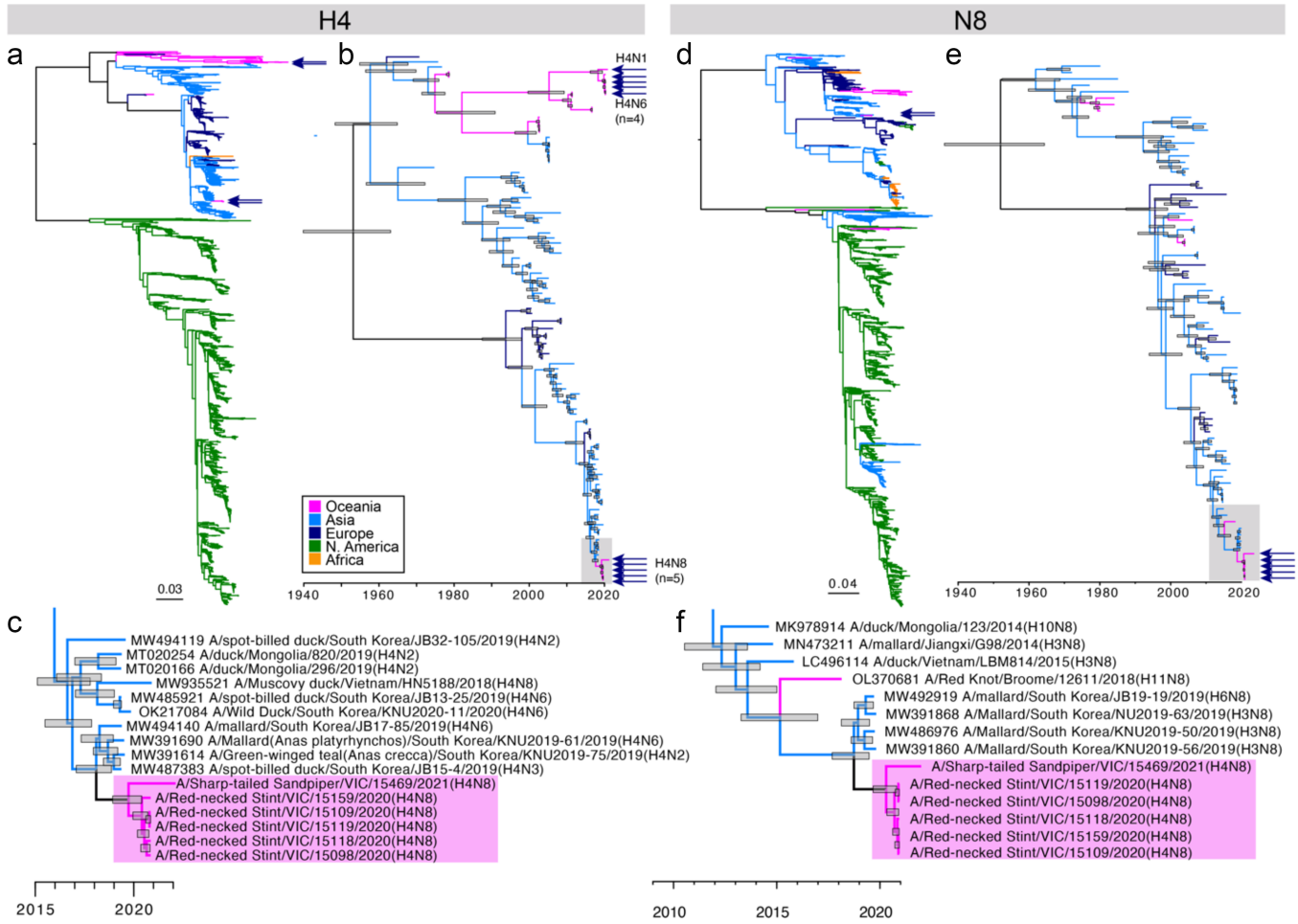

Figure 1. Phylogenetic trees of (a–c) H4 sequences regardless of NA and (d–f) N8 sequences. Phylogenetic trees for the waterfowl clade of H4 and H4-associated NA trees are provided in Supplementary Figs S1 and S2, respectively. (a and d) Maximum likelihood trees comprising all sequences collated for this study. Trees were rooted geographically (i.e. between the 'Eurasian' and 'American' lineages), and the scale bar corresponds to the number of substitutions per site. (b and e) Time-structured phylogenetic trees. The trees comprise select sequences from relevant lineages (here Eurasian lineages containing Australian sequences). (c and f) Expansion of clade containing Australian sequences of interest, highlighted in a grey box in (b and e). In the case of the HA segment, only the clade containing H4N8 viruses has been highlighted in (c). Sequences of interest are indicated by arrows in (a), (b), (d), and (e) and are highlighted in a box in (c) and (f). Node bars correspond to the 95% HPD of node height. Branches are coloured by continent or are black where geographic origin is ambiguous (i.e. deep branches).

The evolutionary histories of the internal segments of these six H4N8 viruses are more complex, but generally fall into clades dominated by viruses detected in Red-necked Stints (Supplementary Fig. S4). These clades are unusual in that they comprise exportation events from Australia back to Asia, which has not been seen with other clades (Wille et al. 2022). In four segments (PB2, PA, NP, and M), the clades were first detected in Australian waterfowl, prior to entering shorebird populations. In the remaining segments, clades were detected in Asian waterfowl, prior to detection in shorebirds in Australia (Supplementary Fig. S4). The