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Spatial spread and emergence of reassortant H5 highly pathogenic avian influenza viruses in Iran

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ABSTRACT

Since 2005, H5Nx highly pathogenic avian influenza (HPAI) viruses of the Goose/Guangdong (Gs/GD) lineage have spread worldwide, affecting poultry and wild birds in Asia, Europe, Africa and North America. So far, the role of Western Asia and the Middle East in the diffusion dynamics of this virus has been poorly explored. In order to investigate the genetic diversity and the role of Iran in the transmission dynamics of the Gs/GD lineage, we sequenced the complete genome of twenty-eight H5Nx viruses which were circulating in the country between 2016 and 2018. We reported the first characterization of the HPAI H5N6 subtype of clade 2.3.4.4B in Iran and gave evidence of the high propensity of the Gs/GD H5 AIVs to reassort, describing six novel H5N8 genotypes of clade 2.3.4.4B, some of them likely generated in this area, and one H5N1 reassortant virus of clade 2.3.2.1c. Our spatial analyses demonstrated that the viruses resulted from different viral introductions from Asia and Europe and provided evidence of virus spread from Iran to the Middle East. Therefore, Iran may represent a hot-spot for virus introduction, dissemination and for the generation of new genetic variability. Increasing surveillance efforts in this high-risk area is of utmost importance for the early detection of novel emerging strains with zoonotic potential.

1. Introduction

Highly pathogenic avian influenza (HPAI) represents one of the major concerns to animal health considering the high morbidity and mortality rates observed in avian species. Since the first detection of the HPAI A(H5) A/goose/Guangdong/1/1996 (Gs/GD/96) strain in China in 1996, multiple lineages have emerged, evolving and co-circulating in different regions and species, acquiring distinct antigenic properties and zoonotic potentials in their evolutionary process (Sims and Brown, 2016; WHO/OIE/FAO H5N1 Evolution Working Group, 2008; World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5N1 Evolution Working Group, 2014). HPAI A(H5) viruses of the Gs/GD/96 lineage have been reported in many Asian countries since 2003 (Sims et al., 2005) and have rapidly disseminated westward, reaching the European and African territories and, on one occasion, also North America (Lee et al., 2018b). The persistent circulation of this lineage has led to the emergence of hemagglutinin (HA) gene variants, classified into ten different clades (0–9) and numerous subclades, from the second to fifth order (Smith and Donis, 2015). HPAI A(H5) viruses of clade 2.3.4 have been circulating in Eurasia as reassortant viruses since 2010, proving their ability to replace the N1 with different neuraminidase (NA) subtypes

Abbreviations: HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza.; Gs/GD, Goose/Guangdong.; IVO, Iran Veterinary Organization.; OIE, World Organization for Animal Health.; WHO, World Health Organization.; FAO, Food and Agriculture Organization.; tMRCA, time to the Most Recent Common Ancestor.; MCC tree, Maximum clade credibility tree.; BF, Bayes Factor.; IVPI, intravenous pathogenicity index.; PB2, polymerase basic protein 2.; PB1, polymerase basic protein 1.; PA, polymerase acidic protein.; HA, hemagglutinin.; NP, nucleoprotein.; NA, neuraminidase.; M, matrix protein.; NS, non-structural protein

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(N2, N5, N6, N8) from unrelated avian influenza viruses. In spring 2016, an HPAI A(H5N8) virus of still unofficially-defined clade 2.3.4.4B was detected in migratory birds in Qinghai Lake in China (Li et al., 2017) and on the border between Mongolia and the Russian Federation (Lee et al., 2017). Since then, the newly emerged virus has caused multiple outbreaks in poultry and wild birds across Europe, Asia, Africa and the Middle East. Reassortant H5 HPAI viruses of clade 2.3.4.4B carrying the neuraminidase gene segment N6 were first detected in late 2017 in Japan, South Korea and the Netherlands and were later reported both in wild bids and in poultry in several European and East Asian countries (Beerens et al., 2018; Kim et al., 2018; Lee et al., 2018a).

In Iran, the first HPAI A(H5N1) epidemic was caused by a virus belonging to genetic clade 2.2.2 and dates back to February 2006 when a high mortality of wild swans was detected in Somaesara, Gilan province in northern Iran, close to Anzali Wetland (Fereidouni et al., 2010; OIE Report, OIE Ref: 4548, Report Date: 26/02/2006, Country: Iran, 2006; Shoushtari Hablolvarid et al., 2007; Smith and Donis, 2015). In January 2008, the Iran Veterinary Organization (IVO) notified to the World Organization for Animal Health (OIE) a H5N1 outbreak which had occurred in December 2007, in a backyard poultry in Babolsar, Mazandaran province in northern Iran (OIE Report, OIE Ref: 6722, Report Date: 27/01/2008, Country: Iran, 2008). Other H5N1 viruses outbreaks, of clade 2.3.2.1, were reported in September 2011 and June 2015 in backyards and farms in Mazandaran province (Ghafouri et al., 2017a, 2017b; Ghafouri et al., 2017a, 2017b). In November 2016, a H5N8 virus of clade 2.3.4.4B was reported for the first time in a commercial layer farm in Tehran province (Ghafouri et al., 2017a, 2017b); 2018 was characterized by the emergence of the HPAI A(H5N6) subtype virus of clade 2.3.4.4B which infected wild ducks in a Natural Park in Gilan Province (OIE Report: Ref OIE: 25874, Report Date: 05/02/2018, Country: Iran, 2018).

