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GENETIC VARIATION OF HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA VIRUSES IN VIETNAM SHOWS BOTH SPECIES-SPECIFIC AND SPATIOTEMPORAL ASSOCIATIONS

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Summary

Domestic poultry act as a reservoir for persistent H5N1 endemicity in Vietnam, and the circulation of poultry flocks across farms and to market is thought to drive the spatial movement and evolution of avian influenza viruses. Using a dataset of complete or nearly full genomic sequences from highly pathogenic H5N1 avian influenza viruses collected in domestic poultry in Vietnam from 2003 to 2007, we explore potential differences in genetic characteristics according to species of isolation and the spatio-temporal characteristics of the viruses. Clustering algorithms and analysis of variance indicate that H5N1 viruses in Vietnam show differences in the amount of genetic change that chicken viruses experience as compared to duck viruses, with duck viruses showing higher rates of molecular evolution on all eight of influenza's gene segments. There also exist distinct patterns of genetic differentiation according to the year in which they were isolated. These findings suggest that genetic evolution of avian influenza viruses is continuous through time but could also be mediated by the species in which the viruses occur, information which has implications for prevention efforts.

Keywords

H5N1 avian influenza; genetic differentiation; poultry; ecology; cluster analysis; analysis of variance

Introduction

Highly pathogenic H5N1 avian influenza was first detected in Vietnam in 2001, and the country was part of a larger endemic emergence of H5N1 across Southeast Asia in 2003 and 2004 (27). Since 2003, Vietnam has remained one of the countries hardest hit by H5N1 avian influenza, with continuing poultry and human infection and mortality (41, 41, 42, 42). H5N1 viruses have undergone rapid evolution in Vietnam since first detection, and since 2003 there have been at least four novel types that have emerged in Vietnamese H5N1 isolates (37). Part of the reason for H5N1's persistence in Vietnam is socio-environmental:

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among Vietnamese there is a preference for live or freshly killed poultry, and a large percentage of rural Vietnamese rear their own backyard poultry flocks (6, 19, 21, 23, 31). Large numbers of susceptible birds, combined with the circulation of birds and people from farms to markets, drive the ongoing H5N1 epidemic (4, 26, 36).

Backyard poultry flocks in Vietnam are composed primarily of chickens and aquatic poultry such as ducks. According to a 2003 livestock census, of Vietnam's 261 million domestic poultry, 73.5% were chickens and 26.3% were aquatic birds (23). Both chickens and ducks in Vietnam are raised by poor rural families as scavenger birds, feeding on insects and other pests, but chickens are confined to the household area while ducks are often taken out of the household into nearby fish ponds and rice fields. Duck populations thus have opportunities for interaction with other domestic duck flocks and with wild or migratory birds in aquatic environments that act as a medium for exchange of viruses. While H5N1 viruses in dried feces quickly lose their infectivity, sometimes in as little as a day, laboratory tests indicate that H5N1 avian influenza viruses can survive in water sources for extended periods of time (2, 5, 12, 25). Ducks infected with H5N1 have been shown to shed viruses not only fecally but also orally, via the trachea (15). Infection can thus be transmitted via feces and saliva through shared water supplies, and ducks have great potential to encounter contaminated water and other environmental surfaces outside the household, while chickens are confined to exposure within the household. Transmission of H5N1 within such domestic poultry could provide the major mechanism by which avian influenza viruses remain endemic in Vietnam (4).

The epidemiology of H5N1 infection differs between chickens and ducks. Prior to 2002, H5N1 infection in domestic ducks was usually asyptomatic or expressed as only minor clinical symptoms (29). Asymptomatic ducks could still shed infective viruses in their feces or saliva, however, for up to two weeks after infection (4, 21). Domestic ducks are thus sometimes referred to as “Trojan horses” of H5N1 infection, unnoticeably sustaining circulation of H5N1 in poultry (16, 21). Since 2002, however, novel genotypes of H5N1 viruses have emerged and been predominant in both domestic and wild birds, and these novel viruses are highly pathogenic in ducks, producing severe systemic infection and lesions in multiple organs (1, 20, 30, 39). In contrast to ducks, chickens experience high (often 100%) mortality after infection with H5N1 viruses, and have symptoms of infection in multiple organs (18, 19). In recent years, H5N1 infections in chickens have become more virulent, as indicated by less time between infection and death (30).

