

1 **Australia as a global sink for the genetic diversity of avian** 2 **influenza A virus**

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5 Running head: Avian influenza in Australia
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76 **Abstract**

77

78 Most of our understanding of the ecology and evolution of avian influenza A virus (AIV) in
79 wild birds is derived from studies conducted in the northern hemisphere on waterfowl, with
80 a substantial bias towards dabbling ducks. However, relevant environmental conditions
81 and patterns of avian migration and reproduction are substantially different in the southern
82 hemisphere. Through the sequencing and analysis of 333 unique AIV genomes collected
83 from wild birds collected over 15 years we show that Australia is a global sink for AIV
84 diversity and not integrally linked with the Eurasian gene pool. Rather, AIV are infrequently
85 introduced to Australia, followed by decades of isolated circulation and eventual extinction.
86 The number of co-circulating viral lineages varies per subtype. AIV haemagglutinin (HA)
87 subtypes that are rarely identified at duck-centric study sites (H8-12) had more detected
88 introductions and contemporary co-circulating lineages in Australia. Combined with a lack
89 of duck migration beyond the Australian-Papuan region, these findings suggest
90 introductions by long-distance migratory shorebirds. In addition, we found no evidence of
91 directional or consistent patterns in virus movement across the Australian continent. This
92 feature corresponds to patterns of bird movement, whereby waterfowl have nomadic and
93 erratic rainfall-dependant distributions rather than consistent intra-continental migratory
94 routes. Finally, we detected high levels of virus gene segment reassortment, with a high
95 diversity of AIV genome constellations across years and locations. These data, in addition
96 to those from other studies in Africa and South America, clearly show that patterns of AIV
97 dynamics in the Southern Hemisphere are distinct from those in the temperate north.

98

99 **Author Summary**

100

101 A result of the ever-growing poultry industry is a dramatic global increase in the incidence
102 of high pathogenicity avian influenza virus outbreaks. In contrast, wild birds are believed to
103 be the main reservoir for low pathogenic avian influenza A virus. Due to intensive research
104 and surveillance of AIV in waterfowl in the Northern Hemisphere, we have a better
105 understanding of AIV ecology and evolution in that region compared to the Southern
106 Hemisphere, which are characterised by different patterns of avian migration and
107 ecological conditions. We analysed 333 unique AIV genomes collected from wild birds in
108 Australia to understand how Australia fits into global AIV dynamics and how viruses are
109 maintained and dispersed within the continent of Australia. We show that the Southern

110 Hemisphere experiences differing evolutionary dynamics to those seen in Northern

111 Hemisphere with Australia representing a global sink for AIV.

112

113

114 **Keywords:** Australia; avian influenza; ecology; evolution; influenza A virus; wild birds

115 Introduction

116 The evolution of avian influenza virus (AIV) is in part driven by the globally booming poultry
117 industry that comprises an estimated three quarters of the global avian biomass [1, 2]. This
118 industry has witnessed a dramatic increase in the incidence of disease outbreaks over the
119 past two decades caused by high pathogenicity avian influenza virus (HPAIV) [3, 4].
120 Despite this, wild birds continue to play an important role in AIV ecology and evolution.
121 Through long distance migration, wild birds have aided in the dispersal of high
122 pathogenicity H5Nx between Asia, Europe, Africa and North America [5]. Conversely, the
123 existence of distinct migratory flyways has constrained viruses into consistent phylogenetic
124 divisions, such as between AIVs detected in the Nearctic and the Palearctic [6].
125 Aggregations of wild birds vary both geospatially and temporally, often leaving their
126 hallmarks on AIV prevalence, diversity and evolution [7]. Importantly, most of our
127 knowledge of AIV ecology and evolution is drawn from studies in temperate northern
128 hemisphere systems [8-15] even though migration patterns and environmental conditions
129 relevant for AIV dynamics differ in the southern hemisphere [7].

130

131 Influenza A virus is a segmented, negative-sense RNA virus and the sole member of the
132 genus *Alphainfluenza* in the family *Orthomyxoviridae* [16]. Wild birds, particularly
133 *Anseriformes* (ducks, geese and swans), and to a lesser extent *Charadriiformes*
134 (shorebirds and gulls), are central reservoirs of AIV, with 16 of the 18 HA (haemagglutinin)
135 and 9 of the 11 NA (neuraminidase) subtypes identified in these taxa [6, 17, 18]. AIVs do
136 not generally cause high morbidity or mortality in their hosts, with the exception of subtype
137 H5 and H7 HPAIVs that emerge in poultry [5, 19-21]. In northern hemisphere systems, the
138 prevalence of AIV peaks in the autumn, driven by the recruitment of immunologically naïve
139 juvenile avian hosts and population congregations associated with migration [8, 9, 18, 22].
140 However, disease dynamics may vary in different global regions due to differences in
141 environmental and host factors [23]. Indeed, despite many parts of Australia being defined
142 as temperate, annual recruitment of immunologically naïve juvenile waterfowl into avian
143 populations is irregular due to highly variable climatic conditions which impact breeding
144 cycles, such that in some years waterfowl may not breed, or breed in small numbers and
145 in other years may attempt to breed multiple times [24]. Unlike the high prevalence of AIVs
146 in temperate northern hemisphere waterfowl, prevalence in Australian waterfowl has
147 consistently been less than 2% with no strong seasonal patterns, however these low
148 prevalence estimates may be driven by the highly aggregated nature of studies [25-32].
149 Furthermore, all Australian waterfowl are endemic and largely nomadic, and do not

150 migrate beyond the Australian-Papuan Region [33, 34]. Indeed, of the key AIV reservoir
151 avian taxa, only members of the *Charadriiformes*, notably the waders (families
152 *Scolopacidae* and *Charadriidae*), migrate and link Australia with Eurasia and North
153 America [35-37]. These species may also be less susceptible to AIV infection than some
154 other species [38]. The ecology of this migratory system has the potential to limit viral gene
155 flow between Eurasia and Australia and, consequently, it is expected that AIV lineages
156 may be evolving independently in Australia compared to other continents [27, 39]. Aside
157 from a small number of studies based on a limited number of AIV sequences [27, 30, 31,
158 39-43], how these distinct features of host-ecology impact AIV evolution in Australia is
159 largely unknown.

160

161 To reveal patterns of AIV evolution in wild birds in Australia, we used low pathogenic AIV
162 (LPAIV) genome data collected over nearly 15 years from all states and territories of
163 Australia to assess (i) the pattern of gene flow between Australia and other continents i.e.
164 a source, sink or combination, (ii) the extent and role played by AIV lineage introduction
165 and maintenance in Australia e.g. local evolution and extinction, and (iii) whether the
166 population dynamics, migration and reassortment of AIVs in Australia differ from those in
167 other geographical locations globally. Studying these processes in Australia, which
168 comprises conditions that are in stark contrast to those found in temperate avian
169 population systems in the northern hemisphere, will provide key insights into the global
170 drivers of AIV ecology and evolution.

171

172 **Results**

173 *Summary of avian influenza viruses sequenced*

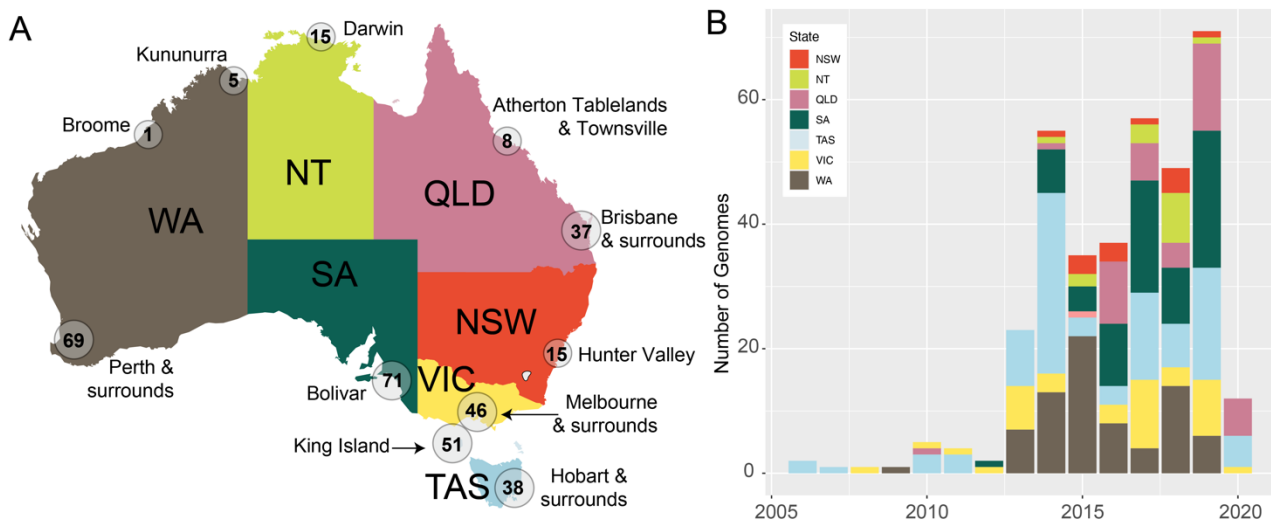
174 The data generated here comprised full or partial genomes of 333 unique LPAIV. Briefly, a
175 total of 397 AIV positive samples collected from 2006 to 2020 were submitted for
176 sequencing (Table S1). We recovered the full AIV genomes from 242 of the samples. In
177 some cases, we recovered partial AIV genomes consisting of gene segments with
178 insufficient sequence length (n=15) or with no sequence (n=76). An analysis of the
179 influence of Ct value and genome completeness is presented in Fig S1. A small number of
180 the virus samples comprised mixed infections (n=20), where two different variants of a
181 segment were detected. Forty-five viruses were sequenced more than once; samples may
182 have been re-sequenced due to poor quality in the initial attempt and/or in cases where
183 both the original sample and the corresponding egg isolate were sequenced.. Our analysis
184 also included additional virus sequences presented in Bhatta *et al.* 2020 (n = 1, individual

185 avian faecal sample in 2018) and Høye *et al.* 2021 (n = 22, combined oropharyngeal
186 cloacal swabs collected in 2014) as the samples were collected as part of the NAIWB
187 surveillance program.

