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Running head: Novel clade of H1 swine influenza A viruses in US swine

Characterization of co-circulating swine influenza A viruses in North America and the identification of a novel H1 genetic clade with antigenic significance

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Characterization of cocirculating swine influenza A viruses in North America and the identification of a novel H1 genetic clade with antigenic significance

- A phylogenetic analysis of North American swine influenza A viruses is presented
- Continual co-circulation of 3 subtypes and 13 genetic clades in the US swine population
- A novel H1 genetic clade, H1γ-2, was detected
- The H1γ-2 clade forms a unique antigenic cluster with functional significance

Abstract

Multiple genetically and antigenically distinct hemagglutinin genes of the H1 and H3 influenza A virus (IAV) subtypes co-circulate in North American swine. This diversity has evolved by repeated transmission of IAVs from humans to swine and subsequent antigenic drift in swine. To understand the evolutionary dynamics of these diverse HA lineages in North American swine, we undertook a phylogenetic analysis of 1576 H1 and 607 H3 HA gene segments, as well as 834 N1 and 1293 N2 NA gene segments, and 2126 M gene segments. These data revealed yearly co-circulation of H1N1, H1N2, and H3N2 viruses, with three HA clades representing the majority of the HA sequences: of the H1 viruses, 42% were classified as H1δ1 and 40.6% were classified as H1γ; and of the H3 viruses 53% were classified as cluster IV-A H3N2. We detected a genetically distinct minor clade consisting of 37 H1 viruses isolated between 2003 and 2013, which we classified as H1**γ-2.** We estimated that this clade circulated in swine since approximately 1995, but it was not detected in swine until 2003. Though this clade only represents 1.07% of swine H1 sequences reported over the past 10 years, hemagglutination inhibition (HI) assays demonstrated that representatives of this clade of viruses are antigenically distinct, and, when measured using antigenic cartography, were as many as 7 antigenic units from other H1γ viruses and therefore vaccines against the

contemporary H1γ viruses are not likely to cross-protect against γ-2 viruses. The long-term circulation of these γ-2 viruses suggests that minor populations of viruses may be underreported in the national dataset given the long branch lengths and gaps in detections. The identification of these γ-2 viruses demonstrates the need for robust surveillance to capture the full diversity IAVs in swine in the USA and the importance of antigenic drift in the diversification and emergence of new antigenic variants in swine, which complicates vaccine design.

Keywords: *influenza A virus; antigenic drift; swine; zoonotic diseases; vaccines; epidemiology*

1. Introduction

Influenza A virus (IAV) was first detected in swine coincident with the 1918 "Spanish flu" (Koen, 1919) and subsequently isolated and characterized as an H1N1 (Shope, 1931). This lineage of swine H1N1, classical-swine H1N1 (cH1N1), was the first of a number of swine IAV lineages to be detected in North America. Though swine are only infected by a fraction of the subtypes found in the natural avian host, phylogenetic methods have identified at least 16 distinct genetic clades within the 3 predominant H1N1, H1N2, and H3N2 subtypes (Kitikoon et al., 2013; Rajao et al., 2014). This diversity has public health and agricultural relevance: the 2009 human pandemic (Garten et al., 2009) was of swine origin, as were variant H3N2 viruses that emerged in 2010-2012 (CDC 2012a; 2012b; Wong et al., 2012), and intervention efforts in swine herds using vaccines are likely to often be equivocal in the face of this extraordinarily high degree of variability in a single mammalian host species (Lewis et al., 2014; Vincent et al., 2008). Consequently, it is necessary to develop a framework that quantifies swine IAV genetic and antigenic diversity; in doing so, emerging viral threats may be identified and objective criteria for updating vaccine composition could be implemented.

During the last 20 years, quantifying swine IAV diversity has been challenged by the incursion of novel subtypes (Nelson et al., 2012; 2014), and the continual process of antigenic drift and shift (Carrat and Flahault, 2007; Webster, 1999). Specifically, in the late 1990s a novel triple-reassortant H3N2 virus was identified in the North American swine population comprised of seasonal human H3N2, avian influenza, and the cH1N1 swine IAV (Olsen, 2002; Zhou et al., 1999). These triple-reassortant viruses co-circulated and reassorted with cH1N1 viruses resulting in additional lineages of H1N1 and H1N2 viruses (Karasin et al., 2002; 2000). Additional lineages of H1N1 and H1N2 viruses derived from human seasonal IAV were detected in the early 2000s and now represent approximately 40% of circulating swine isolates (Anderson et al., 2013; Vincent et al., 2009a). All of these newly introduced lineages, after becoming endemic, continue to generate novel swine IAVs: this is exemplified by the bidirectional transmission of the

H1N1pdm09 from swine-to-human and then back to swine. This genetic lineage donated its matrix gene to a majority of endemic swine IAV in U.S. viruses via reassortment (Anderson et al., 2013). Thus, there are at least 16 genetically defined clades reported in North America (Rajao et al., 2014), with little evidence that the rapid evolutionary diversification is slowing.

Given the importance of controlling IAV in swine agriculture, and the recognition that pigs can act as potential 'mixing vessels' for novel viruses (Ma et al., 2009), the United States Department of Agriculture (USDA) initiated a coordinated surveillance system in 2009. This program has monitored circulating H1N1, H1N2, and H3N2 subtypes through a voluntary and anonymous sample submission process as part of the National Animal Health Laboratory Network (NAHLN). These data allow for the characterization of the evolutionary and seasonal dynamics of IAV (Anderson et al., 2013; Kitikoon et al., 2013; Lewis et al., 2014). In this study, we conducted a comprehensive phylogenetic analysis of North American swine IAV collected from 2009-2014 and characterized an H1 clade that circulated in North America for nearly 20 years despite very low levels of detection.

