Detection and spread of high pathogenicity avian influenza virus H5N1 in the Antarctic Region

Running title: HPAIV in the Antarctic

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Abstract

The Antarctic is the only major geographical region in which high pathogenicity avian influenza virus (HPAIV) has never previously been detected. The current panzootic of H5N1 HPAIV has decimated wild bird populations across Europe, North America and South America. Here we report on the emergence of clade 2.3.4.4b H5N1 HPAIV in the Antarctic and sub-Antarctic regions of South Georgia and the Falkland Islands respectively. We initially detected H5N1 HPAIV in samples collected from brown skuas at Bird Island, South Georgia on 8th October 2023. Since this detection, increased mortalities were observed in brown skuas, kelp gulls, elephant seals and fur seal at multiple sites across South Georgia. We confirmed H5N1 HPAIV in multiple brown skuas and kelp gulls across four different sampling locations in South Georgia. Simultaneously, we also confirmed H5N1 HPAIV in a southern fulmar in the Falkland Islands. Genetic assessment of the virus indicates spread from South America, likely through movement of migratory birds. Here we describe the emergence, species impact and genetic composition of the virus and propose both introductory routes and potential long-term impact on avian and mammalian species across the Antarctic region.

Introduction

Following the emergence and global expansion of A/goose/Guangdong/1/96 (GsGd)-lineage H5 high pathogenicity avian influenza viruses (HPAIV) there have been repeat epizootics in wild birds and poultry populations globally. In the autumn of 2021, the situation escalated considerably with the detection of clade 2.3.4.4b HPAIV subtype H5N1 in Europe. Subsequently, two unprecedented epizootic waves in 2021/22 and 2022/23 with this lineage were associated with mass mortality events in wild birds together with thousands of incursions into poultry[1, 2, 3, 4, 5]. High levels of viral adaptation to wild bird species[6], and increased fitness advantage through continued genetic reassortment[7] likely underpin the broad impact infection has had across many avian species[2]. This wide host range has facilitated the transmission of the lineage across a large geographic area, including from Europe to North America[8, 9], where it has since rapidly expanded its range southward into South America via migratory avian species. Incursion into South American countries, starting in November of 2022, represented the first recorded instances of GsGd-lineage H5 HPAIV in the region[10, 11, 12]. Mass mortality events in the region have been particularly severe and reported across several different bird species in addition to marine mammals[2, 10, 13], highlighting the extensive ecological impact of HPAIV and the ongoing threat it presents to naïve hosts.

The Antarctic region includes the ice shelves, waters, and all the island territories in the Southern Ocean situated inside of the Antarctic Convergence, a marine belt encircling Antarctica where Antarctic waters meet those of the warmer sub-Antarctic. Several islands are located inside the Antarctic region, including South Georgia, while the Falkland Islands among others are located outside the Antarctic Convergence in the sub-Antarctic zone. There have been no previous reports of HPAIV inside the Antarctic region[14]. Antarctica and the sub-Antarctic islands possess unique ecosystems which support the population strongholds of several avian and marine mammal species. The relative isolation of these islands from human populations has provided species across the Antarctic with only limited protection from anthropogenic environmental change[15]. Indeed, wildlife populations in the Antarctic face a broad range of challenges from introduced species[16], to longline fisheries[17, 18], and rapid climate change[19, 20, 21]. Several resident bird species including wandering

albatross (*Diomedea exulans*), macaroni penguins (*Eudyptes chrysolophus*), grey-headed albatross (*Thalassarche chrysostoma*), and white-chinned petrel (*Procellaria aequinoctialis*), are listed as either vulnerable or endangered[22]. Iconic long-lived species with late maturity, such as albatross, exhibit low resilience to rapid increases in population mortality[23]. High mortality disease outbreaks therefore represent a substantial threat to already vulnerable seabird populations[24, 25].