188

189 Overall, unique AIV genomes comprising at least one segment characterised were
190 collected in South Australia (n = 71), Western Australia (n = 75), Tasmania (n = 89,
191 including [41], Queensland (n = 45), Victoria (n = 46, including [40], New South Wales (n =
192 15) and the Northern Territory (n = 15) (Fig 1A). These include those collected from avian
193 cloacal and/or oropharyngeal swab samples or avian faecal samples, and unique
194 genomes were generated from a combination of original samples (n=222) or isolates
195 (n=111). Prior to 2013, there were fewer than five sequenced genomes per year. However,
196 since this time the numbers of virus genomes have steadily increased, with the largest
197 number of genomes sequenced from samples collected in 2019 (n = 71) (Fig 1). This
198 increase coincided with a shift by the NAIWB surveillance program from characterising
199 only H5/H7 viruses towards more comprehensive LPAIV characterisation in Australia. Due
200 to irregular data collection in some states, large numbers of viral genomes were recovered
201 from single sampling events (e.g. Western Australia, Tasmania), whereas in other states
202 we find a more uniform temporal spread of the data (e.g. Victoria) (Fig S2).

203



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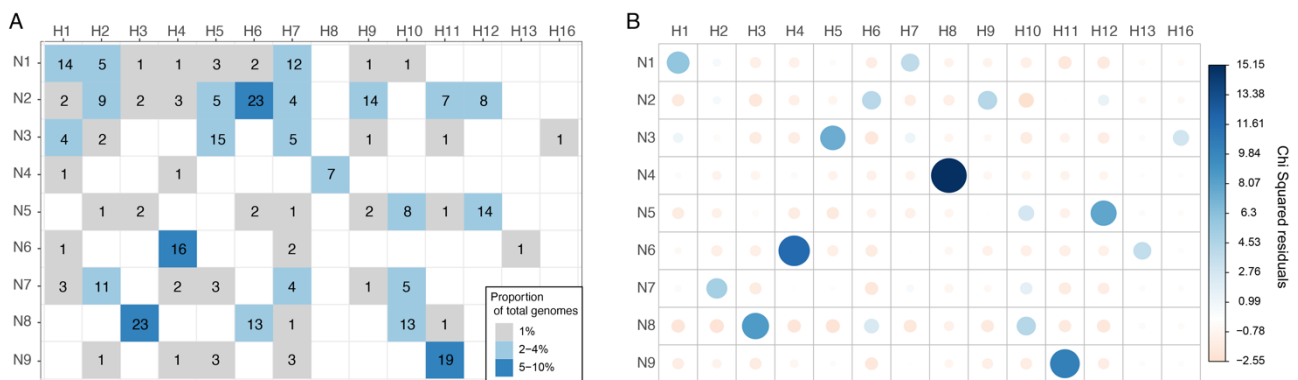
206 **Figure 1. Spatial and temporal distribution of avian influenza genomes used in this study. (A) Map of**
207 **Australia illustrating regional sampling locations. Where sampling locations were within 500km, they were**
208 **merged into a single location. The value within the circle corresponds to the number of unique viral genomes**
209 **comprising at least one segment from each location. States and Territories are as follows: VIC Victoria, NSW**
210 **New South Wales, QLD Queensland, NT Northern Territory, WA Western Australia, SA South Australia and**
211 **TAS Tasmania. (B) Number of genomes per state per year. Colours from panel B correspond to the fill colour**

212 of the state in panel A. This figure includes genomes comprising one or more segments and contains no
 213 duplicates. This figure includes all sequences generated as part of the National Avian Influenza Wild Bird
 214 Surveillance Program, including those recently published in [40, 41]. Metadata is available in Table S1 and a
 215 detailed plot illustrating exact virus sample collection dates and locations can be found in Fig S2.

216

217 Across the data set as a whole, we identified 14 different HA subtypes and all nine
 218 different NA subtypes, comprising 58 HA-NA combinations. We did not detect avian HA
 219 subtypes H14 and H15, and only a single case each of H13 and H16. The most common
 220 subtypes in our data set were H1N1 (n = 14), H3N8 (n = 23), H4N6 (n = 16), H5N3 (n =
 221 15), H6N2 (n = 23), H9N2 (n = 14) and H11N9 (n =19) (Fig 2A). These subtypes each
 222 comprised 5-10% of the subtype combinations. An analysis of HA-NA linkage by
 223 assessing the Pearson's residuals following a Chi-squared test revealed a strong positive
 224 association between H1-N1, H3-N8, H4-N6, H8-N4, H11-N9 and H12-N5 (Fig 2B). These
 225 overrepresented subtypes are comparable to those recovered from intensively sampled
 226 study sites in Europe and North America [8, 10]. In cases in which a HA subtype had
 227 several different NA subtypes, we saw weak positive or weak negative Pearson residuals
 228 (e.g. H7). As our data set largely comprised samples collected from wild bird faeces and
 229 pooled samples, the contribution of avian host species to AIV subtype distribution could
 230 not be determined.

231



232

233 **Figure 2. HA-NA subtype linkage in data generated for this study. (A) The number of each HA-NA subtype**
 234 **combinations (values) and the proportion of the total data set these values represent (shading). (B) A plot of**
 235 **the Pearson residuals of Chi-squared tests. For a given cell, the size of the circle is proportional to the**
 236 **amount of the cell contribution. Positive residuals are in blue and identify HA and NA subtypes for which**
 237 **there is a strong positive association in the data set. Negative residuals are in light pink and show a weak**
 238 **negative association, that is, they are underrepresented in the data set. This figure comprises unique viral**
 239 **genomes with at least one segment.**

240

241 *Australia is a sink for global AIV diversity*

242 Using the data generated here, we first aimed to determine how AIVs in Australia fit into
243 patterns of global genetic diversity. Specifically, we asked (i) whether there was one
244 consistent endemic Australian lineage for each subtype, (ii) how long these endemic
245 lineages have been maintained, and (ii) whether there is connectivity between Australia
246 and New Zealand (sequences mined from GenBank) comprising an “Oceania cluster”
247 within the southern hemisphere temperate zone.

248
249 Our phylogenetic analysis revealed that sequences from Australia tended to fall into
250 distinct Australian lineages, although the number of these lineages varied across subtypes
251 and segments. In the case of the HA segment, sequences from H2-H8 subtypes each
252 comprised a single contemporary lineage (Fig 3, Fig S4-S9). In contrast, H1, H9-H12 had
253 more than one contemporary lineage (Fig 3, Fig S3, S10-S12); Subtypes H9 had three,
254 and H1, H10-H12 each had two, contemporary lineages. In addition to lineages, there was
255 evidence of at least one H10 and two H11 incursions into Australia without subsequent
256 establishment (Fig 3, Fig S11). These differences in the number of lineages concur with
257 the observation that H1-H6 are over-represented at duck-focused study sites as compared
258 to H8-H12 which are under-represented at duck-focused study sites [e.g. 8]. It has been
259 proposed that ducks may not be the central reservoir for H8-H12 [44]; based on
260 phylogenies (Fig 3, Fig S9-S12) there was evidence for more repeated incursions of these
261 subtypes into Australia.

