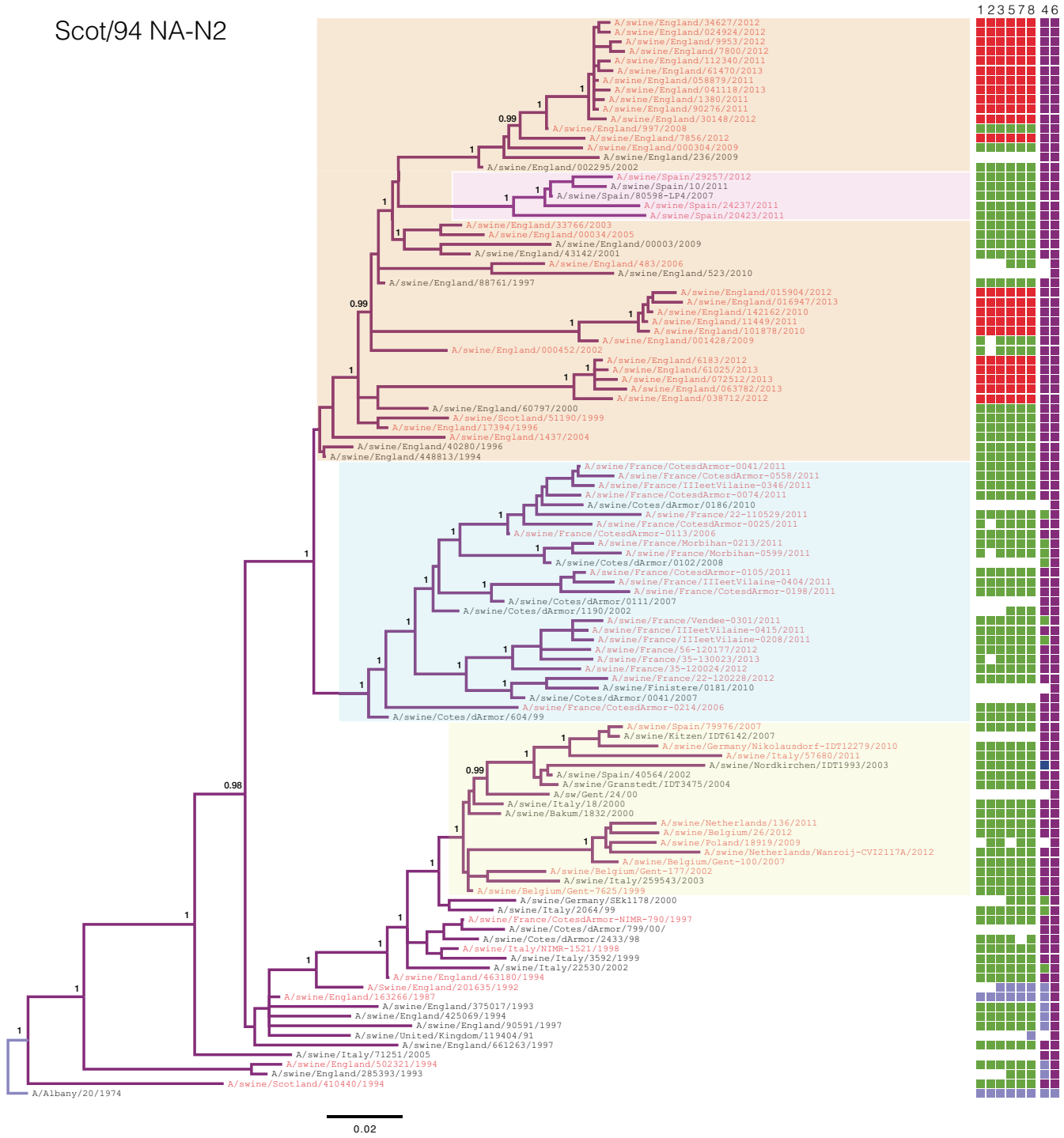

FIG 4 Phylogeny of the Scot/94 lineage N2 gene inferred using Bayesian analysis. Taxa sequenced by members of the ESNIP3 consortium are highlighted in red, while those in black were obtained from the Influenza Virus Resource. Colored squares to the right of each taxon name indicate the corresponding genotypes, with coloring and segment order as described for Fig. 1. White squares indicate that no sequence was available for that segment. Posterior probabilities are given at selected nodes. Colored highlights indicate well-supported circulating clades. The scale bar is given in numbers of substitutions per site.