While geographically isolated, several Antarctic seabird species routinely range between the South Atlantic and Southern Ocean, visiting the South American coast to either forage or overwinter[26]. Brown skuas (*Stercorarius antarcticus*), kelp gulls (*Larus dominicanus*), southern giant petrel (*Macronectes giganteus*), and snowy sheathbills (*Chionis albus*) have previously been identified as potential vectors of infectious pathogens into this vulnerable ecosystem due to their migratory traits, scavenging behaviour, and previously identified roles as carriers of low pathogenicity avian influenza viruses (LPAIV)[27, 28, 29, 30, 31, 32, 33, 34]. Evidence of low pathogenicity avian influenza virus (LPAIV) in the Antarctic region was first detected from serological studies in the 1980s[35, 36, 37]. A range of subtypes have since been reported (H1[37], H3[28], H4[32], H5[38], H6[29], H7[36], H9[28], and H11[30]) including genetic analysis of H4N7[32], H5N5[30, 38], H6N8[29] and H11N2[30, 39, 40] influenza viruses. In contrast to the more prevalent H11N2 viruses, H4-H6 subtypes were found to share high sequence similarity with viruses from continental America, indicating more recent introduction events[29, 30, 32]. Evidence of likely LPAIV transmission to the continent from the Americas demonstrates the high-risk of clade 2.3.4.4b H5 HPAIV introduction to the Antarctic, encouraging researchers in 2022 to employ additional biosecurity measures while maintaining surveillance activities[27, 41]. During the austral summer of 2022/23, sampling and surveillance was conducted at several sites in the Antarctic region, and as of March 2023, HPAIV had not been detected[14].

Here we describe the observation of morbidity and mortality events across different species as well as the positive detection of H5N1 HPAIV in a variety of species in South Georgia, inside the Antarctic region and the sub-Antarctic Falkland Islands. We detail the suspicion, emergence, diagnostic evaluation and clinical presentations of HPAIV in the region. Genetic analysis is used to characterise potential introduction routes and the consequences of HPAIV circulation in this region are considered.

Results

Case description

On September 17th, researchers of the British Antarctic Survey (BAS) on Bird Island, South Georgia, (Figure 1A) discovered a single southern giant petrel showing behaviours indicative of loss of coordination, neurological twitching, and lethargy. This individual was observed being predated and scavenged upon by brown skuas and other southern giant petrels. On 8th October, brown skuas were observed in the same locality showing lethargy, neck spasms, twitching, and an inability to fly, and by 10th October, these individuals had died (Figure 1B). In the following days bird mortality was seen on Bird Island, South Georgia, with the highest number of mortalities occurring at the roosting site of non-breeding birds. Swab samples were collected from the three brown skua (*Stercorarius antarcticus*) on 8th October 2023 which were later found dead on Bird Island, South Georgia (Figure 1B). Mortalities increased rapidly in brown skuas, with ten birds recorded dead by 15th October 2023 and a further twenty birds by 20th October. Further escalation in mortality occurred by 17th November 2023, when 57 skuas were observed to have died at Bird Island, and close monitoring continues.

On 30th October, swab samples were collected from six found dead kelp gull and four found dead brown skua from Hound Bay, South Georgia in addition to four found dead brown skua from St Andrews, South Georgia (Figure 1B). On 31st of October, swab samples were collected from six kelp gulls, and six brown skuas which were found dead in Moltke Harbour, South Georgia. On 3rd November, samples were collected from six found dead kelp gulls and two brown skuas on Harpon, South Georgia (Figure 1B). Alongside avian species, clinical disease consistent with mammalian infection with HPAIV was observed in colonies of elephant seals. Clinical presentation included difficulty breathing, with coughing and short sharp breath intake. Individuals also showed accumulation of viscous fluid around the nasal passage. Swab samples were collected from seven recently deceased southern elephant seals (*Mirounga leonine*) on 31st October from Moltke Harbour, South Georgia (Figure 1B).

Concurrent to the events emerging on South Georgia, on 30th October, a southern fulmar (*Fulmarus glacialoides*) was reported dead in Stanley, Falkland Islands and swab samples were

collected (Figure 1A). Over the next few days mortality was seen in other avian species in Stanley, and samples were collected from a Grey-backed storm petrel (*Garrodia nereis*) and Falkland steamer duck (*Tachyeres brachypterus*) on 6th November.