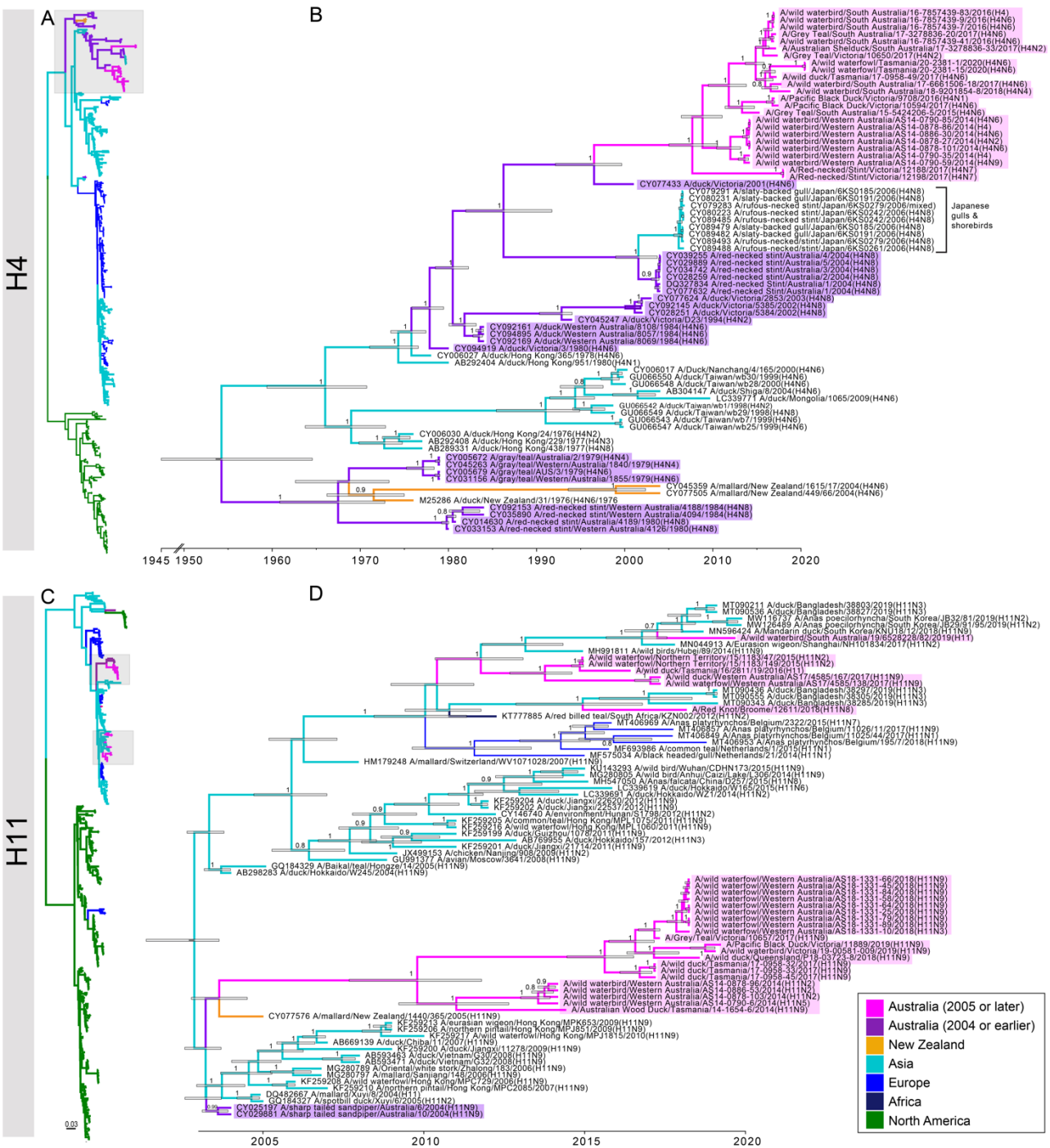
262
263 For many lineages in the time-scaled trees, there was a relatively large time gap between
264 the most recent common ancestor of Australian lineages and the closest reference
265 sequence (Fig 4). For example, in the Australian H1 lineage represented by four viruses
266 from 2013 and 2016 (Fig S3), the time to the most recent common ancestor (tMRCA)
267 ranged from Feb 2011-June 2013 (95% Highest Posterior Density [HPD]; mean at June
268 2012), whereas their date of separation from the closest reference sequence was between
269 1999-2003 (mean at June 2001) (Fig S3). This is most likely due to vast under-sampling in
270 Australia, notably between 2000 and 2012, although sporadic and/or under-sampling of
271 wild birds in Asia may compound this. Critically, this has implications for accurate dating of
272 some Australian lineages as it is unclear how distant the introduction of the lineage to
273 Australia predated the tMRCA of existing diversity (Fig 4). These issues notwithstanding,
274 the tMRCA of contemporary Australian lineages was 2005 or later, suggesting currently

275 established lineages were introduced to Australia relatively recently (Fig 4). This was
276 supported by the fact that most of the older Australian lineages, comprising viruses from
277 the 1970s to 1980s are no longer in circulation. There are some exceptions, such as H7
278 viruses, that had a tMRCA of between Aug 1974 - Aug 1975. This Australian H7 lineage
279 has been associated with eight HPAIV poultry outbreaks in Australia since 1976 [45].
280 Sequence data for this H7 lineage from wild birds has only been available since 2007 due
281 to very limited sampling and sequencing of wild birds in earlier years (Fig S8).

282
283 Notably, sequences from Australia and New Zealand did not consistently fall into the same
284 lineages. There were 42 HA sequences from New Zealand available in GenBank, of which
285 33 had collection dates of 2004 and later, aligning well with the temporal scale of
286 contemporary Australia lineages. Indeed, New Zealand lineages of H1, H2, H5, H6, H7
287 and H10 were each in entirely separate lineages from Australian sequences (Fig S3-S4,
288 S6-S8, S11). This is likely due to limited bird migration involving some shorebird species,
289 between New Zealand and Australia [46].

290
291 Overall, Australia appears to be a sink for Eurasian AIV diversity. Although we identified
292 multiple viral introductions from Eurasia to Australia, in the entire data set there were only
293 two examples of viruses from Australia being introduced to Eurasia. These comprised the
294 H4(N8) subtype (Fig 3B), and one N7 sequence (Fig S21). Notably, each of these events
295 involved the detection of Australian lineage viruses in Charadriiformes (gulls and
296 shorebirds) in Japan. Overall, all viral introductions stemmed from Eurasian lineages with
297 the exception of H10 and H12 that showed introductions from North American lineages.
298 For H8 and one H9 lineage, the most closely related reference viruses were sampled in
299 Europe. However, due to possible under-sampling and/or under-representation of viral
300 diversity in wild birds in Asia it cannot be concluded that these lineages were seeded
301 directly from Europe (Fig S9-S10).

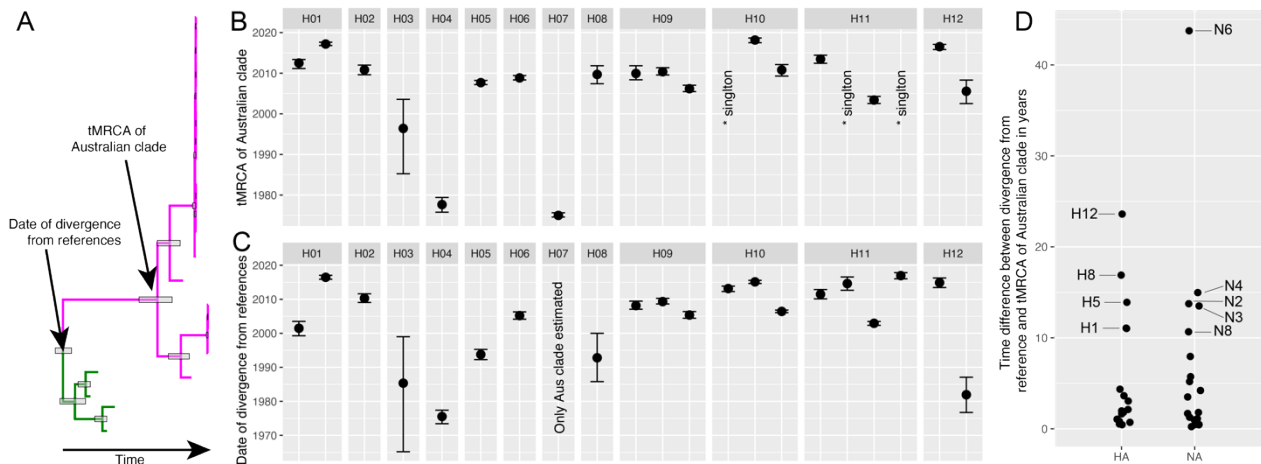
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303

304 **Figure 3. Phylogenetic trees of subtypes H4 and H11.** (A, C) Phylogenetic trees comprising global diversity.
 305 Branches of reference sequences are coloured by continent. Sequences from Australia are coloured in pink
 306 (2005 and later) and in purple (pre-2005), with 2005 marking the year of the oldest sequence in the data set
 307 generated in this study. (B, D) Time structured phylogenetic trees. The trees comprise Australian lineages
 308 (as indicated by grey boxes in A, C) and closest relatives (retrieved by BLAST searches). Branches are
 309 coloured by year and geography as above. Branch labels correspond to posterior clade probabilities of each
 310 node, node bars correspond to the 95% HPD of node height. We selected H4 as it is the only HA subtype for
 311 which there is clear introduction of an Australian lineage virus into Asia (indicated in square parenthesis),
 312 and is an example of an HA segment for which there is only a single contemporary lineage. We selected H11

313 as it is the subtype with the largest number of contemporary Australian lineages (4), of which 2 are
 314 represented by a single sequence. Trees for all other HA subtypes can be found in Fig S3-S14



315
 316
 317 **Figure 4.** The time-scale of AIV evolution by subtype in Australia. (A) A schematic phylogeny demonstrating
 318 the differences between the tMRCA of Australian lineages and the dates of divergence from reference
 319 sequences. (B) The tMRCA distribution of contemporary Australian lineages of the HA segments and (C)
 320 dates of divergence from the reference sequences of all HA lineages. Points represent the node date and
 321 bars the 95% HPD. For segments with multiple lineages, multiple estimates have been provided. Where a
 322 novel introduction is represented by a single sequence the tMRCA was not estimated (here, represented by
 323 “*singleton) but the date of divergence from reference sequences is shown. For H7, we did not estimate the
 324 date of divergence from the closest reference sequences. (D) The time difference between the tMRCA of the
 325 Australian lineages and the date of separation for all HA and NA segments and lineages. Lineages with time
 326 differences of more than 10 years are labelled. All HA and NA trees are presented in Fig 3, Fig S3-S23.