PB2, PA, NP, and NS clades have been circulating in shorebirds in Australia since ~2012, whereas the PB1 and M clades have only been detected in Australian shorebirds more recently (since ~2016). Exportation events from Australia to Eurasia include detections in Red-necked Stints in Japan [e.g. A/*C. ruficollis*/Hokkaido/12EY0172/2012(H4N7)] and, more recently, A/wild bird/Fujian/24/2017(H2N6) (Supplementary Fig. S4). Based on BLAST analysis of the NA segment of A/*C. ruficollis*/Hokkaido/12EY0172/2012(H4N7) (GenBank Accession LC467226), this N7 is most similar to three viruses detected in Red-necked Stints in Australia (GenBank accessions: OL370713, OL370689, and OL370697), suggesting that this NA segment was also exported from Australia. Overall, based on the genome constellations of the H4N8 viruses described here (Fig. 1 and Supplementary Figs S1 and S4; Table 1), it is likely that reassortment occurred within Australian shorebirds following the incursion of novel H4N8 viruses.

Between 2004 and 2017, the now waterfowl-associated H4 Australian lineage was detected in *Calidris* shorebirds in Australia, in addition to both *Calidris* shorebirds and gulls in Asia, including A/*C. ruficollis*/Hokkaido/12EY0172/2012(H4N7), demonstrating circulation in shorebirds with associated export events (Supplementary Fig. S1). However, unlike the internal segments, a cross-host order transmission event occurred followed by widespread circulation in waterfowl with no further detection of this H4 clade in shorebirds (however, this may be affected by undersampling of Australian shorebirds).

Recent incursion and widespread transmission of H10 viruses in Australian birds

The 16 H10 viruses sequenced in this study, comprising H10N2 ($n = 1$), H10N3 ($n = 2$), and H10N7 ($n = 13$), were collected from