Virology and detection

Provision for diagnostic investigation of avian influenza is limited in the region. Local molecular testing at the KEMH Pathology and Food, Water & Environmental Laboratory on the Falkland Islands indicated the presence of avian influenza A H5N1 viral RNA (vRNA) in a Southern Fulmar. This detection, alongside the increase clinical disease and mortalities observed on South Georgia triggered shipment of samples to the International Reference Laboratory for avian influenza, swine influenza and Newcastle disease virus at the Animal and Plant Health Agency (APHA), Weybridge, UK for confirmatory and further diagnostic evaluation. All six oropharyngeal (OP) and cloacal (C) swabs from the three brown skuas from South Georgia collected on 8th October were positive by each of the generic AIV, HPAIV H5 detection and N1-specific rRT-PCR assays (Supplementary Table 1), signifying the presence of HPAIV H5N1 in all three birds. However, infectious virus could not be isolated from any of these samples. Eighteen additional OP and C swab samples from brown skuas (n=12) and kelp gulls (n=6) sampled from four sites across South Georgia between 30th October and 3rd of November also all tested positive for the presence of H5N1 HPAIV vRNA (Supplementary Table 1). Infectious virus was successfully isolated from four birds, including from one kelp gull and two brown skuas from Hound Bay and one kelp gull at Harpon Bay. Nasal and rectal swabs from three elephant seals collected on Moltke Harbour on 3rd November were negative in each assay, and no infectious virus could be isolated from these animals. Within the same period samples collected from a southern fulmar on 30th October in the Falkland Islands tested positive for HPAIV H5N1 vRNA, while a Grey-backed storm petrel and Falkland steamer duck tested negative in each assay.

Genomic and phylogenetic analysis

Three full genome sequences were generated from the initial OP swab samples of the three brown skuas from Bird Island collected on 8th October 2023. Comparison of the three sequences revealed

that they shared 99.86-100% nucleotide identity across all eight influenza viral gene segments. In addition, a single sequence was also generated from the Southern Fulmar collected from the Falkland Islands on 30th October 2023. Comparison of the sequences obtained from South Georgia and the Falkland Islands found that they shared greater than 98.98% nucleotide identity across all gene segments. The Bird Island and Falkland Islands sequences were then combined with representative global H5N1 clade 2.3.4.4b full-genome sequences to assess genetic ancestry (Supplementary Figure 1). The sequences from Bird Island clustered with those of viruses collected from South America, between December 2022 and April 2023, particularly Uruguay, Peru and Chile, across all gene segments. The sequences from Bird Island and the Falkland Islands were genotyped according to the United States H5N1 schema, given the spread of these viruses from North to South America in early 2022[10], and found to be part of the B3.2 genotype[42]. The B3.2 genotype arose in early 2022 in North Dakota as a reassortant formed by the original H5N1 that was transmitted from Europe to North America in late 2021[8] and then obtained gene segments (PB2, PB1, NP and NS) from local North American AIVs[42]. This genotype was reported to have been introduced into South America four times between October 2022 and March 2023[10], and analysis of all publicly available full-genome sequences from South America found that 94% (131 of 140) of H5N1 belonged to this genotype.

To further investigate the introduction of H5N1 HPAIV into South Georgia and the Falkland Islands, representative H5N1 clade 2.3.4.4b HA sequences from North and South America were used to perform time-resolved phylogenetic analysis (Figure 2). This analysis demonstrated distinct, separate introductions of H5N1 into South Georgia and the Falkland Islands, with both sets of sequences sharing a common ancestor with sequences from South America dating between late November 2022 (Falkland Islands) and late January 2023 (South Georgia). However, both sets of sequences produced long branch lengths compared to South American sequences. To further investigate the source of these viruses, discrete trait analysis based upon the country of origin was performed (Supplementary Figure 2), which suggested that the source of HPAIV for both South Georgia and the Falkland Islands was Chile.

Discussion

Since the emergence and global expansion of Gs/Gd-lineage H5Nx HPAIV in 1996, Antarctica and Oceania are the only two continents in which it has not been detected. Moreover, until now, Antarctica remains the only major geographical region in which HPAIV had never been detected.

The island of South Georgia lies in the Southern Ocean inside the Antarctic convergence, a marine belt encircling Antarctica which defines the Antarctic Region. The island is an area of high biodiversity and high conservation priority with multiple species being considered as vulnerable to the incursion of infectious diseases[43, 44, 45]. The Falkland Islands constitute a remote cluster of islands in the South Atlantic Ocean situated approximately 1500km to the west of South Georgia. The Falkland Islands are situated outside of the Antarctic convergence, in the sub-Antarctic region. Both the Falkland Islands archipelago and South Georgia represent key areas that are host to significant avian biodiversity and the presence of HPAIV on these islands represents a significant risk to the populations of susceptible bird species. South Georgia is home to approximately 29 species which breed on the islands and is recognised as an 'Important Bird Area' by Birdlife International[46]. Therefore, any colony or population that comes under threat from an HPAIV outbreak on South Georgia may have direct impact upon the wider population of seabirds. Despite seabird colonies showing space partitioning between colonies[47], there is often a high degree of connectivity between colonies. Often this is due to the movement of nonbreeders or juvenile birds[48]. It is therefore, not unreasonable to suspect that birds on South Georgia may show high connectivity, which may aid the spread of disease, as has been documented previously[33], but also may be evidenced by the rapid collection of samples from different areas within South Georgia. Indeed, in the northern hemisphere it has been found that northern gannets (*Morus bassanus*) increased their connectivity due to high levels of colony prospecting from surviving birds[49].