2. Materials and methods

2.1 *Data generation*

For the years 2009 to 2014, all nucleotide sequences sourced through the USDA Influenza Virus Surveillance System for swine were downloaded from GenBank, the National Center for Biotechnology Information's online sequence repository. These data comprised 2183 HA gene segments, 2127 NA gene segments, and 2126 M gene segments. Viruses were collected from swine in 30 US states (Alabama, Arkansas, California, Colorado, Iowa, Illinois, Indiana, Kansas, Kentucky, Maryland, Michigan, Minnesota, Mississippi, Missouri, Montana, North Carolina, North Dakota, Nebraska, New York, Ohio, Oklahoma, Oregon, Pennsylvania, South Carolina, South Dakota, Tennessee, Texas, Virginia, Wisconsin, and Wyoming) following methods described by Korslund et al. (2013). All sequence data were downloaded from the Influenza Virus Resource on June 9, 2014 (Table S1; Bao et al., 2008).

2.2 *Phylogenetic methods*

From these data, five sequence alignments were constructed using MUSCLE with default settings in MEGA5.2 with subsequent manual correction: an alignment of 1576 H1 and 607 H3 hemagglutinin gene segments, an alignment of 834 N1 and 1293 N2 neuraminidase gene segments, and 2126 matrix gene segments (Edgar, 2004; Tamura et al., 2011). For each alignment, a maximum likelihood tree was inferred using RAxML (8.0.24; Stamatakis, 2006) on the CIPRES Science Center Gateway (Miller et al., 2010) employing a general time-reversible (GTR) model of nucleotide substitution with Γ-distributed rate variation among sites. The starting tree was generated under parsimony methods, with the best-scoring tree and statistical support values obtained with the rapid bootstrap algorithm (1000 replications). Using the H1 phylogeny, H1N1 and H1N2 isolates were assigned to one of six previously described H1 antigenic lineages: H1α, H1β, H1γ, H1δ1, H1δ2, H1pdm09 (Vincent et al., 2009a; 2009b; Lorusso et al., 2011). Similarly, H3N2 isolates were assigned to one of four main clusters based upon the H3 phylogeny, and H3 Cluster IV isolates to one of 6 putative clades (Kitikoon et al., 2013). Within and between clade nucleotide distances for the H1 and H3 antigenic clade designations were calculated in MEGA5.2 (Tamura et al., 2011).

The phylogenetic analyses identified a cluster of 11 sequences that were separated by a high number of nucleotide substitutions from other classic lineage H1 swine IAV. To explore the evolutionary origins of these divergent sequences, a second phylogenetic tree was inferred for 378 classical-swine H1N1 lineage sequences. These data included an additional 45 sequences from GenBank that had high sequence identity using BLASTn (Altschul et al., 1990) to the set of 11 unique sequences identified in the 2009-2014 USDA dataset, 67 Canadian H1α were included to ensure representation of this clade, and we omitted H1N1pdm09 viruses given their unique transmission history between humans and pigs. To study the genome patterns of these viruses, where data was available, we downloaded the internal genes of 378 H1γ-2, and other classic-lineage (H1α, H1β, H1γ) viruses. In addition to sequences already available, we selected 18 isolates for whole genome sequencing following the methods in Bowman et al. (2012) using the Ion 316 v2 chip and Ion PGM 200 v2 Sequencing Kit. We then inferred maximum likelihood trees for

each gene segment using RAxML (8.0.24; Stamatakis, 2006) on the CIPRES Science Center Gateway (Miller et al., 2010) employing a GTR model of nucleotide substitution with Γ-distributed rate variation among sites. Each internal gene segment was then classified to one of two evolutionary lineages: H1pdm09 or TRIG.

To estimate the evolutionary dynamics of the cH1-lineage, we implemented a time-scaled Bayesian approach for the HA alignment. We employed a relaxed uncorrelated lognormal (UCLN) molecular clock, a flexible Bayesian skyline plot (BSP) demographic model (10 piece-wise constant groups), and a general-time reversible (GTR) model of nucleotide substitution with Γ-distributed rate variation among sites. The precision function in BEAUTi was used to sample uniformly within a one-year or one-month window for those viruses for which an exact date of collection was not available. The MCMC chain was run independently 3 times for 200 million iterations with sub-sampling every 20,000 iterations. We used the BEAST package (v1.8.0: Drummond et al., 2012) with the BEAGLE library (v2.0.2: Ayres et al., 2011), incorporating the date of sampling: all parameters reached convergence, assessed visually using Tracer v.1.6. The initial 10% of the chain was removed as burn-in, runs were combined using LogCombiner v.1.8.0, and the evolutionary history was summarized using an annotated maximum clade credibility (MCC) tree using TreeAnnotator v.1.8.0. Posterior probability values were used to assess the degree of support for each node in the tree.