Iran may be considered as an important hotspot for avian influenza virus, as it is crossed by Central Asia, East Africa/West Asia and Black Sea/Mediterranean flyways and is located on the Caspian's southern shores. Nevertheless, there is little information available on the genetic characteristics of the viruses circulating in this area. Aim of this work was to characterize the different HPAI A(H5N6), A(H5N8) and A(H5N1) viral genotypes which were circulating in Iran between 2016 and 2018.

2. Materials and methods

2.1. Complete genome sequencing

The complete genomes of the AIVs identified in Iran between 2016 and 2018 were obtained from the extracted RNA (QIAsymphony DSP Virus/Pathogen Kits and QIAsymphony SP (Qiagen)) of tracheal swabs and tracheas. Genome amplification was performed by using SuperScript III One-Step RT-PCR kit and Platinum Taq High Fidelity kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) with slight modifications to the protocol suggested by Zhou et al. (2009), as previously described (Fusaro et al., 2019; Zhou et al., 2009). Briefly, libraries were prepared by using Nextera XT DNA Sample preparation kit (Illumina, San Diego, CA, USA), quantified using Qubit dsDNA High Sensitivity kit (Invitrogen, USA), pooled in equimolar concentrations and loaded on the Illumina MiSeq instrument.

We assessed read quality using FastQC v0.11.2. We filtered raw data by removing reads with more than 10% of undetermined ("N") bases, reads with more than 100 bases with Q score below 7, duplicated paired-end reads. We than clipped reads from Illumina Nextera XT adaptors with scythe v0.991 (https://github.com/vsbuffalo/scythe) and trimmed with sickle v1.33 (https://github.com/najoshi/sickle). Complete genomes were generated through a reference-based approach. We aligned high quality reads against a reference genome using BWA v0.7.12 67, processing the alignments with Picard-tools v2.1.0 (http://picard.sourceforge.net) and GATK v3.5 68–70. Sequence coverage ranged between 0 and 166,497× fold, with an average value of 9932× fold and a median value of 2549×. We called Single Nucleotide Polymorphisms (SNPs) using LoFreq v2.1.2 (Wilm et al., 2012) and a minimum sequence coverage of 10, finally generating consensus sequences.

Sequences were submitted to the GISAID EpiFlu database (https://platform.gisaid.org) under the accession numbers EPI1701622-EPI1701819 (Supplementary Table S1).

2.2. Phylogeny

The sequences of each gene segment were compared with those from the BLAST search assessed both in GenBank and GISAID (Supplementary Table S2) public databases. Sequences were aligned using MAFFT v. 7 (Katoh and Standley, 2013). Phylogenetic analyses were performed for each gene segment by using the Maximum Likelihood tree reconstruction program IQTREE v1.6.6 (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). The robustness of individual nodes of the phylogenetic trees was obtained performing one thousand ultrafast bootstrap replicates. FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize all the phylogenetic trees obtained. Clusters were defined on the basis of bootstrap values higher than 80%.

The phylogenetic trees for each AIV segment were converted into cladograms to better display the reassortment events; leafs and connecting lines were coloured according to the groups they belong to. Cladograms were represented using the rotations of all nodes which heuristically minimize the number of intersections between connecting lines. To create the tanglegram, we used an in-house pipeline developed at our institute (pipeline available upon request) (Fig. 1a).

2.3. Phylogeography and evolution

Markov chain Monte Carlo (MCMC) analyses were performed using BEAST v1.10.2 package (Drummond and Rambaut, 2007). To estimate the time to the most recent common ancestor (tMRCA) for all the gene segments, we used an uncorrelated lognormal relaxed molecular clock, the HKY85 + Γ4 model with two partitions (1st + 2nd positions vs. 3rd position), base frequencies and Γ-rate heterogeneity unlinked across all codon positions (the SRD06 substitution model) (Shapiro et al., 2006) and a GMRF Bayesian Skyride coalescent tree prior. MCMC chains were run for 100 million iterations and convergence were assessed using Tracer v1.7.1. After the removal of an appropriate burn-in, we generated the Maximum clade credibility (MCC) trees by using TreeAnnotator v1.10.2. FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the MCC trees. For the HA and PA gene segments, we estimated spatial diffusion dynamics among a set of 8 geographical areas, by using a Bayesian discrete phylogeographic approach (Lemey et al., 2009). We used Spread D3 v0.9.6 to estimate Bayes Factor (BF) supports for transitions between discrete locations, and we interpreted the strength of statistical support as positive for $3 < BF < 5$ and strong for $BF > 5$ (Bielejec et al., 2016).

3. Results and discussion

We obtained the complete or nearly complete genome of twenty-six HPAI A(H5N8), one A(H5N6) and one A(H5N1) viruses (Table 1), which were collected from domestic ($n = 25$, commercial poultry farms) and wild ($n = 3$) birds in Iran's northern and western provinces located along Central Asia, East Africa/West Asia and Black Sea/Mediterranean flyways, close to the wetlands surrounding the Caspian Sea and its neighbouring provinces.