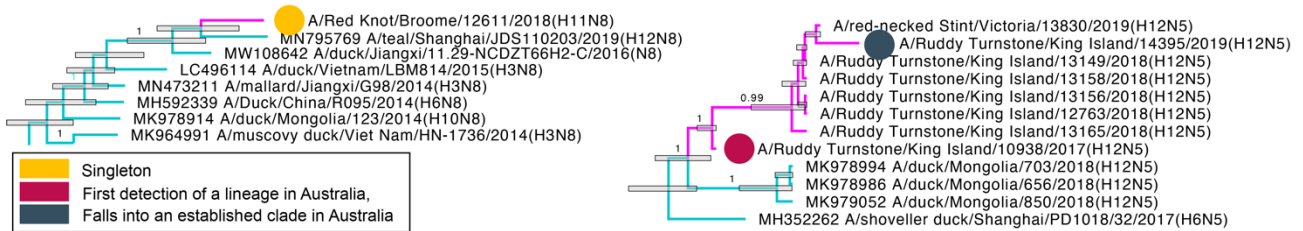
327 328 *Detection of novel virus segments introduced into Australia*

329
 330 The relatively long time difference between the tMRCA of Australian lineages and the
 331 global representative viruses used as reference suggest that the wild bird surveillance
 332 sampling has been unable to detect the index viruses seeding local lineages (Fig 4).
 333 However, a small number of viruses in the data set (n = 18) contained gene lineages
 334 and/or introductions with no further transmission which likely comprise recent introductions
 335 to Australia. These viruses comprised at least one virus gene segment that either
 336 represented the only detection of a novel lineage in Australia (*i.e.* singletons) or comprised
 337 the first detection of an Australian lineage cluster, where the time difference between the
 338 tMRCA of the identified lineage and date of divergence from global references was small
 339 (less than 1 year) (Fig 5). These recently introduced viruses were only detected in the
 340 north of Western Australia, the Northern Territory and Queensland, and from migratory
 341 shorebirds in Tasmania. Migratory birds would likely use these northern locations as initial

342 stopover sites in Australia, highlighting the importance of surveillance of shorebirds in
 343 these regions. Notably, we did not find evidence of a complete “novel” virus genome, that
 344 is all viruses for which whole genome data were available contained at least one gene
 345 segment belonging to an established Australian lineage. For example, A/Ruddy
 346 Turnstone/King Island/10938/2017(H12N5) had 7 segments representing the index
 347 detection of a novel lineage, with only the M segment belonging to an established
 348 Australian lineage. Interestingly, Ruddy Turnstone viruses in 2018 and 2019 had a number
 349 of segments falling into lineages for which A/Ruddy Turnstone/King
 350 Island/10938/2017(H12N5) was basal.

351

		PB2	PB1	PA	HA	NP	NA	M	NS (A)
A/Red Knot/Broome/12611/2018(H11N8)	1 March 2018				H11		N8		
A/wild waterfowl/Northern Territory/15-1183-47/2015(H11N2)	14 Dec 2015				H11		N2		
A/wild waterfowl/Northern Territory/15-1183-149/2015(H11N2)	14 Dec 2015				H11		N2		
A/wild duck/Northern Territory/18-0491-262/2018(H9N1)	8 June 2018				H9		N1		
A/wild waterbird/Queensland/P16-10643-18/2016 (H1N1)	1 Aug 2016				H1		N1		
A/wild waterbird/Queensland/P16-10643-19/2016 (H1N1)	1 Aug 2016				H1		N1		
A/wild waterbird/Queensland/P16-10643-20/2016 (H1N1)	1 Aug 2016				H1		N1		
A/wild waterfowl/Queensland/JCU-234/2018(H1N1)	18 May 2018				H1		N1		
A/wild waterbird/Queensland/JCU-272/2019(H5N9)	9 Aug 2019				H5		N9		
A/Ruddy Turnstone/King Island/10938/2017(H12N5)	30 March 2017				H12		N5		
A/Ruddy Turnstone/King Island/XXXX/2019(H12N2) (n=8)	24-31 Feb 2019				H12		N2		



352

353

354 **Figure 5.** Viruses sequenced in this study that have signatures of recent introduction. For each segment,
 355 coloured tiles correspond to three different statuses: singletons, first detections and well-established
 356 lineages. Singletons represent the only detections of the lineage in Australia. In cases where two viruses
 357 from the same sampling effort were identical and were the only detections of that lineage, they were still
 358 considered a singleton (e.g. the NA segment of the NT/2015 viruses or the NA segment of the 8 H12N2
 359 Ruddy Turnstone viruses from 2019, which have only ever been detected during that sampling event). Only
 360 the A allele NS segment was detected in these viruses. Phylogenetic examples (here excerpts from N8 and
 361 N5) are provided for each status, and branches are coloured as in Fig 3.

362

363 AIV circulation within Australia

364 Given that there are no structured flyways within Australia and birds have nomadic
 365 movements influenced by climate [29, 47], we hypothesized that there would be limited
 366 geographic structure of AIVs within the continent. To assess this, we analysed possible
 367 viral “migration events” (Markov Jumps) between sampled locations using the HA and/or
 368 NA segments comprising Australian lineages with 20 or more sequences (H4, H5, H6, H7,

369 N6, N8), and two independent lineages of the NP segment. These analyses are likely
370 strongly influenced by both small sample sizes and collection biases, such that we do not
371 have sequence coverage across all subtypes for all locations and years. However, we also
372 examined the two larger NP lineages which included substantially more sequences ($n =$
373 197 and $n = 85$ sequences) than any of the subtype specific HA or NA data sets and
374 spanned the entire sampling period and all sample locations.

375

376 Our phylogenetic data revealed potential virus migration events between the sampling
377 locations in the southeastern states (Victoria, South Australia, New South Wales and
378 Tasmania) that occurred consistently across all of the gene segments examined (Fig 6).
379 Using the largest Australian NP gene lineage, we found more than 10 potential migration
380 events between Victoria and South Australia and between Victoria and Tasmania,
381 suggesting high levels of connectivity between these sampling locations. We also found
382 evidence of movement between temperate Western Australia and the southeastern states
383 (Victoria, South Australia, Tasmania), and between Queensland and the southeastern
384 states, although this was only detected in the NP segments and in two of the HA/NA
385 subtypes analysed. As only limited sequences were available from tropical Australia
386 (northern Queensland, Northern Territory and northeastern Western Australia), migration
387 events to/from these locations were not well estimated in our analyses. However, for the
388 largest NP lineage, a number of potential migration events between temperate and tropical
389 Australia were observed (Fig S26). Potential migration events were also detected between
390 the sampled tropical locations. Although it is likely that we have underestimated the
391 migration events due to poor temporal and spatial coverage, the migration events had
392 strong Bayes Factor support (Fig S25). Importantly, these analyses also did not record >1
393 migration event or >10 Bayes Factor between all locations that were included in each tree
394 as a default. For example, despite being included in all eight analyses, we only detected
395 significant migration events (or >10 Bayes Factor) to/from Western Australia in the H6 and
396 N6 lineages, and the two NP lineages (Fig 6, Fig S25).