2.3 ***Antigenic characterization***

Swine antisera against two isolates identified in the H1γ-2 phylogenetic node (A/Swine/South Dakota/A01349306/2013 and A/Swine/Illinois/A01203922/2012) were generated using previously described methods (Lorusso et al., 2011; Lewis et al., 2014). Forty-five cH1-lineage swine viruses were selected as hemagglutinin inhibition (HI) test antigens following methods described in Lewis et al. (2014). In brief, prior to HI testing, sera were treated with kaolin (Sigma-Aldrich, MO, USA), heat inactivated at 56^{o}C for 30 min and adsorbed with 50% turkey red blood cells (RBC) to remove nonspecific inhibitors of hemagglutination. HI assays were performed by testing reference antisera raised against 45 swine H1

viruses according to standard techniques (Lorusso et al., 2011). Serial 2-fold dilutions starting at 1:10 were tested for their ability to inhibit the agglutination of 0.5% turkey RBC with 4 HAU of swine H1 viruses. These data were visualized using antigenic cartography (Lewis et al., 2014; Lorusso et al., 2011; Smith et al., 2004). Antigenic distances (Euclidean distances) were measured between all strains in the antigenic map and from reference antigen A/H1N1/swine/Ohio/511445/2007, selected as the H1γ phylogenetic cluster representative, and from A/CA/4/2009 as the prototype H1N1pdm09 strain. Additionally, to assess the cross-reactivity with currently available vaccines, the H1γ-2 antigens were tested against anti-sera generated in swine with three commercially available fully-licensed swine influenza vaccines as previously described (Kitikoon et al., 2013b). Two pigs were vaccinated with each of the three vaccines and sera tested in the HI assay.

3. Results

3.1 *H1 phylogeny revealed a contemporary H1γ-2 clade*

The H1 phylogeny contained 11 isolates that shared a common ancestor with H1γ and H1pdm09 subtypes, but was located on an independent branch with a long branch length: we designated viruses in this clade as H1γ-2 based on 4.3% within and 11.8% between cluster nucleotide divergence (Table 1) and 100% posterior probability support (Fig. 1: 92% bootstrap support in maximum likelihood phylogeny, Fig. S1). A BLASTn analysis with the H1γ-2 sequences identified 45 additional viruses isolated prior to the inception of the USDA national IAV-S surveillance program with relatively high sequence identity. The historical isolates were added to USDA surveillance data to investigate the evolution of this novel clade. The new H1 data set contained randomly selected H1 sequences from H1α, H1β, and H1γ isolates, as well as the 11 contemporary H1γ-2 and 45 historical H1 sequences with high sequence identity (Fig. 1). This analysis revealed two clades with high statistical support closely related to H1γ viruses. One clade (n=19) (black clade, Fig. 1) does not appear to have persisted in swine herds, circulating only from 2003-2007, whereas the clade we designate H1γ-2 (n=37) demonstrates onward transmission with contemporary isolates. From the time-scaled phylogeny (Fig. 1) we estimate that the time to the most recent common

ancestor (tMRCA) for the H1γ-2 viruses and the most closely related swine IAV H1γ viruses falls between 1994.6 and 1997.6 (95% HPD). This represents an estimated 5-8 years of undetected circulation prior to the first collection of a virus from this clade in Iowa on October 10, 2003. Of the H1γ-2 viruses collected in the USDA surveillance system, HA genes were paired with classical N1 in most cases, with the exception of one 2002-lineage N2. In order to determine whether H1γ-2 HA/NA surface glycoproteins co-evolved, an analysis of the NA genes was conducted. Of the NA data (n=18) that were available, we found 16 H1γ-2 isolates formed a monophyletic group in the NA gene segment phylogeny (Fig. 2). These results suggested H1γ-2 HA and classical N1 genes co-segregated.

The 18 H1γ-2 viruses with sequences for all 8 gene segments were included in phylogenetic analysis of the remaining 6 genes. Historical H1γ-2 isolates (2003-2007) contained an internal gene TRIG constellation (swine (M, NP and NS genes), avian (PB2 and PA genes), and human origin (PB1) genes); however the contemporary H1γ-2 isolates showed evidence of reassortment with H1N1pdm09. Two of the nine contemporary USDA surveillance isolates carried the entire H1N1pdm09 internal gene backbone; five carried a TRIG PB1 gene with the remaining internal genes being from the H1N1pdm09. One of the remaining contemporary isolates, A/swine/Minnesota/A01201429/2011, contained an H1N1pdm09 NP gene with the remaining genes TRIG lineage; and one contained the internal gene TRIG constellation, A/swine/South Dakota/A01349306/2013. Taken together, this unique HA clade is also unique from a whole genome perspective (Fig S4 – S8).

In spite of being under-sampled with sporadic detection, the H1γ-2 had a broad geographic distribution across the midwest, south-east and south-central states: this included Iowa (IA), Illinois (IL), Minnesota (MN), Missouri (MO), North Carolina (NC), Oklahoma (OK), Texas (TX), and South Dakota (SD). H1γ-2 continues to co-circulate in the US in addition to the other contemporary subtypes and cluster types. The gaps in years of isolation and long branch lengths suggest gaps in surveillance in herds that are infected with H1γ-2.

3.2 ***HA, NA and M evolutionary trends in contemporary swine IAV***

From January 2013 to March 2014, 813 HA, 809 NA, and 810 M sequenced gene segments were generated from the USDA IAV-S surveillance system and analyzed in addition to 1075 HA segments, 1049 NA segments, and 1040 M segments reported from 2009 – 2012 that were reported previously (Table S2: Anderson et al., 2013). The 2013 through March 2014 H1 data (n = 590) contained 0.17% H1α (n = 1), 3.56% H1β (n = 21), 49.66% H1γ (n = 293), 0.34% H1γ-2 (n = 2), 37.28% H1δ1 (n = 220), 4.07% H1δ2 (n = 24), and 4.92% H1pdm09 (n = 29) viruses (Fig. S1). The H3 data (n=223) contained 1.35% Cluster IV (n = 3), 76.68% Cluster IV-A (n = 171), 13.90% Cluster IV-B (n = 31), 2.24% Cluster IV-C (n = 5), 3.14% Cluster IV-E (n = 7), and 2.69% Cluster IV-F (n = 6) viruses; no H3 Cluster IV-D HA were detected (Fig. S9 and Table S2). The within and between clade nucleotide distance criteria for delineating clusters proposed in Anderson et al. (2013) were consistent with this extended dataset (Table 1 and 2).