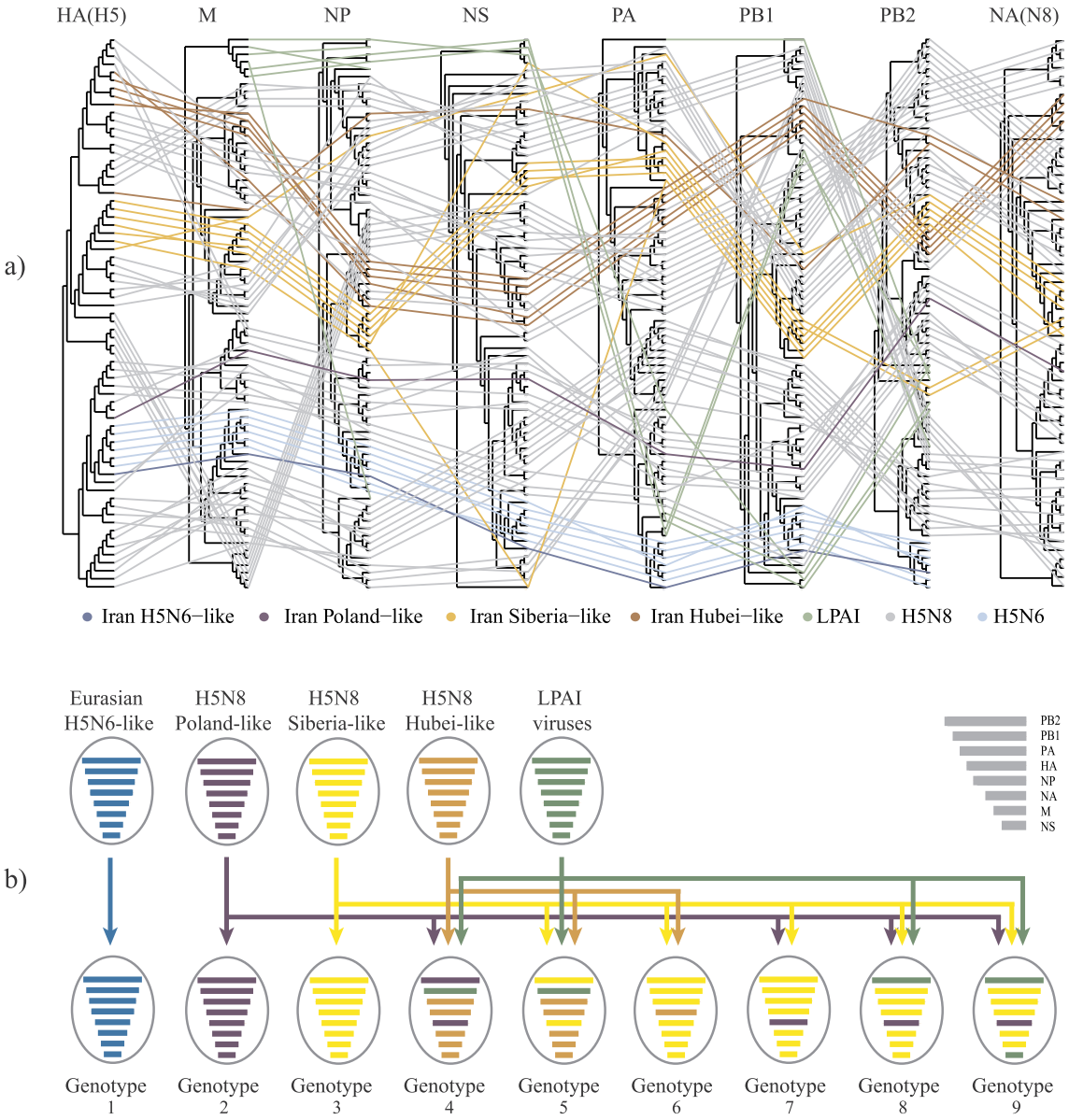

Fig. 1. Reassortments and genotypes. a) Cladograms for each AIV segment; leafs and connecting lines are coloured according to the groups they belong to. b) Genotypes 1–9. Each gene segment is represented by a bar of different length, from top to bottom: polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M), and non-structural protein (NS). Each colour represents a different viral origin: Eurasian H5N6-like virus in light blue, H5N8 Poland-like virus in violet, H5N8 Siberia-like virus in yellow, H5N8 Hubei-like virus in orange, LPAI virus in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1. HA gene analysis

Phylogenetic analysis of the hemagglutinin (HA) gene segment showed that the H5N8 and H5N6 viruses cluster within clade 2.3.4.4B (Supplementary Fig. S1) while the H5N1 belong to the clade 2.3.2.1c (Supplementary Fig. S2).

The viruses of clade 2.3.4.4B identified in Iran belong to four distinct clusters (indicated in violet (i), orange (ii), yellow (iii) and blue (iv) in Supplementary Figs. S1-S11): (i) A/wild_bird/Iran/17RS654–24/2016 (H5N8) clusters with H5N8 viruses collected in Eurasia and Egypt between 2016 and 2018; (ii) nine H5N8 Iranian viruses collected in 2016–2017 from domestic (n = 8) and wild (n = 1) birds cluster with viruses collected in China, Egypt, Israel, Central Africa, Saudi Arabia and India in 2016–2017; (iii) fifteen H5N8 Iranian viruses collected in 2017 from domestic (n = 14) and wild (n = 1) birds cluster with viruses collected in Israel, South Africa, Cameroon, Egypt, Russia, Italy, Korea and India in 2016–2017; (iv) A/gadwall/Iran/18VIR2027–20/2017 (H5N6) clusters with H5N6 viruses collected in Europe and Asia in 2017–2018. The viruses from wild birds show a great heterogeneity and fall into all four different clusters, while all the viruses from poultry belong to two genetic clusters, namely (ii) and (iii). The H5N1 virus A/duck/Iran/17RS654–12/2017 of clade 2.3.2.1c clusters with two H5N1 viruses collected in Iran in 2015, which suggests that the virus has been circulating in the country for at least two consecutive years.