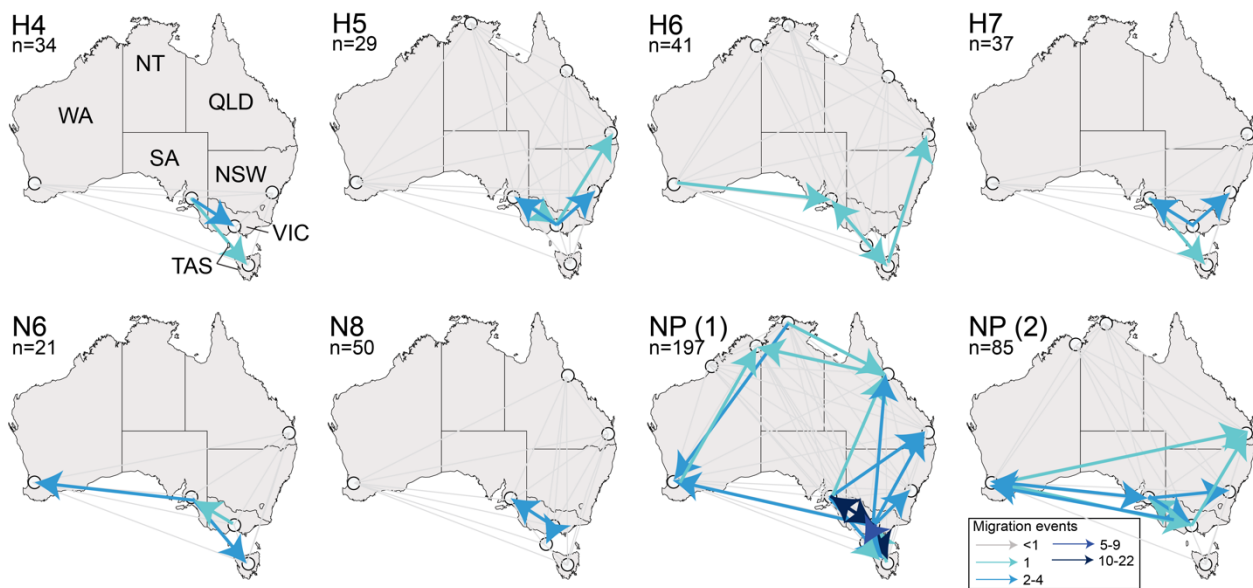
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398 Overall, analysis suggested Victoria was consistently a net exporter as most migration
399 events originated from the state. Specifically, Victoria played a role as a net exporter in H5,
400 H7, N6, N8, and both NP lineages. South Australia also played a role as an exporter (H4,
401 H6, H7, N6, and both NP lineages), although we detected both import and export events
402 from this state across most analyses. Temperate Western Australia was a net importer of
403 AIV, although as with South Australia, we detected both importation and exportation

404 events across the analyses. A positive association between Markov rewards and the
405 number of exportation events may also be evidence of sampling bias. For example, in the
406 case of H4, H5 and H7, Victoria had substantially more sequences available as compared
407 to other sampling locations and was identified as a net exporter. In these cases, the high
408 number of exportations relative to importation events may be due to sampling biases (Fig
409 S27).

410
411 Taking the potential biases in our data set into consideration we did not see consistent
412 source or sink locations for AIV movements but rather detected numerous exportation and
413 importation events in most locations. Further, there was no consistent directionality to
414 patterns of viral dispersal. Rather adjacent locations from which we had many samples
415 were highly connected. These results are consistent with the absence of flyway structure
416 within Australia.

417



418

419 **Figure 6.** Inferred migration events of avian influenza viruses within Australia. Locations included in each
420 tree are marked by a white circle. Specific location names are presented in Fig 1A, and all state names are
421 presented in the first panel and are as follows: VIC Victoria, NSW New South Wales, QLD Queensland, NT
422 Northern Territory, WA Western Australia, SA South Australia and TAS Tasmania. Grey lines correspond
423 potential migration events that were not detected in the analysis (i.e. migration event <1). Blue lines indicate
424 migration events are derived from calculations of state changes (Markov Jumps), ranging from light to dark.
425 Arrows indicate the direction of the migration event. As NP has more than a single discrete Australian
426 lineage, we have generated two independent maps reflecting the 2 largest Australian lineages of NP (Fig
427 S25). Maps illustrating Bayes Factors, also generated using BSSVS can be found in Fig S26, and Markov
428 rewards also generated in this analysis are presented in Fig S27.

429

430

431 *Genomic Reassortment*

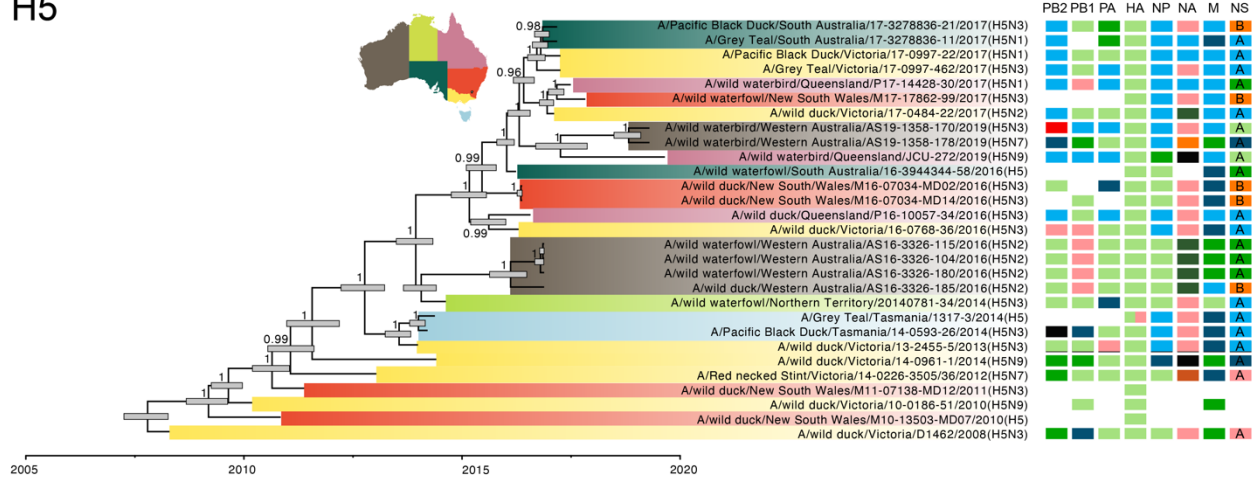
432 Despite a low reported prevalence, multiple lineages and subtypes co-circulated at most of
433 the sampled locations (Fig S28), [e.g. 25]. A number of “mixed” virus samples (*i.e.*
434 samples comprising at least one segment with two different sequences) were also
435 detected through sequencing. These mixed virus samples were often detected from
436 sampling events where a diversity of AIV subtypes were co-detected. The only exceptions
437 were A/wild duck/New South Wales/M15-10737-MD02/2015(mixed) and A/wild
438 waterfowl/Queensland/JCU-78-226/2016(mixed) for which other AIV genomes were not
439 detected in birds collected on the same collection events (Table S2). Next- generation
440 sequencing of the original samples allows for the detection of mixed viruses.

441

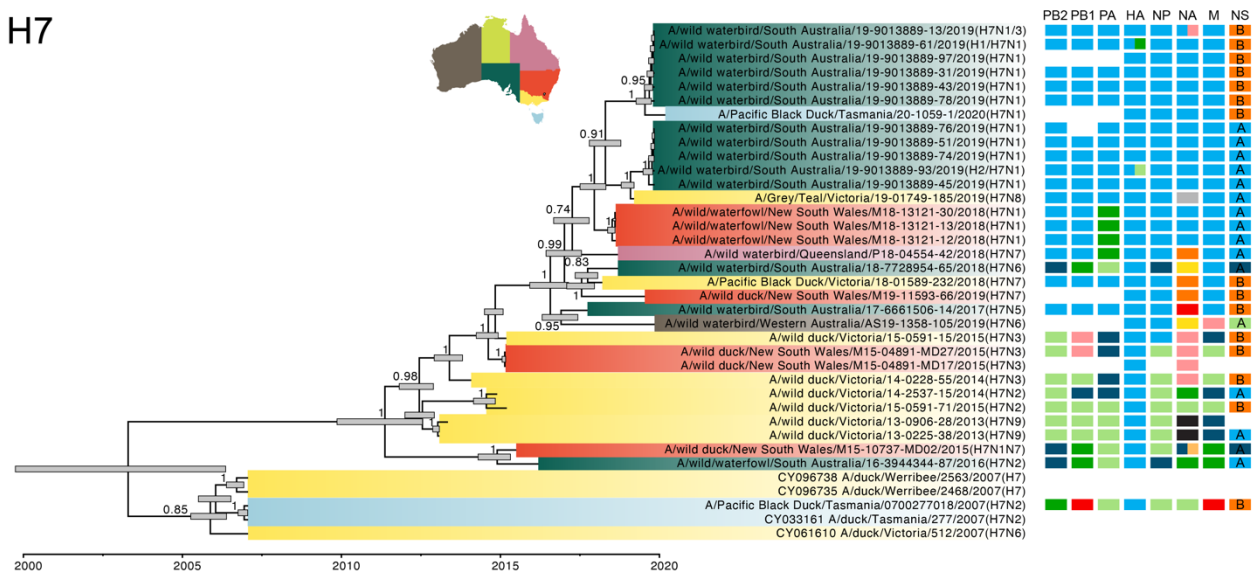
442 Assessment of the diversity of genome constellations indicated prolific reassortment,
443 similar to that found in other locations that have been studied [13, 14]. In the case of the
444 H5 and H7 subtypes of veterinary importance, the LPAIV genome data from wild birds
445 revealed 17 unique constellations from 33 (26 complete) H7 genomes, and 18 unique
446 constellations from 29 (20 complete) H5 genomes (Fig 7). The only virus samples with
447 identical genome constellations were those from the same sampling event and location.
448 However, even within the same sampling event where the same HA-NA subtype
449 combination was detected, there was evidence of genetic reassortment. For example, of
450 the 11 H7N1 virus samples sequenced from a single 2019 sampling event in South
451 Australia, six viruses had an NS B allele while the others had the NS A allele (Fig 7). We
452 found that within the same year, partial genome constellations were shared. For example,
453 in 2018 H7 viruses were collected in New South Wales, Queensland, South Australia, and
454 Victoria. With the exception of A/wild waterbird/South Australia/18-7728954-
455 65/2018(H7N6) these viruses share 5 of 8 segment lineages, with differences in PA, NA
456 and NS. (Fig 7).