All NA genes circulating among US swine are N1 or N2 subtypes. NA genes from N1 subtype viruses (n=341) were categorized as classical swine or H1N1pdm09 lineages, while N2 subtype viruses (n=468) were classified as 1998- or 2002-human seasonal H3N2 lineages (Nelson et al., 2012). N1 genes of classical swine origin accounted for 87.4% (n=298) while N1 genes of H1N1pdm09 origin accounted for 12.6% (n=43) of N1 detections (Fig. S2). N2 genes of the 1998 lineage accounted for 6.0% (n=28) whereas N2 genes of the 2002 lineage accounted for 94.0% (n=440) of N2 detections.

Matrix genes were characterized by phylogenetic analysis as North American swine or H1N1pdm09 lineage (Lorusso et al., 2011). The 2013 through March 2014 M data (n=810) contained 90.7% (n=735) H1N1pdm09 lineage viruses and 9.3% (n=75) North American swine lineage viruses (Fig. S3). A 33.0% increase in pandemic matrix genes was detected compared to 2012, indicating widespread reassortment between viruses carrying the pandemic matrix with endemic swine viruses. While the HA and NA from the H1N1pdm09 have dramatically decreased over recent years since 2009, the M pandemic gene detections continued to increase, suggesting an unknown advantage for this gene.

3.3 *H1γ-2 isolates demonstrated divergent antigenic properties within and between the H1 clusters and with vaccine anti-sera*

We characterized the antigenic inter-relationships among a subset of 45 H1 viruses consisting of reference swine influenza virus strains and representative strains from the emerging H1γ-2 cluster (Figure 3). The antigenic map shows that of the 6 H1γ-2 viruses we characterized in HI assays, only the isolate from Minnesota (A/swine/Minnesota/A01201429/2011) is antigenically similar to the previously circulating H1γ cluster strains, albeit 1.9 antigenic units away from the 2007 H1γ clade reference strain (A/swine/Ohio/511445/2007). The isolate from South Dakota is over 4 antigenic units away from the H1γ reference strain and maps closest to circulating H1N1pdm09 cluster strains from 2009-2011. The Oklahoma isolate (A/swine/Oklahoma/A01134906/2011) and one from Illinois (A/swine/Illinois/A01047930/2011) are antigenically similar to each other, but approximately 7 antigenic units from the reference strain. The second isolate from Illinois (A/swine/Illinois/A01203922/2012) and the isolate from Iowa (A/swine/Iowa/A01510129/2012) are also antigenically similar to each other, again approximately 7 antigenic units from the H1γ reference strain, but in a different trajectory from the IL/11 and OK/11 isolates. We aligned the HA1 coding region of the HA protein and from this we determined a number of potential amino acid substitutions that are likely associated with such dramatic antigenic variation (Table S3). For example, the two pairs of antigenically outlying strains differ from each other at positions E186K and R189Q. These two substitution positions would be equivalent to position 189 and 193 in H3 (Burke and Smith, 2014) and have been shown in H3 to be associated with significant antigenic drift (Koel et al., 2013; Lewis et al., 2014).

Against the vaccine antisera, A/Swine/South Dakota/A01349306/2013 and A/swine/Minnesota/A01201429/2011 demonstrated low but detectable titers with all three vaccines, with reciprocal titers ranging from 20 to 80. However, the remaining four H1γ-2 viruses had reciprocal titers of 20 or less against the three vaccine antisera, suggesting a potential risk of vaccine failure against the novel H1γ-2 clade. These HI data were consistent with the positioning of A/Swine/South Dakota/A01349306/2013 and A/swine/Minnesota/A01201429/2011 closer to H1γ and H1N1pdm09 strains in the antigenic map, whereas the other four demonstrated greater antigenic distance from the

contemporary swine H1 clusters.

4. Discussion

Swine IAV continues to circulate in North America in spite of control efforts, causing considerable morbidity in the swine population (Brown, 2000), and representing a potential risk to the human population (CDC, 2012a; CDC 2012b). Here, we studied the evolution of co-circulating swine IAV using passive surveillance data sourced from across 30 U.S. states. In doing so, we detected a novel clade of H1γ-2 viruses that had sustained transmission in North American swine but went largely undetected for 5-8 years. We demonstrated that these contemporary viruses have unique reassorted genome constellations via 18 whole genome-sequenced H1γ-2. We found that all the isolates predating the surveillance system (9 of 9) contained North American TRIG M, NP, NS and polymerase gene segments, whereas the majority of those collected from 2009-2014 (8 of 9) had reassorted with H1N1pdm09 viruses. In addition, we continued to observe an increase in detection of the pandemic matrix gene segment, suggesting an unknown advantage for viruses acquiring this gene (Nelson et al. 2012; Anderson et al. 2013).