3.2. Reassortant genotypes

The characterization of the complete genome (Supplementary Figs. S1-S11) revealed that the Iranian viruses show distinct gene constellations, likely due to the occurrence of multiple reassortment events.

According to the different gene composition of each analysed virus, we identified 10 distinct genotypes, consisting of eight H5N8, one

Table 1
Sequenced viruses from Iran (2016–2018). Details on genotypes and sequenced gene segments of the analysed viruses.

Subtype	Year	Virus	PB2	PB1	PA	HA	NP	NA	MP	NS	Genotype
H5N8	2016	A/wild_bird/Iran/17RS654–24/2016	√	√	√	√	√	√	√	√	2
H5N8	2016	A/chicken/Iran/17RS654–01/2016	√	√	√	√	√	√	√	√	4
H5N8	2016	A/chicken/Iran/17RS654–03/2016	√	√	√	√	√	√	√	√	4
H5N8	2016	A/chicken/Iran/17RS654–08/2016	√	√	√	√	√	√	√	√	4
H5N8	2016	A/flamingo/Iran/17RS654–18/2016	√	√	√	√	√	√	√	√	5
H5N8	2016	A/chicken/Iran/17RS654–28/2016	n.a.	√	√	√	√	√	√	√	n.a.
H5N8	2016	A/chicken/Iran/17RS654–02/2016	√	√	√	n.a.	√	√	√	√	n.a.
H5N8	2016	A/little_grebe/Iran/17RS654–10/2016	n.a.	n.a.	√	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/17RS654–15/2017	√	√	√	√	√	√	√	√	3
H5N8	2017	A/chicken/Iran/17RS654–26/2017	√	√	√	√	√	√	√	√	4
H5N8	2017	A/chicken/Iran/17RS654–29/2017	√	√	√	√	√	√	√	√	6
H5N8	2017	A/chicken/Iran/17RS654–34/2017	√	√	√	√	√	√	√	√	7
H5N8	2017	A/chicken/Iran/18VIR2027–03/2017	√	√	√	√	√	√	√	√	7
H5N8	2017	A/chicken/Iran/18VIR2027–05/2017	√	√	√	√	√	√	√	√	7
H5N8	2017	A/Crow/Aghakhan/2017	*	*	*	*	*	*	*	*	7
H5N8	2017	A/chicken/Iran/18VIR2027–04/2017	√	√	√	√	√	√	√	√	8
H5N8	2017	A/chicken/Iran/18VIR2027–09/2017	√	√	√	√	√	√	√	√	9
H5N1	2017	A/duck/Iran/17RS654–12/2017	√	√	√	√	√	√	√	√	10
H5N8	2017	A/chicken/Iran/17RS654–37/2017	√	√	√	n.a.	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–01/2017	n.a.	n.a.	√	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–02/2017	n.a.	n.a.	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–06/2017	n.a.	n.a.	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–07/2017	√	n.a.	√	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–08/2017	n.a.	n.a.	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–10/2017	n.a.	√	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–12/2017	n.a.	n.a.	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–14/2017	√	√	n.a.	√	√	√	√	√	n.a.
H5N8	2017	A/chicken/Iran/18VIR2027–15/2017	n.a.	n.a.	√	√	√	√	√	√	n.a.
H5N6	2018	A/gadwall/Iran/18VIR2027–20/2018	√	√	√	√	√	√	√	√	1

√ = sequenced; n.a. = not available; * downloaded from GenBank database.

H5N6 and one H5N1 subtype viruses (Table 1, Fig. 1b, Fig. 2).

Phylogenetic analyses indicated that genotypes 1, 2 and 3 are respectively similar to the existing viruses A/mallard/Republic of Georgia/1/2018(H5N6) (H5N6-like), A/wild duck/Poland/82A/2016(H5N8) (Poland-like) and A/domestic duck/Siberia/50K/2016(H5N8) (Siberia-like), some of which have been previously described (Fusaro et al., 2017; Poen et al., 2019). On the contrary, genotypes 4–9 turned out to have been generated through reassortment events between HPAI A(H5N8) viruses and low pathogenic avian influenza (LPAI) viruses of the Eurasian lineage (Fig. 1, Fig. 2) and were characterized in Iran for the first time. We then observed that the polymerase basic protein 2 (PB2) and non-structural protein (NS) gene segments were the major contributors to the overall genetic diversity among the H5N8 Iranian viruses. PB2 and NS genes of viruses of H5N8-genotypes 4–9 belong to three different genetic groups: Siberia-like, Poland-like, LPAI viruses for PB2; Siberia-like, Hubei-like (A/Cygnus atratus/Hubei/HF-1/2016), LPAI viruses for NS; differently, the remaining genes derive from two viral lineages: Siberia-like, Hubei-like viruses for PA, HA, NA, M; Siberia-like, Poland-like viruses for NP; Siberia-like, LPAI viruses for PB1. Genotypes with the greatest heterogeneity in terms of number of gene segments received from different viral sources are H5N8-genotypes 4, 5, 8, 9, which retain internal genes deriving form 3 different origins: Poland-like, Hubei-like, LPAI viruses for H5N8-genotype 4; Siberia-like, Hubei-like, LPAI viruses for H5N8-genotype 5; Poland-like, Siberia-like, LPAI viruses for H5N8-genotypes 8 and 9. Differently, H5N8-genotypes 6 and 7 reflect a gene constellation similar to Siberia-like viruses for all the gene segments except for the HA gene of H5N8-genotype 6 belongs to Hubei-like viruses and the NP gene of H5N8-genotype 7 belongs to Poland-like viruses (Fig. 2). From a different point of view, Siberia-like gene segments are the most represented of the overall genotypes and contribute to the gene pool of five different genotypes (H5N8-genotypes 5–9). Siberia-like virus has proven to show highly virulent features (IVPI = 2.34) and multi-organ dissemination in experimental infections of chickens (Prokopyeva et al., 2019), which means that it presents genetic characteristics that make it capable of causing serious clinical forms.