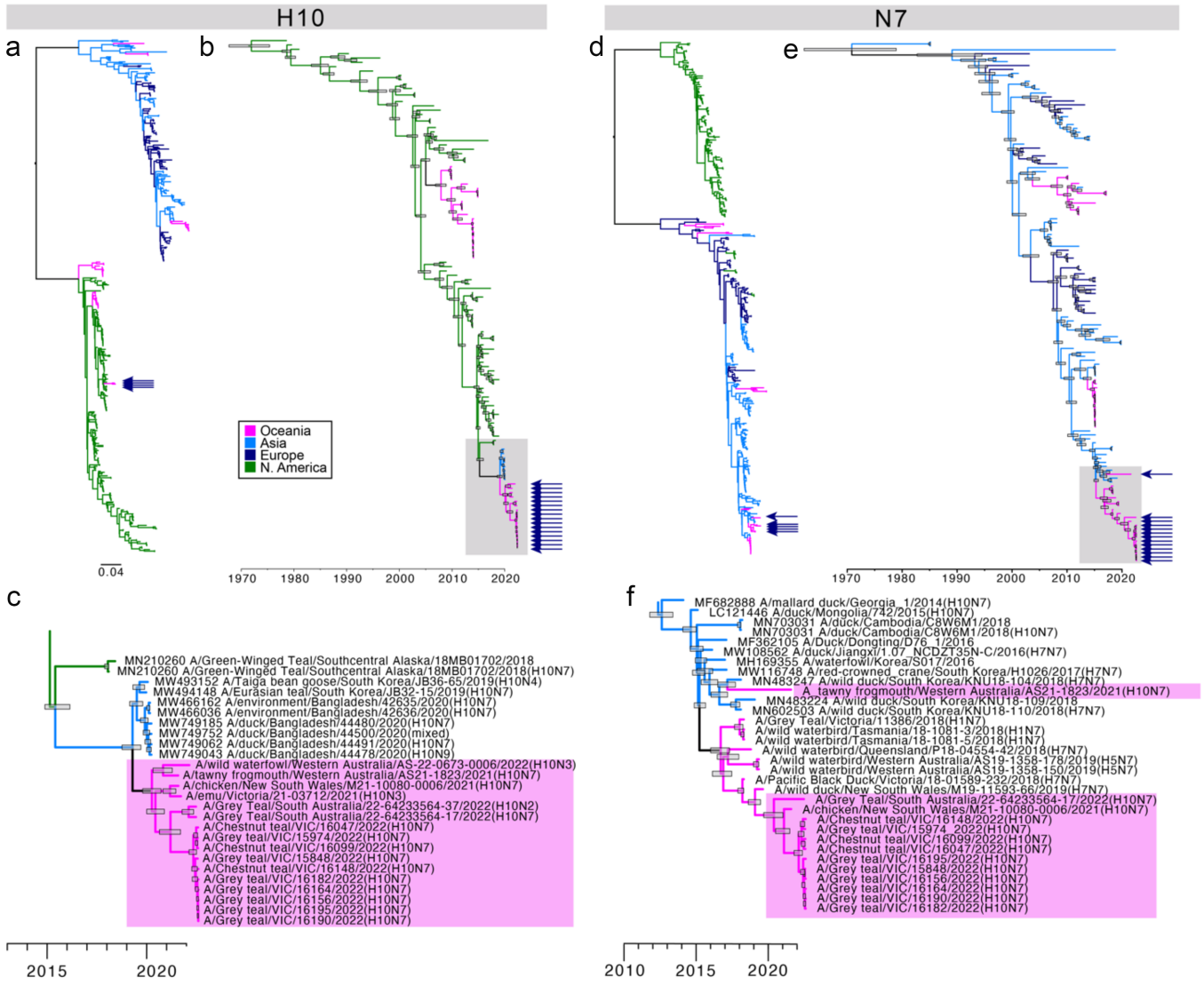

Figure 2. Phylogenetic trees of all (a–c) H10 sequences regardless of NA and (d–f) N7 sequences. A phylogenetic tree for N2 and N3 is provided in Supplementary Fig. S2. (a and d) Maximum likelihood trees comprising all sequences collated for this study. Trees were rooted geographically (i.e. between the 'Eurasian' and 'American' lineages), and the scale bar corresponds to the number of substitutions per site. (b and e) Time-structured phylogenetic trees. The trees comprise select sequences from relevant lineages (here Eurasian lineage with all Australian sequences). (c and f), expansion of clade containing Australian sequences of interest, highlighted in a grey box in (b and e). Sequences of interest are indicated by arrows in (a, b, d, and e) and are highlighted in a box in (c and f). Node bars correspond to the 95% HPDof node height. Branches are coloured by continent or are black where geographic origin is ambiguous (i.e. deep branches).