Further, we demonstrate that not only is there marked antigenic variation within this novel H1γ-2 clade (between 1.9 and 7 antigenic units among the antigenically characterized strains of this novel clade) but importantly, there are also antigenic differences between this novel clade and other currently circulating H1γ and H1N1pdm09 that would be more likely to be included in current swine vaccines. The antigenic distance is consistent with a number of amino acid changes in the HA1 domain compared among the H1γ-2 HA and between H1γ-2 and other swine-lineage H1. The diversity that we document herein presents two distinct problems: first, currently formulated vaccines are likely inadequate as a means of H1γ-2 control in pigs; and secondly, there is a potential risk of swine variants such as these emerging in the human population due to the antigenic distance from seasonal H1 viruses, including H1N1pdm09 and pre-2009 seasonal strains (Nelson et al., 2014). Our data indicate between 2.1 and 4.6 antigenic units separate the H1γ-2 swine strains from the prototypic human H1N1pdm09 strain (A/CA/4/2009). The sustained transmission of the H1γ-2 with years of going unreported despite their widespread geographic distribution

underscores the need for the national surveillance system and increased participation from under-sampled populations.

Previous studies using active and passive surveillance in the U.S. revealed continuous circulation of at least 7 genetic clades of swine IAV in vaccinated and non-vaccinated commercial farms (Anderson et al., 2013; Corzo et al., 2013). In addition, antigenic studies on swine IAV demonstrated that increased levels of genetic diversity have functional antigenic consequences (Feng et al., 2013; Lewis et al., 2014). We demonstrate 3 major phylogenetic clades representing 83% of all co-circulating IAV in North American swine in 2013-2014 alongside an additional 12 diverse but minor milieu of genetic clades. From these data, current swine management and/or IAV control practices appear insufficient to prevent IAV from being persistently transmitted within and between swine production facilities (Rose et al., 2013) and across seasons (Anderson et al., 2013). A potential explanation for the continual circulation is that vaccines may only decrease IAV transmission rather than eliminate it (Romagosa et al., 2011); and further, given relatively mild or subclinical disease presentation, there may be less impetus for implementation of vaccination programs (Bowman et al., 2012a; 2012b). Although this may be financially appealing in the short-term, a long-term vision is needed for controlling IAV in swine: swine IAV represents a substantial economic burden (Rajao et al., 2014); strains with seemingly low disease phenotypes can gain virulence through adaptive mutations or reassortment or contribute to multi-factorial pneumonia when combined with additional pathogens or environmental stressors; the risk of incursion of a newly introduced strain of IAV is relatively high due to the widespread circulation of diverse swine IAV genotypes with potentially dramatic consequences in naïve herds; humans and swine express similar influenza virus-binding α-2,6 sialic acid receptors that can facilitate bidirectional transmission (Imai and Kawaoka, 2012); and swine can play an important role in the generation of novel viruses via reassortment (Nelson et al., 2012).

Currently in North America there are a number of commercially available swine IAV vaccines (reviewed in Reeth and Ma, 2012). While manufacturers disclose the phylogenetic “cluster” of the strains that the product contains, they do not provide genetic or antigenic data. Regardless of the exact constitution

of these formulated vaccines, the marked genetic diversity we document poses a significant problem to their use as an effective means of control as demonstrated by the HI data with the vaccine antisera reported here. Experimental studies in North America and in Europe (e.g., Loving et al., 2013; Van Reeth et al., 2001) revealed that protection against infection can be correlated with the genetic relatedness of the vaccine strain to challenge strains (but see Kyriakis et al., 2010). However, despite the observation that there are broad antigenic clusters in H3 and H1 swine IAV (Feng et al., 2013; Lorusso et al., 2011), it appears that as few as one or two amino acid changes in the HA-1 domain are enough to drive a more than 4-fold reduction in relative cross-reactivity (Lewis et al., 2014). To counter this problem, there are three potential strategies that are not mutually exclusive: the implementation of vaccine platforms that provide broader heterologous protection (e.g., Rajao et al., 2014; Vincent et al., 2012); the generation of more immunogenic vaccines via appropriate dosage and adjuvants; or the regular reconstitution of vaccine formulations to reflect circulating genetic and antigenic diversity. Given that it is unlikely even a highly immunogenic vaccine will protect against all known circulating diversity (Anderson et al., 2012), regular assessment of the predominant antigenic clades will facilitate the appropriate selection of vaccine seed strains and may help prevent human zoonotic events such as variant H3N2 (Wong et al., 2012).

Interestingly, our data revealed a statistically supported clade of 37 viruses that we classify as H1γ-2 that included 11 contemporary isolates (Fig. 1). The H1γ-2 was first identified in Iowa in 2003, with two thirds of the H1γ-2 viruses identified from 2003 – 2007. The H1γ-2 lineage was only detected in IA and TX in 2003, but appeared to have spread throughout the midwest (MN) and south-central (OK, MO) U.S. by 2004. In subsequent years, in addition to SD and IL, H1γ-2 viruses were detected in southeastern states such as NC. Following initial records in 2003, viruses in this clade continued to circulate in swine herds across the U.S. until 2007, followed by a period of no detection between 2008-2009, and then evidence of onward transmission in 2010 – 2013. Millions of hogs are transported within the U.S. in addition to the millions more that are imported from Canada and other counties as part of the integrated North American swine market (USDA-NASS: http://usda.mannlib.cornell.edu/). Likewise, the USA exports thousands of

live pigs to Mexico (as well as mainland China and Russia) each year (USDA-NASS: http://usda.mannlib.cornell.edu/). These movements provide a potential route for the introduction and transmission of genetically distinct swine IAV such as the H1γ-2. Though not explicitly tested, the dissemination of the H1γ-2 clade does not appear to follow swine transportation routes from south-central U.S. and southeastern U.S. to the Midwestern U.S. documented in swine H1δ1 and H1δ2 IAV and porcine reproductive and respiratory syndrome virus (Nelson et al., 2011; Shi et al., 2010). The long branch lengths and absence of detection over long periods of time suggest gaps in surveillance in herds that are infected with H1γ-2. Further, although H1γ-2 was infrequently detected, the persistence of this viral lineage indicates potential co-circulation with a risk of expansion, particularly as these isolates may be more than 7 antigenic units from other contemporary H1γ or other H1 cluster viruses and found in states with relatively high population densities of hogs.