served in European swine only since late 2009. As a result of this recent emergence, the genetic diversity of the virus is low, and the phylogenetic branching in the lineage is incompletely resolved, resulting in polytomies for all of the segments. To place the swine isolates in the context of the human outbreak, a molecular clock phylogeny was estimated for the internal gene cassette, combining all European swine and human isolates. The phylogeny shows the swine isolates interspersed throughout the tree, with an estimated minimum of 32 different transmissions of the virus from humans to swine (see Fig. S2 in the supplemental material). Due to the

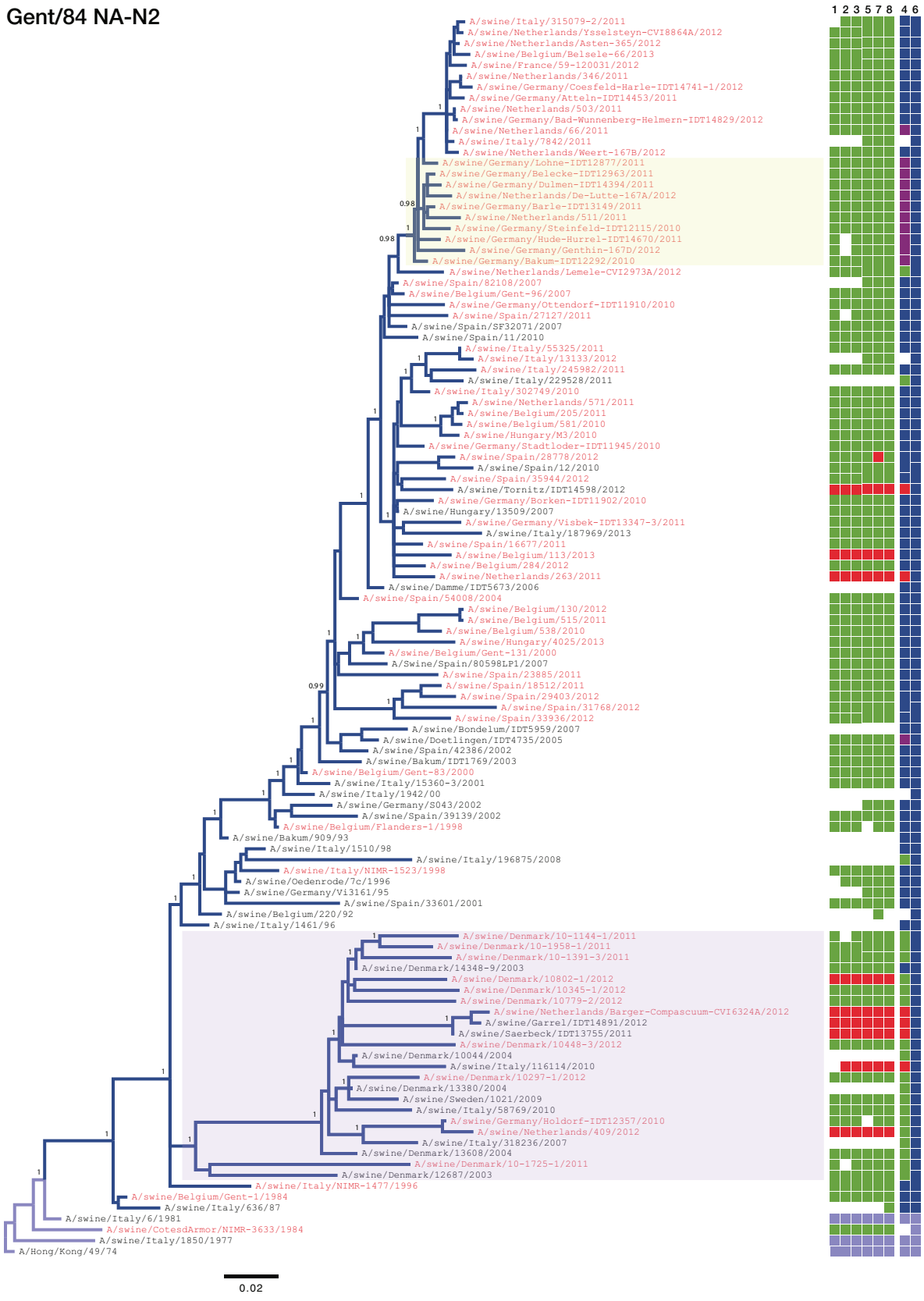

FIG 5 Phylogeny of the Gent/84 lineage N2 gene inferred using Bayesian analysis. Posterior probabilities are given at selected nodes, and the scale bar is given in numbers of substitutions per site. See Fig. 4 legend for other details.