The only virus belonging to H5N1-genotype 10 is the H5N1 virus A/duck/Iran/17RS654–12/2017 which retains the HA, NA, matrix protein (M), nucleoprotein (NP) and polymerase basic protein 1 (PB1) genes of the H5N1 2.3.2.1c clade viruses while the remaining polymerases and the non-structural protein genes show the highest similarity with LPAI viruses of the Eurasian lineage (Supplementary Figs. S2, S5-S11). Long branches separate this virus from the closest ancestors in each phylogeny, which indicates the lack of sequenced viruses from the surrounding areas, a condition that makes it difficult to determine when and where this genotype emerged.

The complete genome was available for a panel of sixteen viruses (fifteen of which sequenced in this study and one recovered from GenBank) (Table 1, S1, Fig. 2). Among the H5N8 viruses, in 2016 three genotypes were co-circulating (H5N8-genotypes 2, 4, 5). In 2017, we observed the co-circulation of multiple subtypes (H5N8, H5N1) and genotypes (H5N8-genotypes 3, 4, 6–9 and H5N1-genotype 10). The only virus collected in 2018 belongs to H5N6-genotype 1, which consists of Eurasian HPAI H5N6 viruses.

Four viruses collected from wild birds out of the 29 Iranian viruses were analysed. Three of them belonged to H5N8-genotypes 2, 5 and 7. The virus A/little_grebe/Iran/17RS654–10/2016 has the same gene composition of viruses belonging to H5N8-genotype 4, except for the PB2 and PB1 gene segments, for which no sequence was obtained. A/little_grebe/Iran/17RS654–10/2016, A/flamingo/Iran/17RS654–18/2016 and A/Crow/Aghakhan/2017 showed a mixed gene composition consisting of genes from Poland-like, Siberia-like, Hubei-like and LPAI viruses (Fig. 2). Interestingly, genotype 4 circulated both in 2016 and in 2017, while genotype 7 was detected only in 2017. Samples collected in 2017 include seven different genotypes: H5N8-genotypes 3, 4, 6–9 and H5N1-genotype 10. Therefore, we reported both the co-circulation of different genotypes during the same year and the co-circulation of one (H5N8-genotype 7) or two genotypes (if we also consider A/little_grebe/Iran/17RS654–10/2016 almost entirely belonging to H5N8-genotype 4), in wild and domestic birds. This great variability may

N.	Subtype	Virus	Genotype	PB2	PB1	PA	HA	NP	NA	MP	NS
1	H5N6	A/chicken/Iran/18VIR2027-20/2018	**1**								
2	H5N8	**A/wild_bird/Iran/17RS654-24/2016**	**2**								
3	H5N8	A/chicken/Iran/17RS654-15/2017	**3**								
4	H5N8	A/chicken/Iran/17RS654-01/2016	**4**								
5	H5N8	A/chicken/Iran/17RS654-03/2016	**4**								
6	H5N8	A/chicken/Iran/17RS654-08/2016	**4**								
7	H5N8	A/chicken/Iran/17RS654-26/2017	**4**								
8	H5N8	A/chicken/Iran/17RS654-02/2016	n.a.								
9	H5N8	A/chicken/Iran/17RS654-37/2017	n.a.								
10	H5N8	A/chicken/Iran/17RS654-28/2016	n.a.								
11	H5N8	**A/little_grebe/Iran/17RS654-10/2016**	n.a.								
12	H5N8	A/chicken/Iran/18VIR2027-12/2017	n.a.								
13	H5N8	**A/flamingo/Iran/17RS654-18/2016**	**5**								
14	H5N8	A/chicken/Iran/17RS654-29/2017	**6**								
15	H5N8	A/chicken/Iran/17RS654-34/2017	**7**								
16	H5N8	A/chicken/Iran/18VIR2027-03/2017	**7**								
17	H5N8	A/chicken/Iran/18VIR2027-05/2017	**7**								
18	H5N8	**A/Crow/Aghakhan/2017***	**7**								
19	H5N8	A/chicken/Iran/18VIR2027-01/2017	n.a.								
20	H5N8	A/chicken/Iran/18VIR2027-02/2017	n.a.								
21	H5N8	A/chicken/Iran/18VIR2027-06/2017	n.a.								
22	H5N8	A/chicken/Iran/18VIR2027-07/2017	n.a.								
23	H5N8	A/chicken/Iran/18VIR2027-08/2017	n.a.								
24	H5N8	A/chicken/Iran/18VIR2027-10/2017	n.a.								
25	H5N8	A/chicken/Iran/18VIR2027-14/2017	n.a.								
26	H5N8	A/chicken/Iran/18VIR2027-15/2017	n.a.								
27	H5N8	A/chicken/Iran/18VIR2027-04/2017	**8**								
28	H5N8	A/chicken/Iran/18VIR2027-09/2017	**9**								
29	H5N1	A/duck/Iran/17RS654-12/2017	**10**								