457

H5



H7



458

459 **Figure 7.** Genome constellations of (A) H5 and (B) H7 viruses. The phylogenies presented are time-
460 scaled Maximum Clade Credibility Trees. Tips are coloured according to Australian state or territory. Scale
461 bar denotes the year of sample collection. Node bars are the 95% HPD of node height, and posterior clade
462 probability is presented on each branch. Adjacent to each tree are coloured tiles where each column of tiles
463 refers to a segment, arranged according to size: PB2, PB1, PA, HA, NP, M, NS. We only included tiles for
464 viruses sequenced in this study and in cases where the tiles are blank, no sequence was available for the
465 segment. Different colours refer to different lineages, whereby tile colour scheme is retained for both H5 and
466 H7 trees. For example, for the NS segment, viruses with an NS B lineage are coloured in orange. The
467 viruses here fall into five different lineage clusters of NS A, and these are presented in two different shades
468 of blue and green and pink. If a virus is a “mixed” infection, segments with two different lineages or subtypes
469 are split to illustrate this.

470

471 Discussion

472 Australia and the Southern Hemisphere have a chequered legacy for research on avian
473 influenza. The first detection of AIV in wild birds was associated with a mortality event in
474 terns off the coast of South Africa [48], and the first descriptions of avian influenza from

475 wild, healthy birds was from the Great Barrier Reef islands of Australia [49-51].
476 Furthermore, one of the most enigmatic subtypes, H15, was initially described in Australia
477 [52, 53]. Through surveillance activities, particularly since 2006, it has become clear that,
478 unlike the northern hemisphere, AIV prevalence in Australia is generally low with no strong
479 seasonal pattern, however prevalence estimates generated from current surveillance
480 methods have large uncertainty. Despite low isolation success, recent studies have
481 demonstrated that AIV detections fluctuated temporally and geographically, and that the
482 full diversity of AIV subtypes circulate on the continent [25, 27, 28, 32, 54]. Early
483 phylogenetic studies on a limited selection of AIV subtypes and sporadic sequence data
484 suggested the potential for Australian specific lineages, and detections of intercontinental
485 reassortants [30, 39, 41-43]. Despite these findings, understanding of AIV evolution in wild
486 bird populations of the southern hemisphere lags behind that of the northern hemisphere
487 due to low sampling rates and characterisation of virus data [54, 55]. This study is the first
488 to comprehensively assess AIV evolution across all detected subtypes in Australia, the
489 outcomes of which demonstrate the importance of the globally varying characteristics of
490 bird migration on AIV dynamics.

491
492 A key observation was that AIV in Australia are characterised by infrequent enduring
493 introductions followed by decades of isolated circulation. Hence, Australia appears to be a
494 sink for AIV genetic diversity and not closely linked to the Eurasian virus gene pool. This
495 dynamic is mirrored in Africa and South America. Although southern hemisphere AIV
496 lineages sit within lineages originating in the northern hemisphere, our results reinforce
497 findings from a growing number of studies demonstrating that AIV lineages from the
498 temperate north are sporadically introduced to the southern hemisphere [41-43, 56-59].
499 Specifically, sequences generated in the Neotropics fall into lineages within the Nearctic
500 lineage, and Afrotropical and Australasian lineages are generally part of the Palearctic
501 lineage. In contrast, export events from the southern hemisphere into the temperate north
502 have very rarely been reported [57, 60]. Once introduced, lineages circulate in isolation in
503 the southern hemisphere until extinction. Data from both South America and Australia
504 illustrate that in some cases, lineages have been maintained in isolation for decades.
505 Rimondi *et al* 2018 reported that a unique PB2 lineage has been circulating in South
506 America for ~ 100 years. Similarly, lineages such as those of the Australian H7 subtype
507 viruses, have been circulating in the country continent for more than 50 years, although
508 precisely dating the divergence of these lineages is challenging due to the sparsity of AIV
509 sequence data prior to 1980. Despite long-term isolation, we demonstrated that many

510 lineages that circulated in the 1980's have become extinct and have been replaced,
511 perhaps due to competitive exclusion as seen in other locations [61].

512

513 Waterfowl migration influences viral evolution, and Australia as a sink for AIV diversity is
514 likely driven by a lack of waterfowl migration between Australia and Asia, particularly
515 across the Wallace Line [33]. AIV are predominately distributed by waterfowl, with key
516 evidence described for the flyway system of North America [62], the rapid movement of
517 HPAIV H5Nx viruses across the globe coinciding with waterfowl migration patterns [5], and
518 prevalence peaks in Africa coinciding with the arrival of migratory Palearctic waterfowl [63].
519 In Oceania, waterfowl species are endemic to the Australo-Papuan region [33]. It is
520 therefore more likely that the limited introduction of novel AIV lineages to Australia are due
521 to long-distance migratory shorebirds flying from their northern hemisphere breeding
522 grounds along the East Asian Australasian Flyway to Australia for the duration of their non-
523 breeding season [64]. This is notably reflected in the larger number of novel introductions
524 detected in subtypes such as H9, H10 and H11, and fewer detectable introductions of
525 subtypes typically associated with waterfowl, especially ducks (e.g. H4). Our finding of
526 virus gene lineages originating from both Eurasia and North America further supports that
527 migratory shorebirds play a key role in introducing AIVs into Australia. Alaska is part of the
528 East Asian Australasian flyway [64] and shorebird species such as Sharp-tailed
529 Sandpipers (*Calidris acuminata*) [65], Ruddy Turnstone (*Arenaria interpres*) [35] and Bar-
530 tailed Godwit (*Limosa lapponica*) [66] migrate from Alaska to Oceania. AIV genomes that
531 we identified to contain novel viral introductions were detected in shorebird samples
532 including from locations where shorebirds may stop during southward migration, such as
533 Broome, Western Australia [67]. Like Australia, the Amazon rainforest forms a major
534 barrier to waterfowl migration in the Nearctic-Neotropical system [68, 69]. As such, in a
535 similar manner to Australia, the movement of AIVs from the Nearctic to the Neotropics is
536 mostly likely carried by long-distance shorebird migrants [57, 70, 71]. A large evolutionary
537 study similarly showed the importance of shorebirds in introducing viruses to South
538 America, as many of the recently characterized virus detections from shorebirds belonged
539 to the main North American shorebird-associated lineages rather than divergent South
540 American lineages [57].

541

542 Within Australia, we found no evidence of directionality in the movement of the AIV gene
543 pool within Australia. As Australian waterfowl are nomadic rather than strictly migratory,
544 there are no key migratory flyways within the continent. Rather, ducks have “erratic”

545 movement patterns across the continent which are heavily dictated by the availability of
546 water [47]. Therefore, consistent patterns of AIV movement between specific locations
547 would not be expected. However, we did observe high connectivity (*i.e.* the number of
548 strongly supported viral migration events detected within phylogenies) between the
549 southeastern locations. Across this region, movements of waterbirds tracking water within
550 and between the large Murray-Darling and Lake-Eyre basins may form the natural links.
551 For example, satellite tagged Grey Teals (*Anas gracilis*) moved widely across the Murray-
552 Darling basin, utilizing permanent and temporary watercourses in Victoria, New South
553 Wales, and South Australia. Some of these tagged individuals connected the Murray-
554 Darling with sites in Queensland and Northern Territory with flights of over 1200km [47].
555 While ducks are likely the major driver of virus movement within Australia, there are also a
556 number of nomadic waders that similarly move long distances in search of water for
557 breeding and foraging [72]. Unfortunately, due to low prevalence and a sampling regime
558 not designed to investigate these dynamics, we were unable to infer the fine scale patterns
559 of virus movement.

560

561 Our data and analyses are central for placing future Australian AIV genome sequences
562 and studies within the local and global context. Some recent studies have reported the
563 possible detection of novel intercontinental reassortants where AIV segments were
564 reported to be more closely related to lineages from Eurasia and North America compared
565 to those from Australia [40, 41]. Here we clarified that these viruses are not necessarily
566 recent intercontinental reassortments but belong to pre-established lineages in Australia
567 [40, 41]. For appropriate outbreak response and biosecurity policy development it is
568 crucial to accurately assign the source of AIV detected in poultry or wild birds as potential
569 novel introductions of “exotic” viruses or their derivative reassortants, especially in the
570 presence of reassortment promiscuous lineages such as clade 2.3.4.4 HPAIV H5Nx
571 associated with current epizootics in the Northern Hemisphere that may have devastating
572 consequences for the local poultry industry. This, combined with the potential roles of
573 shorebirds in introducing AIV lineages [37] to Australia has implications for wild bird AIV
574 surveillance and risk assessments for wild bird, poultry and human health.