This connectivity and the interlinkages between avian and mammalian species in a 'single ecosystem' having been identified across the Antarctic region means that the virus may be readily spread across the region. Circumpolar and trans-Pacific migrants such as Gray-headed albatross (*Thalassarche chrysostoma*)[50], White-chinned petrel (*Procellaria aequincotialis*)[51], Northern and

202 southern giant petrels may facilitate this spread. Indeed, phylogeographic analysis has suggested a
203 dynamic geneflow between southern Atlantic populations and Macquarie island[52], and as such the
204 threat of transmission to New Zealand and Australasia must be considered.

205 From a mammalian infection standpoint there have been several reports globally of wild
206 aquatic mammals, including seals, being infected with H5Nx HPAIV since 2020, where infection has
207 been attributed to the predation of sick or dead infected birds[10, 53, 54]. Information to date suggests that
208 HPAIV infection in seals often leads to a neurological presentation with infrequent detection of viral
209 material being detected through standard swab sampling activities[53]. This may explain the lack of
210 influenza A vRNA detection in elephant seal swab samples taken from this study, despite the
211 consistency of clinical presentation seen in elephant seals with that reported elsewhere. Certainly, the
212 timing of mortality and clinical signs exhibited by elephant seals are consistent with HPAIV infection.
213 Unfortunately, invasive sampling was prohibited in this study due to a lack of personal and respiratory
214 protective equipment to safely undertake such sampling and invasive sampling of avian and
215 mammalian species remains challenging to undertake in areas where appropriate facilities are lacking.

216 A further conundrum that will likely significantly impact upon the course of infection and
217 onward spread of viral infection across the region is the limited options for carcass disposal and
218 environmental clean-up. The Antarctic region is one of the most remote environments on earth and is
219 the location of enormous breeding colonies of various avian species that may be susceptible, and
220 succumb, to infection with HPAIV. Where mortality events occur, the opportunity for scavenging
221 animals exists to predate upon carcasses and become infected. Carcass removal is not an option.
222 Further the potential for virus survival in this cold environment is increased and it may be that
223 infectious virus remains for longer periods in carcasses preserved by the local climate. Local ecology
224 of species could also influence the scale of impact throughout Antarctica. Although all species remain
225 vulnerable to large scale infection events, it is possible that the density of animals may preclude some
226 species from rapid spread[55]. For example, wandering albatross nest at low density (approximately
227 0.0022 nests per m^2)[56], which could limit spread between breeding individuals. However, non-
228 breeding birds congregate in groups to display and dance[57] which may provide opportunities for
229 disease spread. Similar ecological considerations must be made when considering burrow nesting

230 species (such as white-chinned petrel, diving petrel, and prion species), which nest in separated
231 burrow systems and may limit spread. Penguins are also susceptible to HPAIV, and mortality has
232 been observed following infection[58]. Penguin species nest in high densities (dependent upon species
233 ranging between 0.25 - 1.7 nests per m^2)[59, 60], and if HPAIV does enter penguin colonies, it could
234 show rapid infection and spread. If the virus does start to cause mass mortality events across penguin
235 colonies, it could signal one of the largest ecological disasters of modern times. Activities within the
236 region are ongoing to track mortality events and autonomous authorities are on high alert to signal the
237 potential for incursions across the broader area.
238 Genomic analysis of the sequences obtained from South Georgia and the Falkland Islands
239 suggested separate, distinct introductions of the B3.2 HPAIV genotype into the two locations. The
240 B3.2 genotype emerged in early 2022 in the midwestern United States of America as a reassortant
241 formed following coinfection with the original H5N1 that was transmitted from Europe to North
242 America in late 2021[8] with a North American virus from which the novel genotype emerged,
243 containing the PB2, PB1, NP and NS gene segments of the North American AIVs[42]. This genotype
244 has been demonstrated to have been introduced into South America four times between October 2022
245 and March 2023[10]. Analysis of all available full-genome sequences from South America demonstrated
246 that 94% (131 of 140) of H5N1 HPAIV sequences corresponded to this genotype. Given the close
247 geographical proximity of South Georgia and the Falkland Islands to South America, and that wild
248 bird species are known to migrate between the mainland and these islands, it is not surprising that the
249 B3.2 detected as the cause of the disease events. The phylogenetic analyses undertaken demonstrated
250 that the viruses detected in South Georgia and the Falkland Islands shared common ancestors with
251 those detected in mainland South America from late 2022 to early 2023. However, the long branch
252 lengths observed across all gene segments suggest unsampled evolutionary ancestry. There are also
253 only a limited number of sequences deposited in public databases from H5N1 HPAIV detections in
254 South America during summer 2023. Taken together, this highlights the importance of real-time
255 global data sharing as a key tool in understanding the emergence and spread of these viruses. The
256 current lack of publicly available data precludes a conclusive assessment of potential incursion routes