* downloaded from GenBank database

n.a. not assigned

clade 2.3.4.4B H5N6-like

clade 2.3.4.4B Poland-like

clade 2.3.4.4B Siberia-like

clade 2.3.4.4B Hubei-like

LPAI

clade 2.3.2.1c H5N5-like

clade 2.3.2.1c H5N1-like

n.a.

Fig. 2. Gene composition of 29 Iranian viruses. The different viral sources are indicated at the bottom of the figure. H5N6-like origin refers to the gene composition of H5N6 viruses of clade 2.3.4.4B; Poland-like origin refers to the gene composition of A/wild duck/Poland/82A/2016(H5N8) (clade 2.3.4.4B); Siberia-like origin refers to the gene composition of A/domestic duck/Siberia/50 K/2016(H5N8) (clade 2.3.4.4B); Hubei-like origin refers to the gene composition of A/Cygnus atratus/Hubei/HF-1/2016 (clade 2.3.4.4B); H5N1-like origin refers to the gene composition of H5N1 viruses of clade 2.3.2.1c; H5N5-like origin refers to the gene composition of H5N5 viruses of clade 2.3.2.1c; LPAI origin refers to Low Pathogenic Avian Influenza viruses belonging to different subtypes; n.a. refers to not available gene segments.

indicate different viral introductions by means of migratory birds and the occurrence of reassortment events with viruses circulating in the wild resident population. However, we cannot completely exclude that poultry trade and legal or illegal trade of wild birds may have played an important role in the virus introduction into the country. Of note, viruses with the same gene compositions of A/Crow/Aghakhan/2017 (genotype 7) and A/little_grebe/Iran/17RS654–10/2016 (hypothetical genotype 4) were also identified in domestic birds. This finding, as well as the high genetic diversity of viruses circulating in the domestic and wild bird population in Iran, supports the hypothesis of wild species as the main source of the virus introduction into Iran, as previously suggested for other geographic areas (Bevins et al., 2016; Fusaro et al., 2019; Hill et al., 2015; Lycett et al., 2016; The Global Consortium for H5N8 and Related Influenza, 2016; Verhagen et al., 2015).

The time to the most recent common ancestor (tMRCA) estimated for each gene segment suggests that H5N8-genotypes 2, 3, 4, 7 had emerged between the end of 2015 and January 2017, in the period of massive circulation of clade 2.3.4.4B in Eurasia. Differently, BEAST analyses estimated the tMRCA of H5N6-genotype 1 between September 2016 and November 2017 (Fig. 3).

It has been shown that the time and direction of the intercontinental viral spreads overlapped with fall bird migrations. Iran is located at the centre of the most important bird migration corridors to/from Africa and to/from Asia. Northern Iran is part of the temperate zone and with the arrival of the cold season it hosts a large number of migratory birds (Ahmadpour et al., 2016; Parchizadeh and Williams, 2018). The viruses from Iran we have described were identified in autumn/winter 2016–2017 and autumn/winter 2017–2018, the season when wild birds migrations occur.

To investigate the dynamics of virus incursions in Iran, we performed the phylogeographic analysis of the H5N8 and H5N6 subtype viruses (clade 2.3.4.4B) for the HA gene, which is the most variable gene. The results were confirmed using the PA gene, coding for the internal polymerase acidic protein, which showed a high genetic diversity among the analysed samples. MCC trees of HA and PA gene segments reflected the same topology (Fig. 4, S12, S13). This investigation revealed the occurrence of at least 5 different viral introductions (BF > 3) in the country mainly from Central Asia and Europe, which might have derived from different wild birds incursion. We also identified a viral spread from Iran to Egypt and Israel (Figs. S12-S13), which could suggest that wild birds which had stopped in Iran continued their journey to East Africa spreading the H5 virus. These observations could suggest that Iran served both as a sink and source of the H5N8 virus.