5. Conclusion

In this study, we identified a novel clade of H1 viruses that we classified as H1γ-2 due to their divergence from the major clade of H1γ viruses approximately 20 years ago. These viruses appear to be endemic in North American swine, are geographically widespread, and have reassorted with other endemic swine IAV and H1N1pdm09. In addition, we demonstrate that current vaccine formulations are unlikely to protect against these H1γ-2 viruses due to substantial antigenic drift from H1γ viruses during many years of independent evolution. These data highlight the importance of the passive USDA Influenza Virus Surveillance System in swine for detecting minor variants that present a threat to swine and potentially to human health, and for providing a more complete understanding of the evolutionary dynamics of IAVs in North American swine (Jhung et al., 2013; Ma et al., 2009; Vincent et al., 2013).

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Figure legends

Figure 1. Phylogenetic relationships of classic lineage swine H1. Time-scaled Bayesian MCC tree inferred for the HA (H1) sequences of 378 swine IAVs. Isolates are color-coded and labeled by the HA lineage. H1α are cyan; H1β are blue; H1γ are green; and H1γ-2 are purple. The clade in black is closely related to H1γ viruses, but appears not to have persisted in swine herds, circulating only from 2003-2007. Posterior probabilities >0.9 are included for key nodes; phylogeny with tip labels included is presented in Figure S10.

Figure 2. Maximum-likelihood phylogeny of NA N1 gene swine influenza A viruses collected as part of the United States Department of Agriculture surveillance system from 2009-2014. These data represent 782 viruses, along with an additional 45 putative H1γ-2 viruses that predated the surveillance program were also (total n=827). The branches are colored by NA genetic lineage; N1 NA classic swine are colored gray; N1 NA H1N1pdm09 viruses are colored red; the H1γ-2 viruses are identified and colored purple. The scale bar represents nucleotide substitutions and tree is midpoint rooted for clarity. A phylogeny with tip labels included is presented in the supplementary materials.

Figure 3. 3D antigenic map of swine influenza A (H1N2 and H1N1) from 2009–2013. The relative positions of isolates (colored spheres) and antisera (open grey cubes) were computed (A) such that the distances between isolates and antisera in the map correspond with the least error to measurements in the HI assay (Smith et al. 2004). The white scale bar represents 1 unit of antigenic distance, corresponding to a twofold dilution of antiserum in the HI assay. The strains are colored by phylogenetic cluster: H1α (pink), H1β (cyan), H1γ (green), H1N1pdm09 (red) and H1γ-2 (purple). The strain names for the numbered purple isolates are shown in the legend.

565

566 Table 1. Average percentage pairwise nucleotide distances within and between H1 antigenic clades

		Between (%)					
	Within (%)	H1α	H1β	H1γ	H1γ-2	H1pdm09	H1δ1
H1α*	14.5						
H1β	5.1	18.9					
H1γ	2.4	17.8	13.2				
H1γ-2	4.3	19.7	15.8	11.8			
H1pdm09	1.7	18.4	14.1	9.40	13.2		
H1δ1	3.0	43.8	40.0	41.0	41.6	41.6	
H1δ2	4.7	44.3	40.0	40.0	40.2	41.7	11.3

567 *H1α is represented by 2 isolates in USDA surveillance data from 2009-2014 (see Table S2);

568 consequently, these data may not represent true diversity.

569

570 Table 2. Average percentage pairwise nucleotide distances within and between cluster IV H3 antigenic

571 clades designated using the terminology of Kitikoon et al. (2013).