across multiple states in Australia, including Western Australia, South Australia, New South Wales, and Victoria from 2020 to 2022. The viruses were recovered from both national active surveillance of wild birds and passive or general surveillance (Supplementary Table S1). Seven different genome constellations were recovered, with novel incursion events recorded for six segments. Only the PA and NP segments were exclusively from enduring Australian lineages (Fig. 2 and Supplementary Figs S2–3; Table 1).

All 16 HA sequences fell into the same H10 lineage (mean MRCA March 2020, 95% HPD 19 September 2019 to 20 August 2020). While these viruses fell into the broader North American clade of viruses, a viral incursion event in Asia occurred prior to their introduction in Australia, with detections in Bangladesh and South Korea in 2020 (Fig. 2). Three different NA subtypes were detected: N2, N3, and N7 (Fig. 2 and Supplementary Fig. S2; Table 1). Both N3 and the majority of N7 sequences (12/13) fell into established Australian N3 and N7 lineages (Fig. 2 and Supplementary Fig. S2; Table 1). A single N7 sequence [A/Tawny Frogmouth/WA/21-1823/2021(H10N7)] and the N2 sequence [A/Grey Teal/South Australia/22-64233564-37/2022(H10N2)] represented novel incursions (Table 1; Supplementary Fig. S2 and Fig. 2), with the date of divergence of reference sequences being January 2017 (95% HPD May 2016 to 18 August 2017) and December 2017 (95% HPD January 2017 to Oct 2018), respectively. In both cases there was a time lag of ~4 years between the date of divergence from the most closely related sequences in GenBank and detection in Australia, suggesting potential cryptic circulation for several years prior to detection. Alternatively, this time lag may reflect the lack of sampling and/or available sequence data from Asia (Supplementary Table S4).

Focusing on the segments likely comprising novel incursions into Australia (PB2, PB1, HA, NA, M, NS), the mean MRCA estimates range from March to June 2020 (Supplementary Fig. S3 and Table S4) and the dates of divergence from the

closest sequences in reference databases range from 2019 to 2020. Among the H10 viruses, a number of different genome constellations were present, with a variable mix of novel and established Australian lineages, despite all sharing the same HA clade. This indicates different reassortment histories within Australia. For example, despite being detected on opposite sides of Australia and in different hosts and captivity settings, A/chicken/ NSW/M21-10080-0006/2021(H10N7) and A/tawny frogmouth/WA/21-1823/2021(H10N7) share a genome constellation across 6/8 segments, with differences in the NA and M segments (Fig. 2 and Supplementary Fig. S3; Table 1). Furthermore, while 9 of the 10 H10N7 viruses collected from *Anas* ducks at the same site in Victoria across 3 weeks in June and July 2022 share the same genome constellation, one virus, A/Chestnut teal/Victoria/16148/2022(H10N7), possessed a different novel Eurasian lineage PB2 segment demonstrating the potential for different reassortants to circulate in the same locations within the same host populations (Fig. 2 and Supplementary Fig. S3; Table 1).

Given that H10 viruses have spilled over into mammals repeatedly, we interrogated the genomes for potential markers of mammalian adaptation. The H10 HA sequence of all viruses contained the amino acid motif GlnSerGly at residues 226–228 (H3 numbering), indicating avian-like receptor binding preference (Chen et al. 2014). There was no evidence for other reported mammalian HA or PB2 mutations in our sequences, which are present in human or seal H10 viruses. Fluserver reports contained no amino acid changes consistent with mammalian adaptation.