substantially more difficult. Multiple disciplines globally continue to monitor the situation in Antarctica to see whether fears of ecological disaster in the region will be realised.

Methods

Virological detection

On the Falkland Islands, initial diagnostic assessment of samples was undertaken at the KEMH Pathology and Food, Water & Environmental Laboratory utilising the QIAamp Viral RNA Mini Kit (Qiagen) and the Oasig OneStep RT-qPCR kit for H5N1 (Genesig). A preliminary diagnosis was made of avian influenza H5N1 infection. Following reports of increasing mortalities and the observation of disease consistent with HPAIV infection in avian and mammalian species in South Georgia, oropharyngeal (OP) and cloacal (C) swabs collected from birds were submitted to the Animal and Plant Health Agency (APHA)-Weybridge for laboratory virological investigation. Total nucleic acid was extracted from all samples[61] for testing by a suite of three AIV real-time reverse transcription polymerase chain reaction (rRT-PCR) assays consisting of the Matrix (M)-gene assay for generic influenza A virus detection[62]; an assay for specific detection of HPAIV H5 clade 2.3.4.4b[61] and an N1-specific rRT-PCR to confirm the neuraminidase type[63]. A positive result was denoted in each case by a Cq value ≤36.0. The samples were also screened for avian paramyxovirus type 1 (APMV-1) by an rRT–PCR assay targeting the large polymerase (L) gene[64] where a positive result was denoted by a Cq value ≤37.0. All amplifications were carried out in an AriaMx qPCR System (Agilent, United Kingdom). The OP swabs and the C swabs were separately pooled for attempted virus isolation in 9- to 11-day-old specific pathogen-free (SPF) embryonated fowls' eggs (EFEs) according to the internationally recognised methods[65].

Whole-Genome Sequencing and Phylogenetic Analysis

For whole-genome sequence analysis, the extracted vRNA was converted to double-stranded cDNA and amplified using a one-step RT-PCR using SuperScript III One-Step RT-PCR kit (Thermo Fisher Scientific). The primers used were as follows: Optil-F1 5'-TTACGCGCCAGCAAAAGCAG-3',

Optil-F2 5'-GTTACGCGCCAGCGAAAGCAGG-3' and Optil-R1 5'-GTTACGCGCCAGTAGAAACAAG-3' that have been previously described[66, 67]. PCR products were purified with Agencourt AMPure XP beads (Beckman Coultrer) prior to sequencing library preparation using the Native Barcoding Kit (Oxford Nanopore Technologies) and sequenced using a GridION Mk1 (Oxford Nanopore Technologies) according to manufacturer's instructions. Assembly of the influenza A viral genomes was performed using a custom in-house pipeline as described previously[7] but adapted for nanopore sequence reads. All influenza sequences generated and used in this study are available through the GISAID EpiFlu Database (https://www.gisaid.org). All H5N1 HPAIV clade 2.3.4.4b sequences available in the EpiFlu database between 1st September 2020 and 27th October 2-23 were downloaded to create a sequence dataset. As North America and Europe were over-represented in this dataset, these were sub-sampled to maintain representative sequences using PARNAS[68]. The remaining dataset was separated by segment and aligned using Mafft v7.520[69], and manual trimmed to the open-reading frame using Aliviewversion 1.26[70] The trimmed alignments were then used to a infer maximum-likelihood phylogenetic tree using IQ-Tree version 2.2.3[71] along with ModelFinder[7] and 1,000 ultrafast bootstraps[72] For the time-resolved and mugration analysis, all HA sequences available from South America, and representative from North America were combined with the sequences from Bird Island and the Falkland Islands and used to infer a maximum-likelihood phylogenetic tree as described above. The resulting tree was then used for ancestral sequence reconstruction and inference of molecular-clock phylogenies using TreeTime[73]. Phylogenetic reconstruction with discrete trait analysis of the country of origin using the mugration model, was also performed in TreeTime using the default settings. Phylogenetic trees were visualised as described previously[72] or using FigTree v1.4.4. Nucleotide identity between sequences was determined as described previously[72]. Sequences were genotyped according to the USDA schema, using the GenoFLU tool (https://github.com/USDA-VS/GenoFLU)[42].