Given these divergent household ecologies and influenza epidemiologies, we sought to answer whether the genetic characteristics of H5N1 avian influenza viruses were correlated with the species in which those viruses occurred. Specifically, did the amount of genetic change that has taken place between Vietnamese viruses and their ancestral virus vary according to species of isolation? Are there differences in the effect of species when considering the spatial and temporal characteristics of viral isolates? Cluster analysis and multiple analysis of variance (MANOVA) were conducted to explore these questions. Our results show that H5N1 viruses exhibit distinct patterns of genetic differentiation according to the year in which they were isolated and that chicken viruses are associated with lower amounts of genetic change than are duck viruses. To our knowledge, this work represents the first spatial and temporal analysis of varying genetic characteristics of highly pathogenic H5N1 avian influenza viruses in Vietnamese domestic poultry.

Materials & Methods

Data for the study consisted of 110 highly pathogenic H5N1 avian flu viruses isolated in Vietnam between 2003 and 2007 (Figure 1). Viruses were either publicly available in online

repositories or collected by the National Centre for Veterinary Diagnostics (NCVD) of Hanoi, Vietnam. For each of the 110 isolates there is a complete or nearly complete genetic sequence as well as the year of isolation and the province in which the virus was found. At least one gene segment of these viruses belong to a single genetic lineage originating in Hong Kong in 2002, and all 110 viruses belong to a single VN3 genotype, whose HA gene belongs to Clade 1 (8, 28, 37, 40). While the exact collection sites of the publicly available viruses are unknown, the NCVD collaborates with the Vietnamese Department of Animal Health's regional offices to detect H5N1 outbreaks in backyard poultry flocks, commercial farms and live bird markets.

Using the putative ancestral virus (A/duck/HongKong/821/2002(H5N1)) as the point of reference, geographic, temporal and genetic distance measures were created for the viral dataset. Viruses were geocoded to the latitude and longitude of the centroid of the province of isolation using a geographic information system (GIS) in order to calculate the distance between the province centroid and Hong Kong's centroid. Temporal distance between the Vietnamese viruses and the progenitor virus was calculated as the number of years since the isolation of the Hong Kong virus in 2002. Eight genetic distance measures, one for each of the influenza viruses eight gene segments, were calculated using PATRISTIC. A patristic distance is the length of the branches connecting two nodes of a phylogenetic tree, and indicates the amount of genetic change that exists between those two nodes (10).

In addition to the distance measures, each virus was assigned a species designation. The species of isolation was determined by the universal virus identification code (e.g. “Ck/VN/19/03” is a virus isolated in a Vietnamese chicken in 2003). Viruses isolated in chickens were assigned “1”, ducks assigned “2”. Within the dataset, 53 viruses were isolated in chickens and 57 in ducks.

Cluster analysis using a model clustering technique was carried out using the *mclust* package in R v.2.9.2 (11). Cluster analysis was used to assess whether viruses grouped according to genetic characteristics would reflect the species designations of the viruses. In model clustering, the eight genetic distance measures are used to partition the 110 viruses such that within-cluster likeness and between- cluster difference is maximized. The model cluster algorithm investigates a variety of shape and size constraints for the clusters, including equal and unequal volume, equal and unequal shape, and spherical, diagonal or ellipsoidal orientation, and returns indications of how well each of these ten models fit the dataset across different numbers of clusters. The number of clusters that viruses will be divided into, the shape of the clusters and the optimum cluster assignment is determined by the highest Bayesian Information Criterion (BIC). The BIC is calculated given the log-likelihood, the dimensionality of the data (8 gene segments) and the number of mixture components (110 viral isolates), and varies greatly according to the model type and number of clusters.

Once viruses were assigned to clusters based on their genetic characteristics, those cluster assignments were mapped in the GIS according to geographic location of viral isolation. Maps of clusters were stratified both by year of viral incidence and species of isolation. This allowed us to assess whether the cluster assignments generated in the model clustering algorithm expressed spatial, temporal or species-specific patterns.