Discussion

We provide compelling evidence of shorebirds forming an important vector for intercontinental spread of avian influenza viruses between Asia and Australia and demonstrate key differences in the outcomes of viral introduction events resulting in contrasting opportunities for introduction, establishment, distribution, and evolution of avian influenza viruses on the Australian continent.

The data generated here provide evidence for the recent incursion of two LPAIV subtypes into Australia, with contrasting patterns of establishment and spread following their introduction. Australia is a sink for viral diversity, such that avian influenza lineages circulate on the continent, in isolation, for many decades. However, viral incursions do occur as shown by the recent (2005–15) MRCA of most HA lineages found in Australia, and these lineages were unrelated to historic sequences (from the 1980s) (Wille et al. 2022). Incursion events of H10 into Australia have been previously described, with epidemiology consistent with our findings. Specifically, an H10 virus from the North American lineage entered Australia in 2007/08, was detected in wild birds in Victoria and Tasmania, and was subsequently detected in chickens in Queensland and New South Wales in 2010 and 2012, respectively (Vijaykrishna et al. 2013, Hoye et al. 2021). Here, we describe a new introduction of North American lineage H10, with initial detections occurring within the space of 2 months (June to August 2021), in backyard domestic chicken, farmed emu, and captive Tawny Frogmouth in a wildlife zoo from both western and eastern states of Australia. In addition to an expanded avian host range, H10 has caused outbreaks in numerous mammalian species including seals (Bodewes et al. 2015, 2016, Krog et al. 2015), mink (Berg et al. 1990), and humans (Arzey et al. 2012). Indeed, the H10 lineage described in 2012 in New South Wales (Vijaykrishna et al. 2013, Hoye et al. 2021) was also detected in poultry abattoir workers (Arzey et al. 2012). The human health risk posed by the current introduced avian H10 lineage in Australia is unknown but is expected to be low.

While it is likely that this novel H10 lineage is now established in Australia due to widespread detections, a viral introduction event does not necessarily result in the establishment of the lineage or uptake of all parts of its genome. Indeed, in the case of Australia where viruses are likely introduced by shorebirds, there are three possible outcomes: (i) viral introduction followed by extinction prior to detection or redetection; (ii) maintenance in shorebird hosts without transmission into other bird species, notably waterfowl; and (iii) transmission into the waterfowl population, resulting in either co-circulation or competitive exclusion of previously circulating viruses. There have been a number of instances of novel lineages detected in shorebirds, but with no evidence of their establishment. For example, Hurt et al. (2006) described H11 viruses in Sharp-tailed Sandpipers detected in 2004, and a recent analysis of all Australian sequences demonstrated that no other viruses have been detected in this lineage since (Wille et al. 2022).

In the invasive-species literature, there is ample appreciation that the successful spread of an invader (or viral lineage) is preceded by the translocation, introduction, and establishment of that invader and that all four steps need to be completed effectively for the invasion to be successful (e.g. Kolar and Lodge (2001). In making risk assessments and in the development of mitigation strategies, it is important to distinguish between these processes. Using LPAIV incursions into Australia by migratory shorebirds as an example, whether the translocation step is successful depends on the prevalence of the virus in the source population, the volume of migrants, and how it affects the host's migration success (Risely et al. 2018). Importantly, successful introduction may depend on how the infection affects host fitness (e.g. illness leading to increased predation risk by dead-end hosts); the number, density, and suitability of potential hosts at the location of introduction; and the suitability of the new environment for the virus to survive outside the host. Whether the virus will establish at the new location depends on any temporal variation in these conditions, which may or may not result in an establishment bottleneck. For instance, densities of migratory shorebirds in Australia are very low during the Australian winter and high host specificity of the virus will thus reduce its chance of establishment, with host switching being a prerequisite for lineage establishment in Australia. Finally, successful invasion or spread of the virus to other parts of the continent also depends on how successful the virus is in surviving with the typical biotic and abiotic conditions in Australia, which may vary (dramatically) over time (Dalziel et al. 2016).