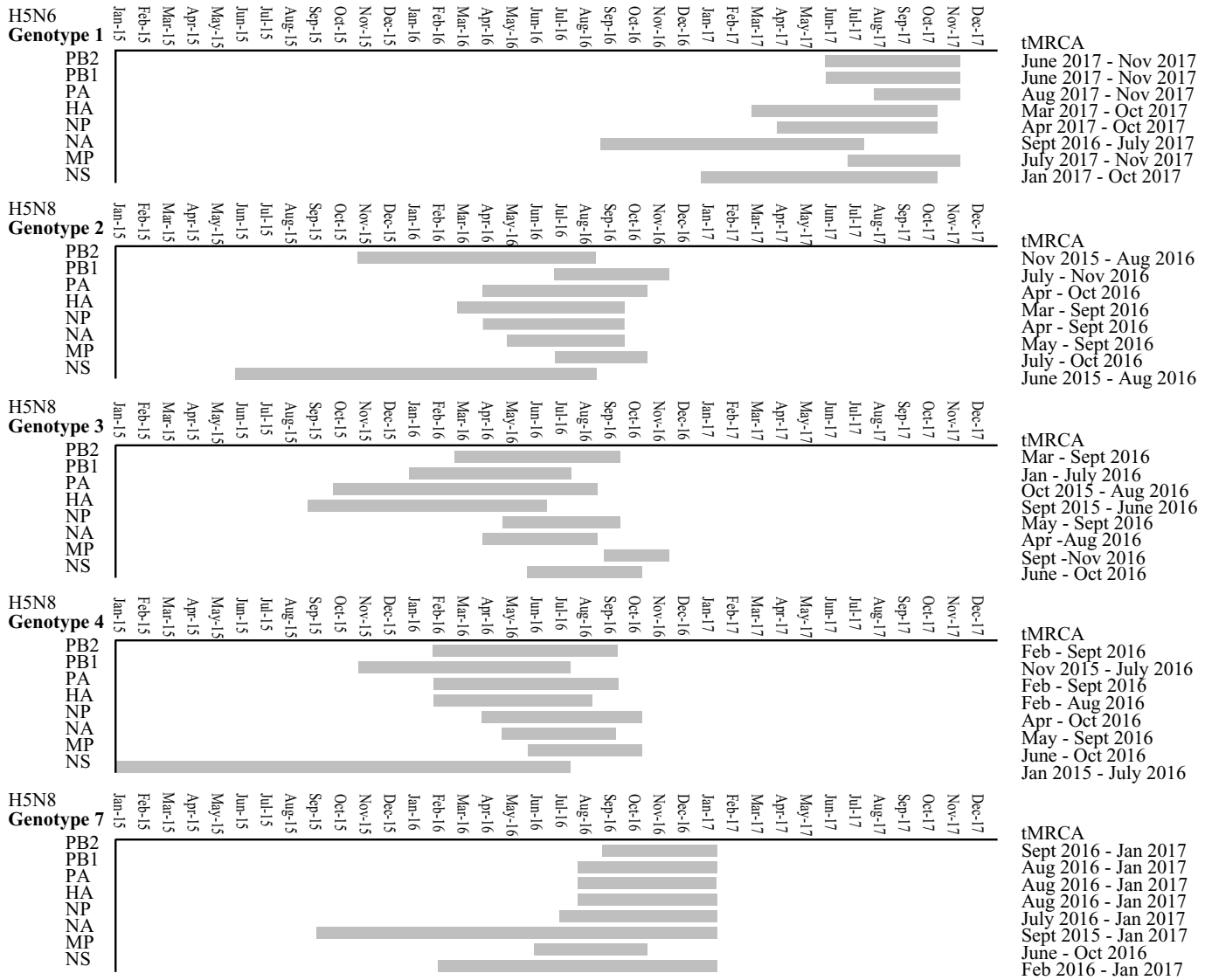

Fig. 3. Time to the most recent common ancestor (tMRCA). TMRCA for each gene segment of genotypes from 1 to 9.