575

576 In sum, we revealed that the evolution of AIV in Australia differs from patterns found in the
577 northern hemisphere. These reflect differences in environmental conditions influencing bird
578 ecology, notably in AIV host competency and movement patterns, and taken together

579 should be integrated into improved risk assessments of potential AIV spillover into poultry
580 and the distribution of exotic or potentially zoonotic AIV lineages into Australia.

581

582 **Methods**

583 *Ethics Statement*

584 All capture and sampling of wild birds carried out by Deakin University was conducted
585 under approval of Deakin University Animal Ethics Committee (permit numbers A113-
586 2010, B37-2013, B43-2016, B39-2019, B03-2020), Philip Island Nature Park Animal Ethics
587 Committee (SPFL20082), Wildlife Ethics Committee of South Australia (2011/1,
588 2012/35,2013/11) and Department of Primary Industries, Parks, Water & Environment
589 Animal Ethics Committee of the Tasmanian Government (5/2019-20). Banding was done
590 under Australian Bird Banding Scheme permit (banding authority numbers 2915, 8000,
591 8001). Research permits were approved by Department of Environment, Land, Water and
592 Planning Victoria (10005726, 10006663, 10007534, 10008206, 10009534), Department of
593 Primary Industries, Parks, Water & Environment of the Tasmanian Government (FA11255,
594 FA13032, FA14110, FA15270, TFA14065, TFA15269, TFA16256, TFA17018, TFA18088,
595 TFA19044), Office of Environment and Heritage New-South Wales (SL101252),
596 Department of Environment, Water and Natural Resources South Australia (M25919-
597 1,2,3,4,5), Department of Environment and Conservation of Western Australia (SF008456,
598 SF009067, BB003100, BB003163, BB003312), Department of Parks and Wildlife of
599 Western Australia (08-001825-1) and Parks and Wildlife Commission of the Northern
600 Territory (51604, 58510)

601

602 Capture and sampling carried out by Agriculture Victoria Research was done in
603 accordance with permits by the State Government of Victoria Research Permit under
604 Wildlife Act 1975 (FF380519 Permit No: 10004073, FF383165 Permit No: 10005321,
605 FF383294 Permit No: 10006640, FF383493 Permit No: 10007877, FF383578 Permit No:
606 10008927), and Animal Ethics Research Project Permit (AEC 2019-04).

607

608 Sampling undertaken by the Northern Australia Quarantine Strategy was undertaken in
609 accordance with a Licence to take Fauna (SF006970) from the Department of
610 Environment and Conservation (WA) and permits from Department of Agriculture, Forestry
611 and Fisheries (QLD – now Department of Agriculture and Fisheries) (CA 2013/07/703) and
612 Department of Agriculture and Fisheries (QLD) (CA 2016/07/980). Permits for collection of
613 faecal samples were not required from Parks and Wildlife (NT) or DAFWA (WA).

614

615 For samples collected by Department of Primary Industries, Parks, Water and
616 Environment, Tasmania and Primary Industries and Regions, South Australia, Department
617 of Primary Industries and Regional Development, Western Australia; James Cook
618 University; NSW Department of Primary Industries; University of Technology Sydney; or
619 Biosecurity Queensland, Department of Agriculture & Fisheries, permits were not required
620 for the collection of environmental faecal samples or for samples collected
621 opportunistically from carcasses.

622

623 Cloacal samples collected from a wild bird as part of a mortality event investigation by
624 Department of Primary Industries and Regional Development, Western Australia, is also
625 exempt from a permit.

626

627 *Sample collection and screening*

628 All samples were collected from wild birds or from wild bird faeces since 2006, as part of
629 the National Avian Influenza Wild Bird Surveillance Program (NAIWB). Details of sample
630 collection and screening methods can be found in [25]. No HPAIV were detected in wild
631 birds through the duration of this study.

632

633 *Next generation sequencing*

634 Viral RNA was extracted with MagMAX™-96 viral RNA isolation kit (Thermo Fisher
635 Scientific, Waltham, MA) from avian faecal swab samples, avian swabs and embryonated
636 chicken egg isolated virus samples according to manufacturer's instructions. Positive
637 samples with an influenza A matrix gene qPCR Ct of ≤ 30 were selected for influenza A
638 virus targeted next generation sequencing (NGS). The AIV genome segments were
639 amplified using the SuperScript™ III one-step RT-PCR system with high fidelity Platinum™
640 Taq DNA polymerase (Thermo Fisher Scientific) and universal influenza A virus gene
641 primers as previously described [73]. Sequencing was performed on the Illumina MiSeq
642 NGS platform (Illumina, San Diego, CA) with up to 24 samples pooled per sequencing run
643 by use of dual-index library preparation and the Nextera XT DNA Library Preparation kit
644 and 300-cycle MiSeq Reagent v2 kit (Illumina), according to manufacturer's instructions.
645 Sequence reads were trimmed for quality and mapped to respective reference sequence
646 for each influenza A virus gene segment using Geneious Prime software
647 (www.geneious.com) (Biomatters, Auckland, NZ).

648

649 For a small subset of AIV sequences generated by Agriculture Victoria (Table S1), RNA
650 was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), AIV genome
651 amplified [74, 75] and Illumina sequencing libraries prepared using PerkinElmer
652 NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (Perkin Elmer, Waltham, MA, USA). The
653 libraries were sequenced using a S4 NovaSeq flow cell with the MiSeq 600-cycle v3 kit.
654 The sequences were assembled through the iterative refinement meta-assembler (IRMA)
655 pipeline using the default FLU parameters [76].

656

657 *Data availability*

658 Assembled consensus AIV sequences have been deposited in GenBank (accession
659 OL369937-OL372235, OL450375- OL450392) (Table S1).

660

661 *Statistical analysis*

662 We analysed sample and sequence metadata for completeness using R 4.0.2 integrated
663 into RStudio 1.3.1073 and the *dplyr()*, *Hmisc()*, *reshape2()*, and *ggplot2()* packages. To
664 compare the differences in Ct values of samples and sequencing “completeness” we used
665 a generalized linear model and a summary of results is presented in Fig S1.

666

667 *Phylogenetic analysis*

668 Full-length reference sequences for all AIV segments and subtypes were downloaded from
669 the Influenza Research Database (<https://www.fludb.org/>). Our sequence search was
670 limited to samples from North America, Europe, Asia and Oceania. Overall, for each of the
671 HA and NA trees, final data sets contained ~500 sequences (+/-20), and for internal
672 segments data sets contained 800-900 sequences. For the internal segment sequences,
673 reference sequences did not include the poultry adapted subtypes H5N1, H7N9, H9N2 or
674 other AIV sequences from poultry. In addition to sequences from Australia generated in
675 this study, we also included all sequences from Oceania (Australia and New Zealand) in
676 GenBank, including partial sequences. Australian H10 sequences [30] that were not
677 available in the Influenza Research Database or GenBank were downloaded from GISAID
678 (<https://www.gisaid.org/>).

679

680 Sequences were aligned using MUSCLE v3.8.425 [77] integrated within Geneious Prime.
681 Sequence alignments were cleaned to remove any obviously problematic sequences,
682 including those containing many ambiguous bases, insertions or deletions, and respective
683 data sets were trimmed. Global phylogenetic trees were estimated using the maximum

684 likelihood (ML) method incorporating the most appropriate model of nucleotide substitution
685 estimated using Smart Model Selection in PhyML v3.0 [78, 79]. Trees were visualised
686 using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>). From these global trees we
687 were able to infer the number of independent introductions into Australia, as well as the
688 number of local genome constellations, and used this information to assess the pattern
689 and frequency of segment reassortment.

690

691 *Time-scaled phylogenetic analysis*

692 Time-structured phylogenetic trees of all contemporary (those lineages circulating in 2005
693 or later) Australian lineages of HA, NA and nucleoprotein (NP) sequences, were estimated
694 using the Bayesian Markov chain Monte Carlo method available in BEAST 1.10.4 [80].
695 Prior to the BEAST analysis ML trees were used to determine the degree of clock-like
696 behaviour of each data set by performing linear regressions of root-to-tip distances against
697 year of sampling using TempEst [81]. All data sets exhibited a strong positive correlation
698 between genetic divergence and sampling time, with correlation coefficients ranging from
699 0.8-0.99 and R^2 values ranging from 0.66-0.99. Using BEAST, time-stamped data were
700 analysed under the uncorrelated lognormal relaxed molecular clock [82] and the SRD06
701 codon-structured nucleotide substitution model [83]. We selected the uncorrelated
702 lognormal relaxed clock following comparisons of the marginal likelihood of the strict and
703 uncorrelated lognormal relaxed molecular clocks for a subset of trees (H4, H5, N6, N8,
704 and two NP lineages) using path/stepping-stone sampling [84]. The Bayesian skyline
705 coalescent tree prior was used as this likely reflects the complex epidemiological dynamics
706 of AIV [85]. Three independent analyses of 100 million generations were performed, which
707 were then combined in LogCombiner v1.8 following the removal of a 10% burn-in.
708 Convergence was assessed using Tracer v1.6 (<http://tree.bio.ed.ac.uk/software/tracer/>).
709 Maximum credibility lineage trees were generated using TreeAnnotator v1.8 and visualized
710 in FigTree v1.4.