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Author contributions

Conceptualisation: ACB, JJ, AB, EMF, ZF; formal analysis: AB, JJ, SMR, KF, ACB, AMPB; investigation: SMR, JLJ, DdS, FB, MB, RH, AMPB, JPD, BM; resources: ACB, JJ, IHB, ZF, EMF; writing—original draft, ACB, JJ, AMPB, SR; writing—review and editing: ACB, JJ, APMB, AB, ZF, EMF, SMR, IHB. All authors have read and agreed to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Additional information

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Figure Legends

Figure 1. Geographical distribution of H5N1 HPAIV detections from South Georgia. (A) Map showing the locations of South America (blue), the Falkland Islands (red), South Georgia (green) and the Antarctic peninsula (yellow). **(B)** Map showing the location, species, and date of sampling of avian and mammalian species in South Georgia.

Figure 2. H5N1 HPAIV transmission from the South American continent to Falkland Islands and South Georgia. (A) Representative haemagglutinin (HA) sequences H5N1 HPAIVs from North and South America were combined with those obtained from South Georgia and the Falkland Islands and used to generate a time-resolved maximum-likelihood phylogenetic tree and coloured according to origin location.

Supplementary Figure 1. Representative global H5N1 HPAIV sequences collected since 1st September 2020 were combined with the sequences generated from samples collected from South Georgia and the Falkland Islands and used to infer maximum likelihood phylogenetic trees. Tips are coloured according to region of origin, and the sequences from South Georgia and the Falkland Islands are indicated.

Supplementary Figure 2. Discrete trait analysis of representative H5N1 clade 2.3.4.4b from North and South America. Branches are coloured according to the inferred source country.

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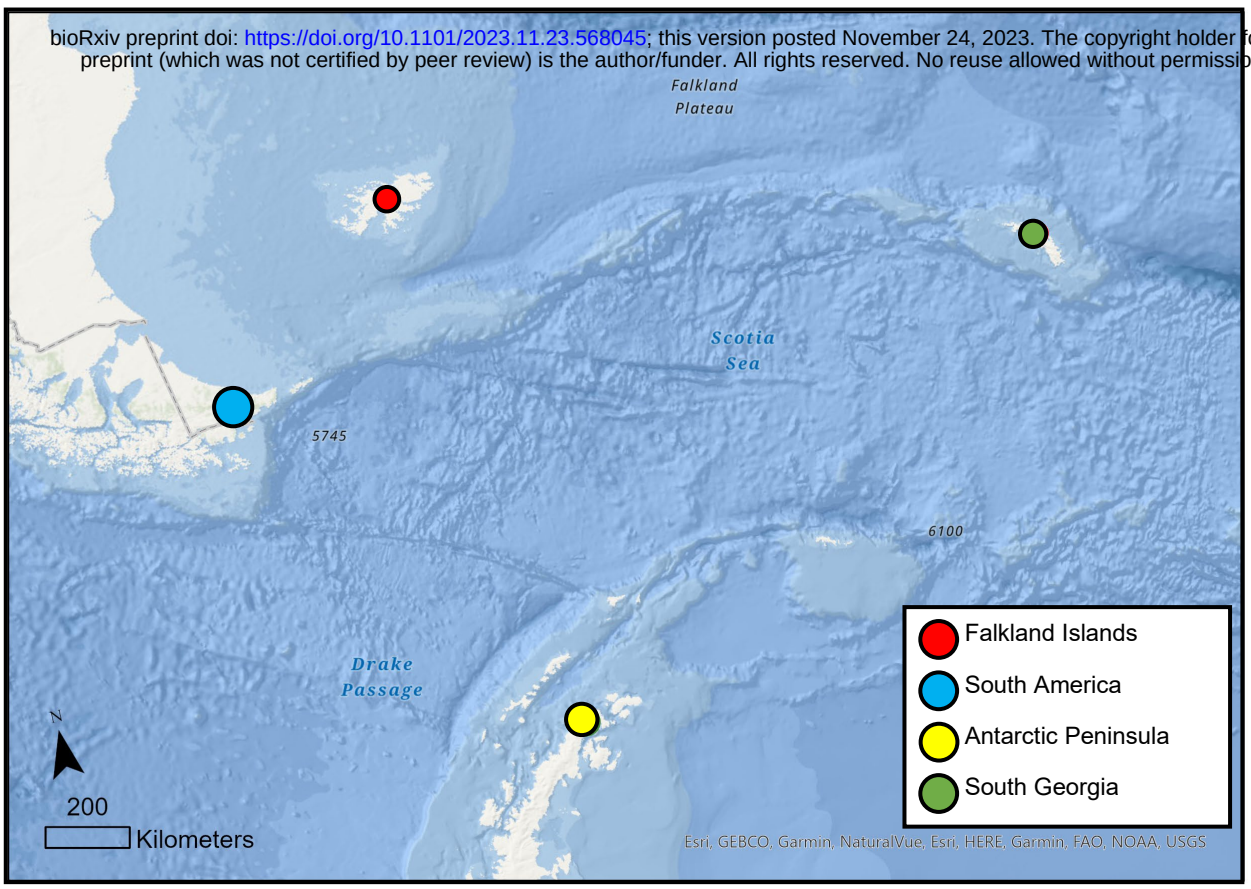
Figure 1