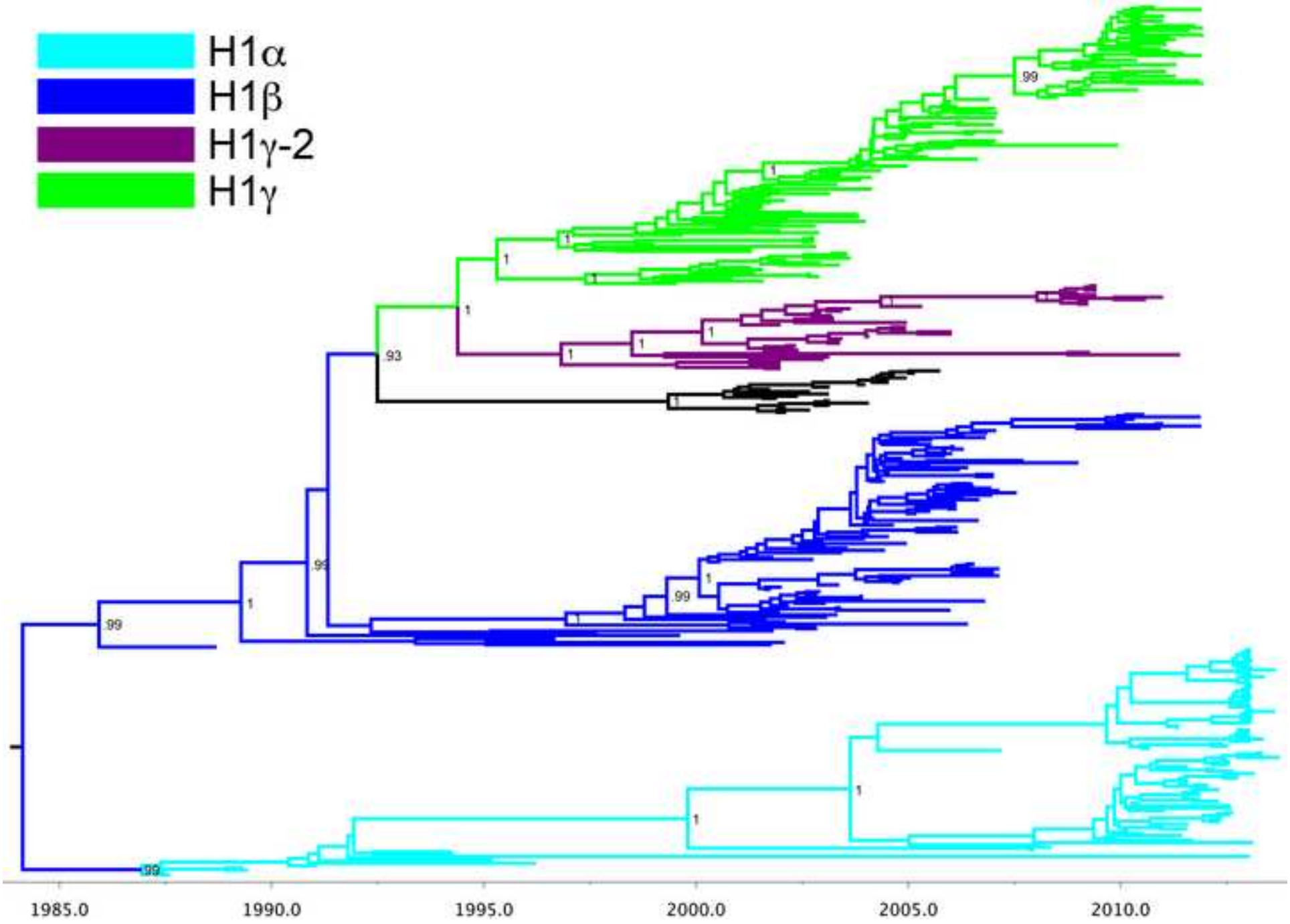
572

		Between (%)					
	Within (%)	IV	IV-A	IV-B	IV-C	IV-D	IV-E
IV	4.0						
IV-A	1.3	5.9					
IV-B	3.4	5.8	6.3				
IV-C	1.4	6.6	7.0	7.1			
IV-D	4.1	5.4	6.1	6.0	6.8		
IV-E	1.3	5.6	5.6	6.1	6.9	5.5	
IV-F	1.3	6.5	6.6	7.3	7.6	6.6	6.7

573

574

Figure 1


H1α
H1β
H1γ-2
H1γ
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2000.0
2005.0
2010.0