711

712 *AIV phylogeography*

713 We selected the HA, NA and NP internal segments as representatives to investigate the
714 phylogeography of AIVs in Australia. Importantly, the selected HA or NA subtypes
715 comprised Australia-specific lineages with >20 sequences (containing sequences from no
716 other continent). For the NP segment, we selected two lineages that comprised only
717 sequences from Australia. Discrete trait analysis was performed using the asymmetric
718 substitution model, and social networks were inferred with Bayesian Stochastic Search

719 Variable Selection (BSSVS) [86]. The extent and pattern of virus movement between
720 locations were determined using Bayes Factor analysis generated by Spread3 [87]. We
721 considered Bayes Factors of greater than 10 to be strong support of virus movement
722 between the locations sampled, and greater than 100 to be decisive support [88, 89] within
723 the necessary constraints imposed by sampling bias. The mean number of migration
724 events were inferred by logging/counting the transitions between states along the
725 phylogenetic branches (Markov Jumps) [90]. We also calculated the time spent in the
726 states between two transitions (Markov Rewards) to ensure that rewards were not strongly
727 correlated with export events, thus providing some insight into the effect of sampling bias
728 in our dataset.

729

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760
761

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1024

1025 **Figure Captions**

1026

1027 **Figure 1. Spatial and temporal distribution of avian influenza genomes used in this**
1028 **study.** (A) Map of Australia illustrating regional sampling locations. Where sampling
1029 locations were within 500km, they were merged into a single location. The value within the
1030 circle corresponds to the number of unique viral genomes comprising at least one segment
1031 from each location. States and Territories are as follows: VIC Victoria, NSW New South
1032 Wales, QLD Queensland, NT Northern Territory, WA Western Australia, SA South
1033 Australia and TAS Tasmania. (B) Number of genomes per state per year. Colours from
1034 panel B correspond to the fill colour of the state in panel A. This figure includes genomes
1035 comprising one or more segments and contains no duplicates. This figure includes all
1036 sequences generated as part of the National Avian Influenza Wild Bird Surveillance
1037 Program, including those recently published in [40, 41]. Metadata is available in Table S1
1038 and a detailed plot illustrating exact virus sample collection dates and locations can be
1039 found in Fig S2.

1040

1041 **Figure 2. HA-NA subtype linkage in data generated for this study.** (A) The number of
1042 each HA-NA subtype combinations (values) and the proportion of the total data set these
1043 values represent (shading). (B) A plot of the Pearson residuals of Chi-squared tests. For a
1044 given cell, the size of the circle is proportional to the amount of the cell contribution.
1045 Positive residuals are in blue and identify HA and NA subtypes for which there is a strong
1046 positive association in the data set. Negative residuals are in light pink and show a weak
1047 negative association, that is, they are underrepresented in the data set. This figure
1048 comprises unique viral genomes with at least one segment.

1049

1050 **Figure 3. Phylogenetic trees of subtypes H4 and H11.** (A, C) Phylogenetic trees
1051 comprising global diversity. Branches of reference sequences are coloured by continent.
1052 Sequences from Australia are coloured in pink (2005 and later) and in purple (pre-2005),
1053 with 2005 marking the year of the oldest sequence in the data set generated in this study.
1054 (B, D) Time structured phylogenetic trees. The trees comprise Australian lineages (as
1055 indicated by grey boxes in A, C) and closest relatives (retrieved by BLAST searches).
1056 Branches are coloured by year and geography as above. Branch labels correspond to
1057 posterior clade probabilities of each node, node bars correspond to the 95% HPD of node
1058 height. We selected H4 as it is the only HA subtype for which there is clear introduction of

1059 an Australian lineage virus into Asia (indicated in square parenthesis), and is an example
1060 of an HA segment for which there is only a single contemporary lineage. We selected H11
1061 as it is the subtype with the largest number of contemporary Australian lineages (4), of
1062 which 2 are represented by a single sequence. Trees for all other HA subtypes can be
1063 found in Fig S3-S14

1064

1065 **Figure 4. The time-scale of AIV evolution by subtype in Australia.** (A) A schematic
1066 phylogeny demonstrating the differences between the tMRCA of Australian lineages and
1067 the dates of divergence from reference sequences. (B) The tMRCA distribution of
1068 contemporary Australian lineages of the HA segments and (C) dates of divergence from
1069 the reference sequences of all HA lineages. Points represent the node date and bars the
1070 95% HPD. For segments with multiple lineages, multiple estimates have been provided.
1071 Where a novel introduction is represented by a single sequence the tMRCA was not
1072 estimated (here, represented by “*singleton) but the date of divergence from reference
1073 sequences is shown. For H7, we did not estimate the date of divergence from the closest
1074 reference sequences. (D) The time difference between the tMRCA of the Australian
1075 lineages and the date of separation for all HA and NA segments and lineages. Lineages
1076 with time differences of more than 10 years are labelled. All HA and NA trees are
1077 presented in Fig 3, Fig S3-S23.

1078

1079 **Figure 5. Viruses sequenced in this study that have signatures of recent**
1080 **introduction.** For each segment, coloured tiles correspond to three different statuses:
1081 singletons, first detections and well-established lineages. Singletons represent the only
1082 detections of the lineage in Australia. In cases where two viruses from the same sampling
1083 effort were identical and were the only detections of that lineage, they were still considered
1084 a singleton (e.g. the NA segment of the NT/2015 viruses or the NA segment of the 8
1085 H12N2 Ruddy Turnstone viruses from 2019, which have only ever been detected during
1086 that sampling event). Only the A allele NS segment was detected in these viruses.
1087 Phylogenetic examples (here excerpts from N8 and N5) are provided for each status, and
1088 branches are coloured as in Fig 3.

1089

1090 **Figure 6. Inferred migration events of avian influenza viruses within Australia.**

1091 Locations included in each tree are marked by a white circle. Specific location names are
1092 presented in Figure 1A, and all state names are presented in the first panel and are as
1093 follows: VIC Victoria, NSW New South Wales, QLD Queensland, NT Northern Territory,

1094 WA Western Australia, SA South Australia and TAS Tasmania. Grey lines correspond
1095 potential migration events that were not detected in the analysis (i.e. migration event <1).
1096 Blue lines indicate migration events are derived from calculations of state changes
1097 (Markov Jumps), ranging from light to dark. Arrows indicate the direction of the migration
1098 event. As NP has more than a single discrete Australian lineage, we have generated two
1099 independent maps reflecting the 2 largest Australian lineages of NP (Fig S25). Maps
1100 illustrating Bayes Factors, also generated using BSSVS can be found in Fig S26, and
1101 Markov rewards also generated in this analysis are presented in Fig S27.

1102

1103 **Figure 7. Genome constellations of (A) H5 and (B) H7 viruses.** The phylogenies
1104 presented are time-scaled Maximum Clade Credibility Trees. Tips are coloured according
1105 to Australian state or territory. Scale bar denotes the year of sample collection. Node bars
1106 are the 95% HPD of node height, and posterior clade probability is presented on each
1107 branch. Adjacent to each tree are coloured tiles where each column of tiles refers to a
1108 segment, arranged according to size: PB2, PB1, PA, HA, NP, M, NS. We only included
1109 tiles for viruses sequenced in this study and in cases where the tiles are blank, no
1110 sequence was available for the segment. Different colours refer to different lineages,
1111 whereby tile colour scheme is retained for both H5 and H7 trees. For example, for the NS
1112 segment, viruses with an NS B lineage are coloured in orange. The viruses here fall into
1113 five different lineage clusters of NS A, and these are presented in two different shades of
1114 blue and green and pink. If a virus is a “mixed” infection, segments with two different
1115 lineages or subtypes are split to illustrate this.

1116

1117 **Supporting information captions**

- 1118 Figure S1. The effect of Ct value of original samples on sequencing success
- 1119 Figure S2. Temporal distribution of sampling dates.
- 1120 Figure S3. Phylogeny of H1
- 1121 Figure S4. Phylogeny of H2
- 1122 Figure S5. Phylogeny of H3
- 1123 Figure S6. Phylogeny of H5
- 1124 Figure S7. Phylogeny of H6
- 1125 Figure S8. Phylogeny of H7
- 1126 Figure S9. Phylogeny of H8
- 1127 Figure S10. Phylogeny of H9
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- 1129 Figure S12. Phylogeny of H12
- 1130 Figure S13. Phylogeny of H13
- 1131 Figure S14. Phylogeny of H16
- 1132 Figure S15. Phylogeny of N1
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