A



B

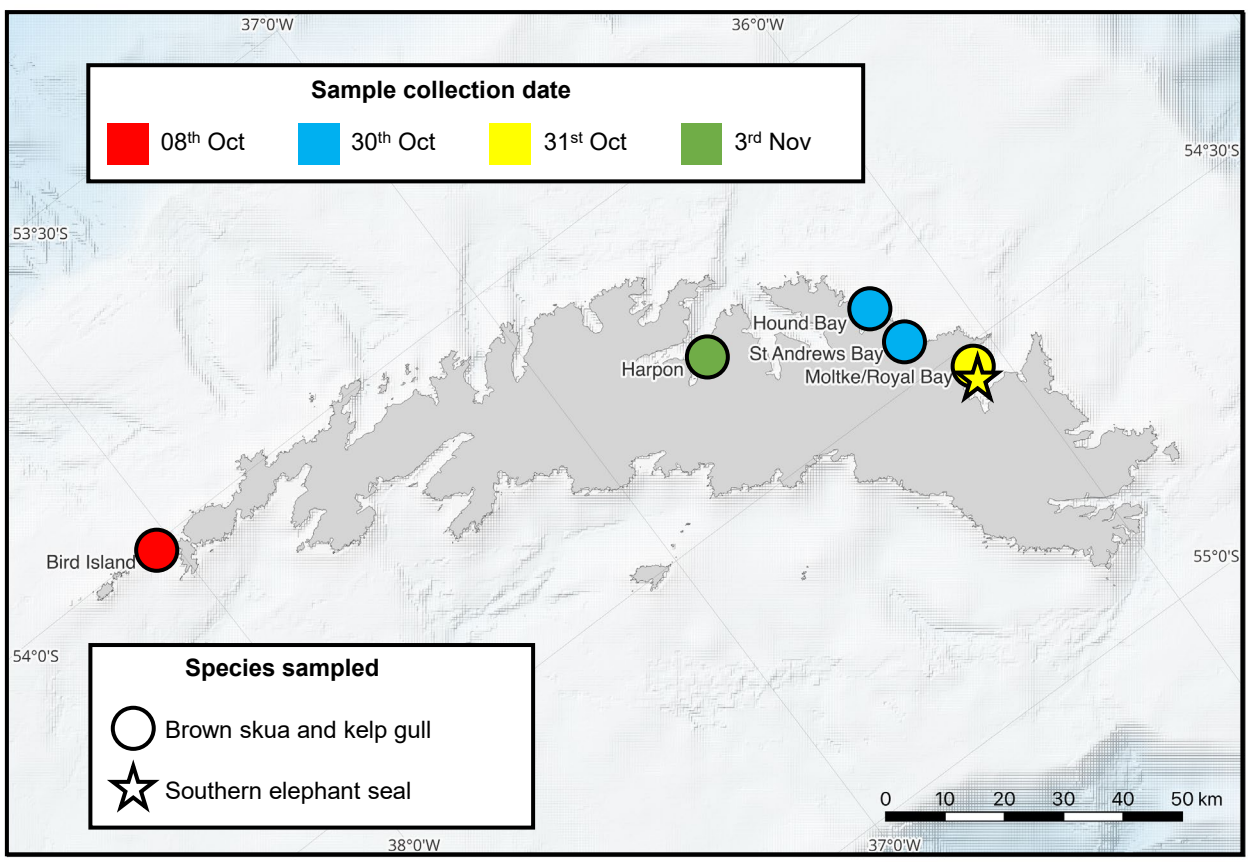
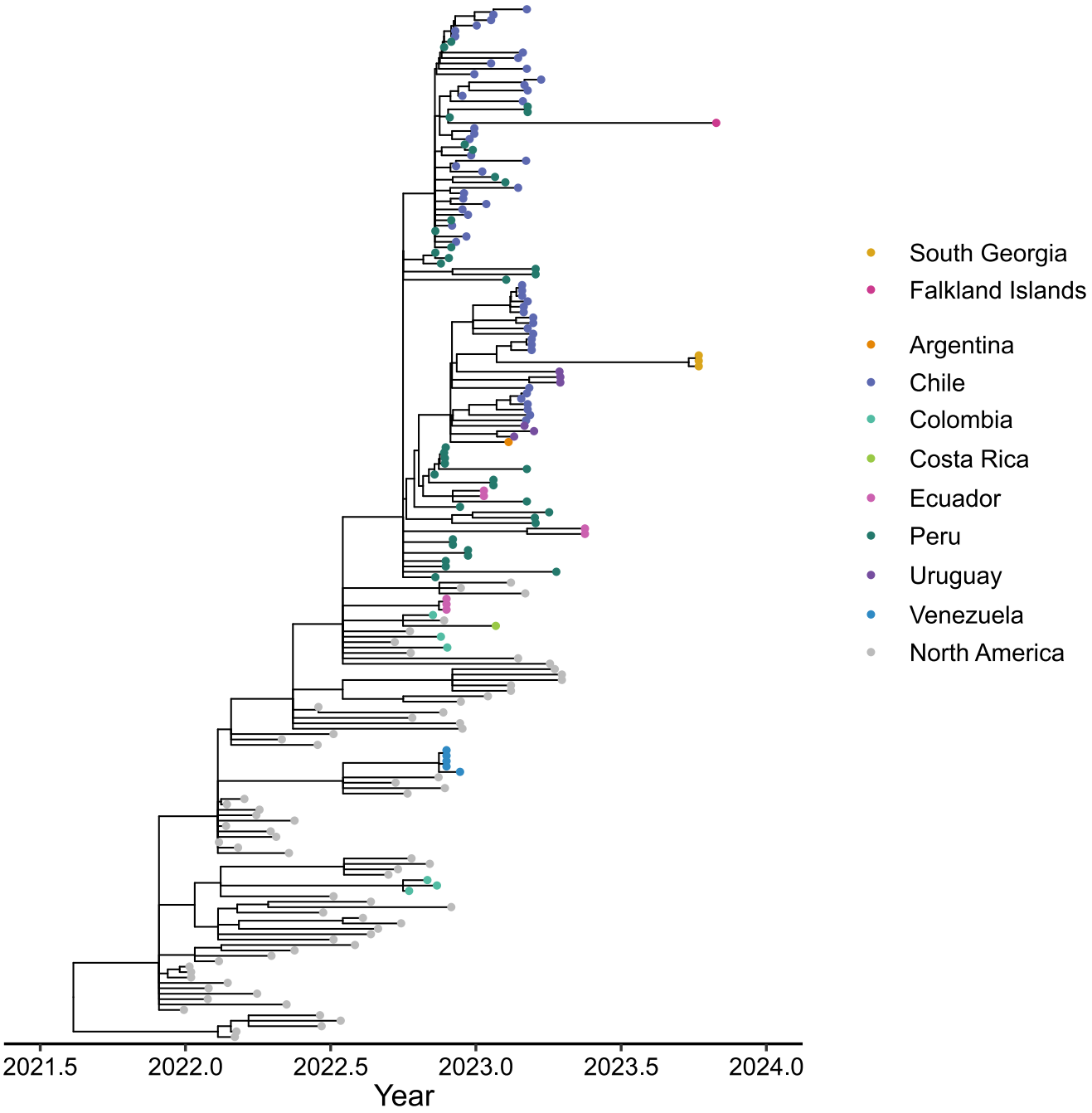


Figure 2


South Georgia
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Argentina
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