Evidence for the long-term maintenance of LPAIV exclusively in shorebirds without spread into waterfowl is provided by the internal segments of the H4N8 viruses described here. Not only have these internal segments been circulating in shorebirds for a number of years, but there is also evidence of exportation events with detection in *Calidris* shorebirds in Asia. A recent study demonstrated that viral exchange between shorebirds and ducks was uncommon relative to the rate of exchange within each host order (Hicks et al. 2022). This is reflected in phylogenetic studies of shorebird-specific clades, such as those discrete clades of H11 and H12 viruses circulating in North American shorebirds (Wille et al. 2018). However, cross-order virus transmission does occur, as shown by the long-circulating H4 lineage detected in ducks. Historically, this lineage has circulated in waterfowl (1980–2003, 2014–present) and shorebirds (2004–17), resulting in a complex pattern of interspecies transmission, and the propensity for viruses dispersed intercontinentally in shorebirds to enter waterfowl populations. Furthermore, viruses found in Ruddy Turnstones (*Arenaria interpres*) in Australia had genome

constellations with complex origins—not only geographically but also in host origin—as a result of reassortment (Hoye et al. 2021, Wille et al. 2022). It is, however, important to take into consideration the low number of viral sequences generated each year, such that we may not have the power to detect cross-species transmission if they do not further transmit through populations.

Finally, there are two potential outcomes once viruses have emerged in wild waterfowl populations: co-circulation or competitive exclusion of the previously circulating viruses, as shown by H6 viruses in North America (Bahl et al. 2009). We suggest that the patterns we observed in H10 are consistent with competitive exclusion. That is, neither the H10 lineage reported by Vijaykrishna et al. (2013) and Hoye et al. (2021) nor an H10 lineage of Eurasian origin reported in Wille et al. (2022) have been detected in wild birds since 2015 and 2019, respectively. Therefore, these lineages have likely been replaced by the novel H10 lineage reported here; alternatively, they persist in locations or populations which have not been sampled adequately. In addition to lineage replacement through competitive exclusion, novel lineages reassort with locally circulating viruses, resulting in genome constellations comprising segments that have been circulating in Australian wild birds for decades as well as segments recently introduced to Australia, as described both in this study and elsewhere (Hoye et al. 2021, Wille et al. 2022). The timing of the H10 incursion may provide further insight into why viral spread, both spatially and across host species, was so frequent following emergence in waterfowl populations. Studies of LPAIV in Australia have demonstrated that high rainfall is associated with increased viral prevalence in wild birds (Ferenczi et al. 2016, Wille et al. 2023), which in turn is associated with outbreaks in poultry (Ferenczi et al. 2021) due to the high spillover potential from wild birds. In 2020, there was a shift to La Niña conditions in Australia, resulting in high rainfall following a number of years of drought (Australian Government Bureau of Meteorology 2023), leading to a dramatic increase in juvenile waterfowl.

The understanding of viral incursion and consequent dispersal is of critical importance for both LPAIV and HPAIV. The global HPAIV H5N1 panzootic is affecting wildlife on all continents except Australia (Oceania) (Klaassen and Wille 2023, Wille et al. 2024). A greater understanding of the rates and risk factors for viral incursion, establishment, and spread across space, time, and host community of LPAIV, such as presented here, may play a key role in informing risk assessment and response strategies for potential HPAIV incursions. Critically, our study highlights that shorebirds should be monitored for viral incursion, which will reveal whether the virus has established in the local waterfowl population (Wille et al. 2024). Studies of viral movement within Australia (Wille et al. 2022), as well as the understanding of environmental factors on LPAIV virus ecology in waterfowl (Wille et al. 2023) and spillover risk to poultry (Ferenczi et al. 2021), are critical to response planning.

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Supplementary data

Supplementary data is available at *VEVOLU Journal* online.

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None declared.

Data availability

Sequences generated in this study have been deposited in GenBank (accession numbers are available in Supplementary Table S1). XML files for BEAST analyses are available on GitHub (https://github.com/michellewille2/H4_H10).

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