TABLE 1 Statistical support for the association of host species with ancestry for the HA-H1, NA-N1, and PB2 segments of the A(H1N1)pdm09 lineage[a]

Segment	Statistic	Observed value			Null value			Significance (*P*)
		Mean	Lower 95% CI	Upper 95% CI	Mean	Lower 95% CI	Upper 95% CI	
HA-H1	AI	6.369	5.287	7.432	9.022	8.019	10.084	0.039
	PS	35.842	34	37	40.909	39.477	41.750	<0.01
NA-N1	AI	4.605	3.561	5.714	8.134	7.264	9.017	<0.01
	PS	32.886	31	35	45.541	44.012	46.573	<0.01
PB2	AI	4.883	3.987	5.804	6.925	5.951	7.792	<0.01
	PS	28.680	28	30	38.070	36.591	38.858	<0.01

[a] AI, association index; PS, parsimony score; CI, Bayesian credible interval.

limited sampling of A(H1N1)pdm09 in swine, it is not clear from the molecular clock phylogeny whether the virus circulated enzootically in European swine or whether it was maintained through continual short-lived introductions from humans. To assess this issue statistically, Bayesian phylogenies containing human and swine isolates were inferred for the A(H1N1)pdm09 PB2 segment (see Fig. S3). Statistical testing of the association between the host species and the phylogenetic relationship ancestry using BaTS showed that the swine isolates clustered more often than expected by chance, suggesting that some of the introduced A(H1N1)pdm09 virus had been circulating within swine in Europe (Table 1). The BaTS test was repeated for the HA-H1 (see Fig. S4) and NA-N1 (see Fig. S5) segments, with the results again showing significant clustering of the swine isolates (Table 1). Together, these results suggest that both the internal genes and the external genes of the A(H1N1)pdm09 virus are clustering more than expected by chance.

Reassortant genotype of public-health interest. A genotype of particular note is the triple reassortant isolated in Spain in 2012 containing Gent/84 external glycoproteins and EA internal genes but an A(H1N1)pdm09 matrix segment (A/swine/Spain/28778/2012). This genetic makeup is comparable to that of the North American A(H3N2)v strain that has been associated with multiple swine-to-human zoonoses in North American swine fairs (44); both contain human-derived H3 and N2 glycoproteins that have since evolved within swine, and both contain internal gene cassettes with an acquired pdm09 matrix protein. This constellation therefore poses a potential public health risk, particularly as its external glycoproteins have been antigenically evolving within swine for over 30 years, so humans will likely be immunologically naive against the virus. Because of the possible public health interest in this genotype, this isolate was reextracted and resequenced, confirming its makeup.

DISCUSSION

The genomic characterization of 290 European swIAV, of which 243 were sequenced as part of this study, is reported here. These genomes define the diversity of swIAV across Europe between 2009 and 2013, during which time the A(H1N1)pdm09 lineage was introduced back into swine through reverse zoonosis. The introduction of this new swIAV lineage into European swine increased the complexity of the circulating genotypes, resulting in an increase in the number of reassortment possibilities. In total, 23 different genotypes were observed among 278 genomes from European swine. In contrast, a study by Liang et al. in southern China over a comparable time period found 29 genotypes among 387 genomes (21). This reduced reassortment diversity in European swIAV is a result of both a smaller number of genotypes and a bias toward fewer genotypes. Furthermore, reassortment involving the IGC was rare in European swIAV, with just three (1%) isolates found to contain a reassortant IGC. In contrast, reassortment of the internal segments was observed frequently in southern China, with multiple reassortant genotypes persisting in the swine population. This is likely to be due to the presence of the TR lineage in China, in addition to the EA and pdm09 lineages, because the majority of IGC reassortment events observed by Liang et al. involved the TR lineage (21). Only one EA/pdm09 IGC reassortant—an EA genotype with an A(H1N1)pdm09 matrix gene—was isolated recurrently in China which was also isolated in this study (genotype M). This reassortment difference extends to North America, where the TR lineage first arose and is the predominant lineage; although the frequency of interlineage reassortment within the internal gene segments was lower than for southern China, it was still considerably higher than in Europe (24).