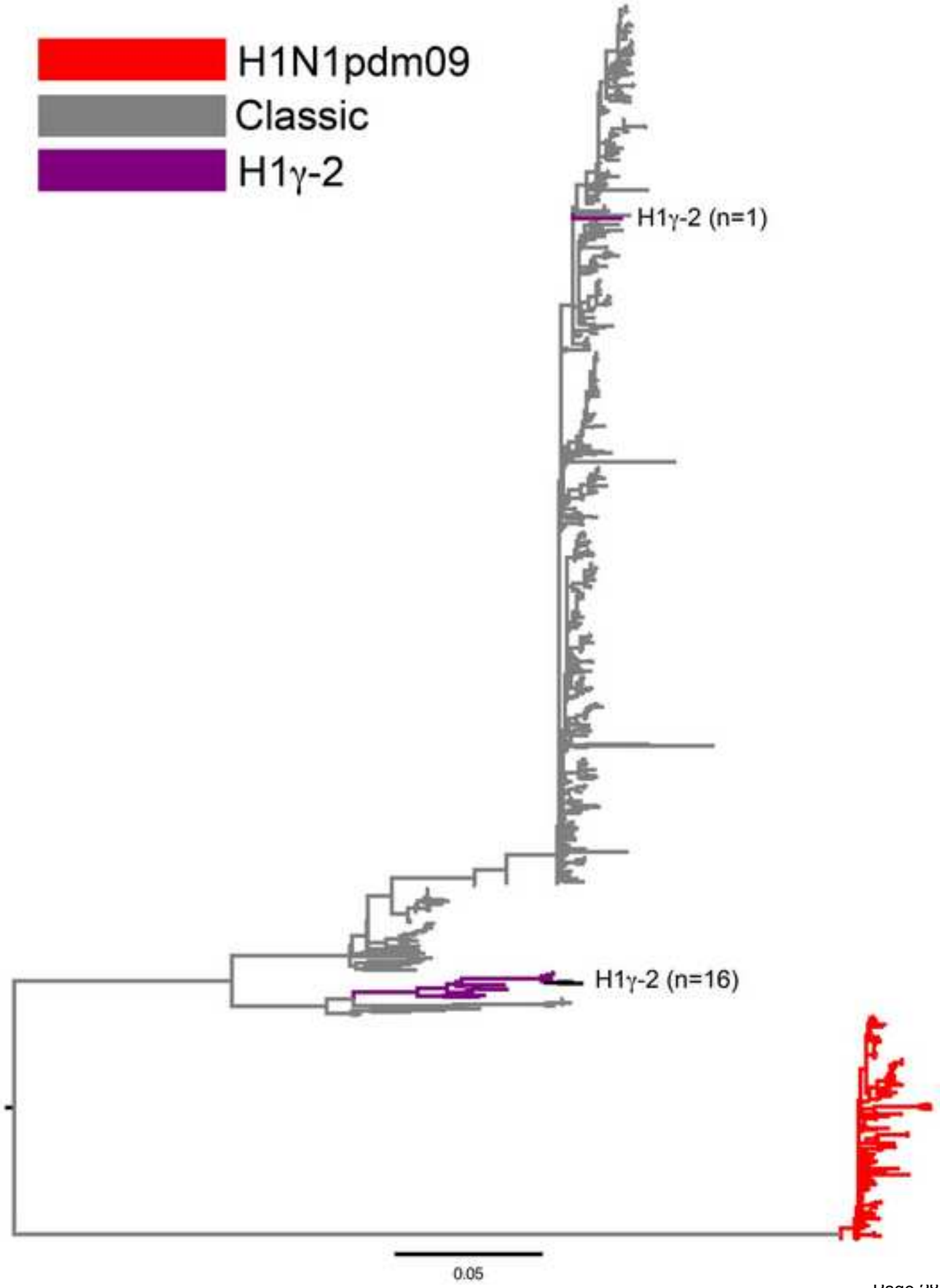
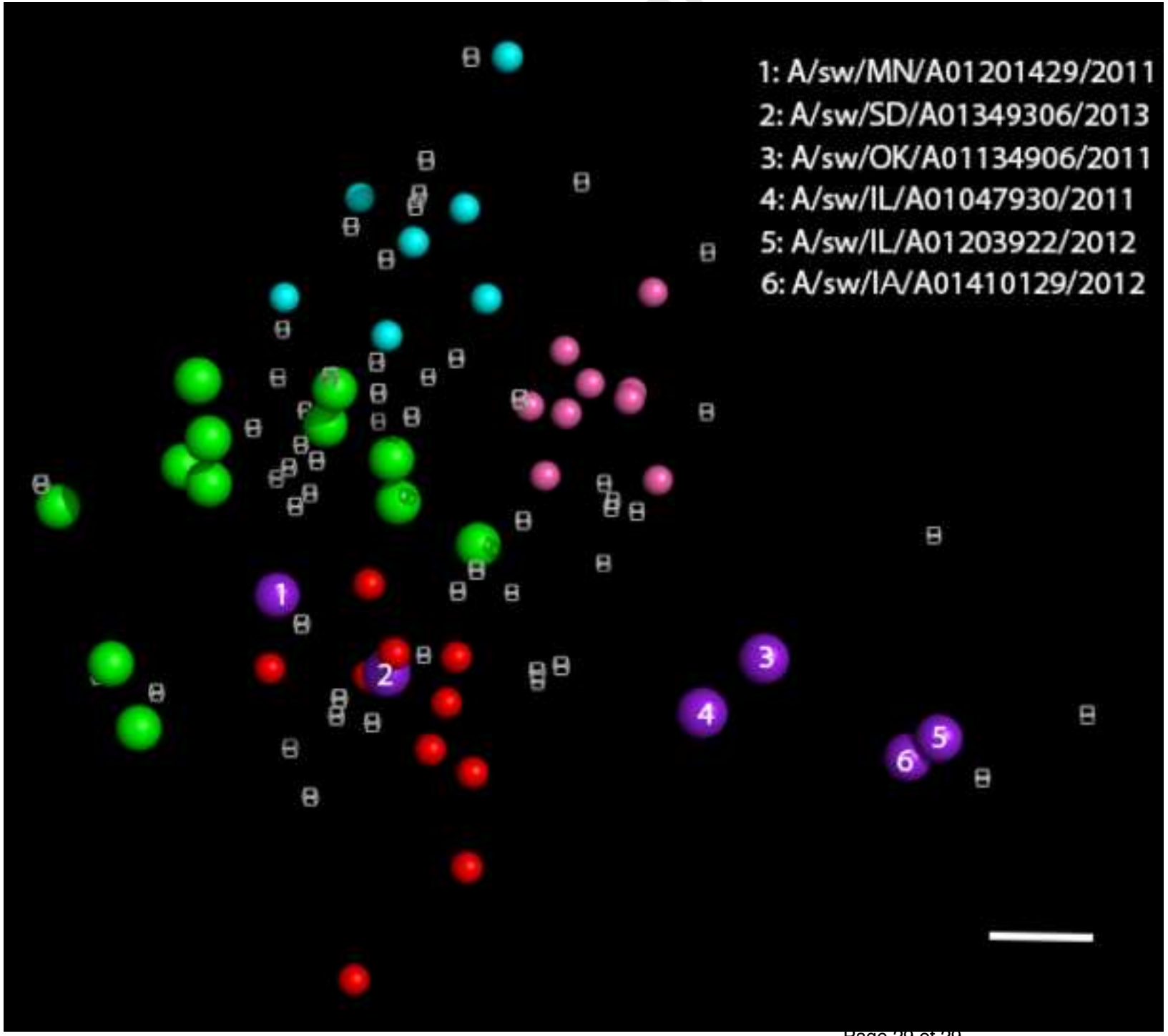


Figure 3


1: A/sw/MN/A01201429/2011
2: A/sw/SD/A01349306/2013
3: A/sw/OK/A01134906/2011
4: A/sw/IL/A01047930/2011
5: A/sw/IL/A01203922/2012
6: A/sw/IA/A01410129/2012
1
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