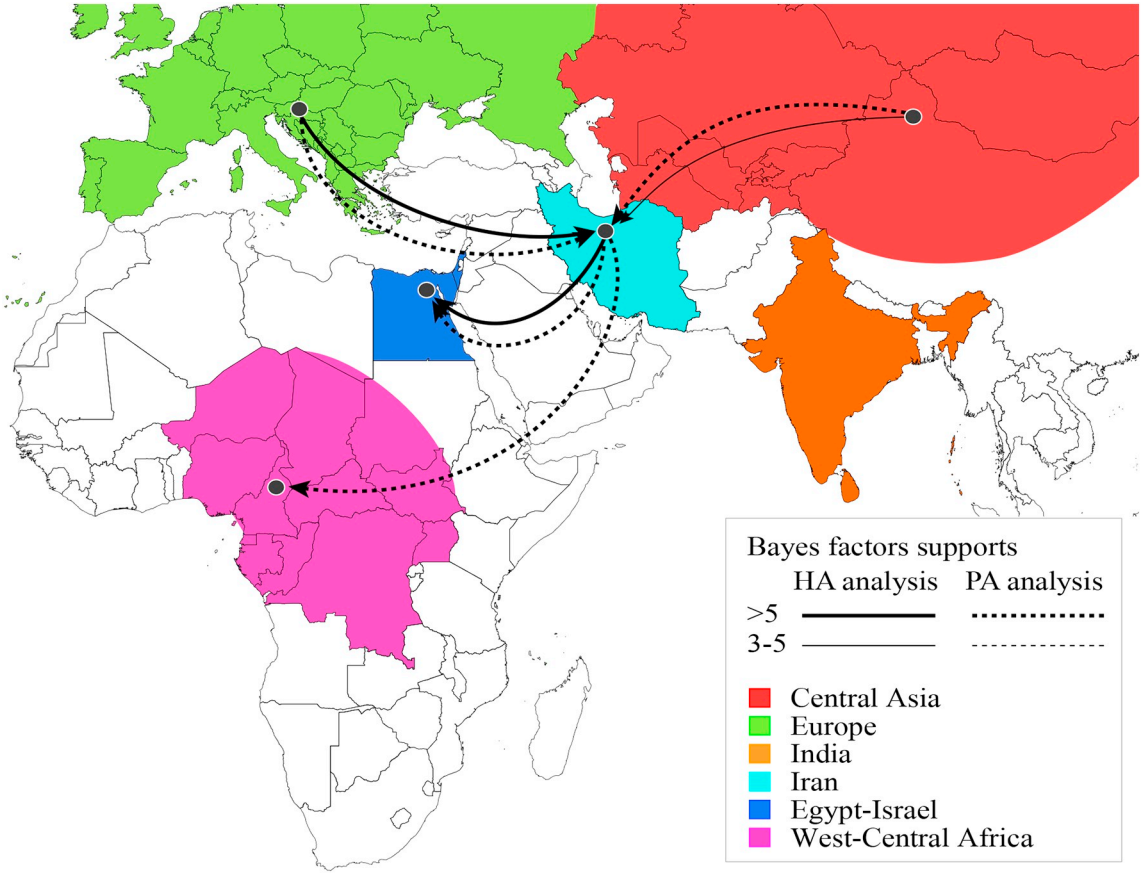

Fig. 4. Migration rates of the HPAI H5 viruses of clade 2.3.4.4B. The map shows statistically supported non-zero rates for H5 and PA gene segments of H5 viruses of clade 2.3.4.4B. The thickness of the lines (HA analysis) and dashed lines (PA analysis) is proportional to the relative strength by which rates are supported: strong (BF > 5, thick lines) and positive (3 < BF < 5, thin lines). Each country is labelled by the same colour used in the annotated phylogenetic trees (Supplementary Figs. S1-S13).

The molecular analysis of the Iranian viruses revealed that the NA protein of four H5N8 viruses collected in 2016 and six H5N8 viruses collected in 2017 possess a mutation at aminoacid position 44 (Ile44Thr or Ile44Ser). This mutation creates a new potential N-glycosylation site which may also affect antigenic and other properties of this strain. In detail, the motif at positions 44–46 changed from NGI to NGT or NGS. In addition, the NA protein of three H5N8 samples collected in 2017 lacks a potential glycosylation site at position 67–69 (NTN).

4. Conclusions

In this study, we compared the origins and genetic characteristics of different H5 viral genotypes of AIVs identified in Iran between 2016 and 2018. Our results underline that Gs/GD H5 AIVs have a high propensity to reassort with co-circulating LPAI and HPAI viruses, and in Iran this generated six novel H5N8 genotypes (H5N8-genotypes 4–9) of clade 2.3.4.4B and one H5N1 reassortant virus (H5N1-genotype 10) of clade 2.3.2.1c. Some of the identified viruses had likely been introduced from different countries as already existing genotypes (H5N6-genotype 1 and H5N8-genotypes 2–3), while some others could have been generated on site through reassortment events involving local wild or domestic birds.

This study reports the first characterization of the HPAI A(H5N6) subtype in Iran (H5N6-genotype 1). Phylogenetic analyses demonstrated that this virus clustered with H5N6 viruses circulating in Europe and Asia, and was unrelated to the zoonotic H5N6 Asian strains that had caused infections in humans. The reconstruction of the spatial dispersal of the Iranian viruses collected between 2016 and 2018 identified different viral introductions from Central Asia and Europe and spatial spread of the virus from Iran to the Middle East. The presence of the Black Sea/Mediterranean flywaywhich runs through the northern Iranian territory may have contributed to this westward spread. This geographic area is characterized by lagoons and wetlands and represents a wintering site of great attraction (Fereidouni et al., 2010; Sinka-Karimi et al., 2015). Several works have supported the role of migratory birds in the dispersal of HPAI H5 viruses, on the basis of phylogenetic analyses and epidemiological investigations. Moreover, poultry outbreaks were frequently recorded simultaneously or immediately after the identification in the same area of HPAI H5 infections in wild birds, suggesting that wild birds may have been the most likely source of AI introduction into poultry (Bevins et al., 2016; Hill et al., 2015; Lycett et al., 2016; Si et al., 2009; Trovão et al., 2015; Verhagen et al., 2015). These observations together with the evidence of a great heterogeneity in the wild birds viral genomes we sequenced, which belong to four different genotypes, suggest that wild birds may have acted as main carrier of the virus in the country. Even though this is the most likely scenario, poultry trade must also be taken into account. In 2016–2017, Iran imported live chickens and turkeys from Europe, in particular from Germany, Italy, France, the Netherlands and the United Kingdom, where HPAI H5 viruses of clade 2.3.4.4B had been extensively circulating (UN-FAO: faostat3.fao.org, accessed on 14 April 2020). Therefore, we cannot completely exclude the role of poultry trade in the virus spread.

In Iran, 31 provinces host approximately 65,000 villages populated by families which keep birds for different purposes (Fallah Mehrabadi et al., 2016). AI infected wild birds can be considered a risk factor in rural poultry, being the interaction of wild birds with free-ranging poultry easy to occur. Moreover, human activities and poor biosecurity

practices may contribute to the spread of AIVs infections.

This work highlights that Iran appears to be an area at risk for virus introduction and dissemination and for the generation of genetic variability. Our observations suggest that enhanced monitoring in wild birds in wetland ecosystems coupled with the application of increased biosecurity measures in poultry holdings are needed to mitigate the risk of virus introduction and spread.

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Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. According to the national legislations regulating animal experimentation, no ethical approval or permit was required for collecting and processing the type of samples examined for this study.

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