Despite 23 distinct genotypes being found in European swine, only four (A, B, C, and P) were found to be circulating across the whole of Europe. A further six (D, E, F, G, Q, and R) were found in geographically constrained areas; i.e., they were highly frequent in a single country, with occasional outbreaks in other countries. The remaining 13 genotypes were isolated sporadically and infrequently, suggesting that these were perhaps less-fit reassortants that were identified due only to the extent of surveillance. It is likely that regular whole-genome-sequencing-based surveillance of human, swine, and avian influenza will provide a more compelling catalogue of the diversity and fitness landscape of IAV reassortants than is possible through *in vitro* studies.

The four genotypes circulating throughout Europe include the three lineages that have been enzootic and prevalent in European swine for at least 19 years: EA $H1_{av}N1$, Gent/84 H3N2, and Scot/94 $H1_{hu}N2$. However, these genotypes have different frequencies and dynamics across mainland Europe. While $H1_{av}N1$ was found at a high frequency in all countries, the prevalences of H3N2 and $H1_{hu}N2$ were inversely related. These observations are consistent with the data obtained through preliminary subtyping of the ESNIP3 samples (25). Differences between the lineages were also observed in phylogenetic analysis of the two lineages' glycoprotein segments; the $H1_{hu}N2$ phylogeny showed greater genetic diversity at any point in time through the presence of long-lived lineages that circulate independently in different countries (Fig. 4). Con-

versely, the H3N2 lineage had less genetic diversity at any point in time due to its rapid turnover of short-lived lineages that were geographically diffuse (Fig. 5). This dynamic relationship between the two lineages is similar to the relationship between the A/H1N1 and A/H3N2 subtypes in humans (45) or between the B/Yamagata and B/Victoria lineages in humans (46).

The differing phylogenies of the $H1_{hu}N2$ and H3N2 lineages give an insight into their epidemiological dynamics. The ladder-like phylogeny of H3N2 suggests that the lineage is under strong selective pressure, against which the virus fixes advantageous mutations rapidly along the trunk of the tree, with loss of the side branches that do not contain the variation (47). The cocirculation of multiple subclades of the $H1_{hu}N2$ lineage within each country, however, suggests that the virus is not subject to the same intense selective pressures as the H3N2 lineage. This could be due to reduced cross-reactive immunity in swine between the $H1_{hu}N2$ subclades. This difference in selective pressure on the two lineages may explain why Gent/84 H3 was found only in conjunction with Gent/84 N2, whereas Scot/94 H1 was apparently more able to reassort with the NA of other lineages (Fig. 1). Furthermore, this inverse relationship between H3N2 and $H1_{hu}N2$ may be influenced by evolutionary dynamics and selection—in swine populations in countries such as France and the United Kingdom, the emergence of $H1_{hu}N2$ correlated with the disappearance of H3N2. Possibly immunity to N2 had an influence, favoring selection of the lineage with greater diversity and an opportunity for selection of fitter viruses in swine. The cause of the apparent absence of such pressures in other major swine-producing countries (Belgium, Germany, The Netherlands, and Spain) where H3N2 and $H1_{hu}N2$ coexist is unclear, though it may be due to differences in their swine production systems compared to those of the United Kingdom and France. The latter have a relatively low pig density (<90 heads/hectare of agricultural area in 2010) compared to the former (>90 heads/hectare). The Netherlands, Belgium, Spain, and Germany have among the highest swine densities in Europe, with The Netherlands having had as many as 704 heads/hectare in 2010 (http://www.fao.org/docrep/017/i3138e/i3138e07.pdf). A higher swine density has been previously shown to increase the risk of seroprevalence for influenza and as such may be associated with the cocirculation of the two genotypes (48). However, a more formal statistical assessment of the predictors is needed and will require examination of different industry structures, production systems, and vaccination usage to better understand the underlying factors influencing virus evolution.

The A(H1N1)pdm09 virus that emerged in humans in early 2009 was the third-most-frequent genotype found in swine across Europe. The first confirmed disease outbreak in European pigs was in September 2009, but it is highly possible that the virus crossed to pigs from humans earlier, after its emergence in Europe in April (43). In this study, we have estimated that at least 32 separate introductions of the A(H1N1)pdm09 virus from humans into swine have occurred in the period though 2013, and we find phylogenetic evidence that the virus circulates endemically among swine. This finding is in contrast to those of the study conducted in southern China, where the A(H1N1)pdm09 virus did not persist after each introduction and its internal genes were maintained in swine only through reassortment with the HA and NA genes of other enzootic lineages (21). However, we also observed replacement of the A(H1N1)pdm09 external glycoproteins through reassortment with enzootic lineages, notably acquiring the H1 and N2 from the $H1_{hu}N2$ lineage (genotype C) in the United Kingdom or acquiring the N2 from the H3N2 lineage (genotype B) in Germany. The prevalence of these A(H1N1)pdm09 reassortants has previously been noted (49–52) and suggests that, as in southern China, the internal genes are highly compatible with glycoprotein segments from enzootic lineages and that the circulation of the A(H1N1)pdm09 in European swine therefore increases the reassortment potential for European swIAV.

Rates of infections in swine of the A(H1N1)pdm09 virus differed across Europe; mainland Europe was found to have an average frequency of 8%, which is in agreement with the 9% proportion found from the preliminary subtyping of European swine (25). Swine in the United Kingdom, however, have shown a near-complete replacement of their enzootic $H1_{av}N1$ and $H1_{hu}N2$ viruses with A(H1N1)pdm09 and pdm09-$H1_{hu}N2$ viruses. Phylogenetic analysis of the Scot/94 H1 and N2 segments showed that at least four separate sublineages of EA-$H1_{hu}N2$ each replaced their EA IGC with the pdm09 one, consistent with the idea that the pdm09 IGC is fitter in swine. The relative proportions of the $H1_{av}N1$ and $H1_{hu}N2$ subtypes have remained the same since the replacement of the EA lineage by the pdm09 one; a serological study of United Kingdom swine between 2008 and 2009 showed that $H1_{hu}N2$ was the predominant subtype, detected in 45% of all farms, with the $H1_{av}N1$ subtype found in approximately 21% of farms (53). Consistent with this, we showed here that, since the introduction of the pdm09 virus in United Kingdom swine, the frequency of the pdm09-$H1_{hu}N2$ is approximately 54% whereas that of the pdm09 virus is 27%. The reasons for the difference in prevalence between mainland Europe and the United Kingdom are unclear and warrant further investigation.

A triple reassortant genotype containing an EA IGC with a pdm09 matrix gene and Gent/84 H3 and N2 segments (genotype N) was observed in a single Spanish pig (A/swine/Spain/28778/2012). Acquisition of the pdm09 matrix protein by enzootic swIAV has been previously noted in China (21) and also in the United States, where the resultant A(H3N2)v genotypes were able to infect humans (54, 55). Genotype N has a genetic makeup similar to that of A(H3N2)v, and, importantly, its external glycoproteins are derived from the Gent/84 H3N2 lineage that has been evolving in swine since the early-to-middle 1970s (1). As such, humans are likely to be immunologically naive against this virus, and it thus poses a potential public health risk. Given the level of reassortment observed in European swine, further surveillance efforts should be sought to track the emergence and potential spread of such genotypes with human-pandemic potential. Whole-genome sequencing of swIAV isolates is an important aspect of this surveillance effort, without which the dynamics of the circulating lineages cannot be determined. Furthermore, it is only through whole-genome sequencing that the rare, but potentially important, reassortants involving the IGC can be observed. However, the limited IGC reassortment indicates that preliminary subtyping of the HA and NA segments is still suitable for routine surveillance of European swIAV.

This report reveals that the emergences and drivers of virus evolution in pigs differ at the global level. The factors favoring virus emergence and selection are complex, but we show that establishment of new genotypes and lineages is complex and less frequent at the population level. Whole-system analyses performed at the virus host level, together with analysis of the influ-

ence of natural or vaccine-derived immunity, require further investigation.

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