To ascertain whether the results observed in the cluster analysis were statistically significant, MANOVA was performed using the *stats* package in R to simultaneously assess the degree of variation present in the eight genetic distance measures attributable to three potential sources: species type (coded as a factor rather than a categorical variable to avoid the statistical implication that 2 (“duck”) is numerically more valuable than 1 (“chicken”)),

geographic distance and temporal distance (24). Interaction plots were generated to show differences in genetic distances according to species and temporal distance for the eight gene segments.

Results

In partitioning the 110 viruses into clusters, the BIC score of 8127.612 indicated that an eight-cluster partitioning of the data with varying cluster volume and shape and orientation along the coordinate axes provided the best fit (Figure 2). The cluster assignment of all 110 viruses across the eight genetic segments is shown in Figure 3, with the clear grouping of viruses near to one another in genetic space assigned to the same cluster.

When the cluster assignments were mapped according to the species of isolation and province of isolation, stratified by year, distinct temporal patterning but indistinct species patterning was observed. Cluster assignments for chicken H5N1 isolates are mapped in Figure 5, while duck H5N1 isolates are shown in Figure 6. In 2003, chicken viruses were assigned to cluster 1 & 2, and are found only in northern Vietnam. In 2004, chicken isolates are grouped into clusters 1 and 2, while duck isolates are also grouped into cluster 3. In 2005, chicken viruses fall into clusters 3, 4, 5 and 6. Duck viruses in 2005 are assigned to clusters 1, 3, 4, 5 and 6. In 2007, both duck and chicken viruses are assigned to cluster 7, the group with the second highest amount of genetic change from the progenitor virus, but only duck viruses in southern Vietnam are found in the highest cluster of genetic distances, cluster 8.

The genetic characteristics of each viral cluster are closely aligned with the temporal pattern described above. The average genetic distance for each of the eight gene segments in each of the eight clusters is shown in Table 1. Clusters 1 and 2, observed primarily in 2003 and 2004, represent the viral isolates with the lowest genetic distance (.09882 and .07976 respectively), indicating they are closest genetically to the Hong Kong progenitor virus. Viruses taking place in 2005 were assigned to clusters 3 to 6, which have medium-scale average genetic distances (from .11995 to .14602). Viruses isolated in 2007 are furthest away from the progenitor virus in both time and in genetic space, with total average distances of .17028 for cluster 7 and .16839 for cluster 8. It is only in this cluster with the greatest total average genetic distance, cluster 8, that ducks and chickens are assigned to different viral groups.

In the overall MANOVA model (see Table 2), there is statistically significant variation in mean genetic distance across all 8 gene segments according to the species of isolation, as well as according to geographic and temporal distance. Individual ANOVA results for each of the eight gene segments (Table 3) show that there exist significant differences in genetic distance according to species designation. Temporal distance is also a strong axis of variation, with statistically significant differences in genetic distances when stratified by year of incidence (as measured by temporal distance). The F statistics, an indication of how strongly the null hypothesis is rejected, are much higher for the temporal distance model than for the species model. Four of the gene segments also exhibited significant differences in genetic distance according to the amount of geographic distance from the progenitor virus in Hong Kong.

Interaction plots (Figure 7) of mean genetic distances according to species type and temporal distance demonstrate how the genetic profiles of the 110 H5N1 viruses vary according to both gene segment and species of isolation. The scale of genetic distance for the HA and NA gene segments are larger than for the other gene segments, indicating greater genetic change

on those two sections of the influenza genetic code between the progenitor virus and the Vietnam H5N1 dataset.

Variation in genetic distance for chickens versus ducks is seen in the divergence of the Type 1 and Type 2 lines, this divergence occurs in different years for each gene segment. The HA gene segment, for instance, appears to have less variation according to species than does the PB1 or the MP gene segment (average genetic distances for each gene segment according to species are shown in Table 4). The strong influence of time is also shown in the interaction plots, with mean genetic distance spiking as time between the isolation of the progenitor virus and year of isolation in Vietnam increases.

Discussion

The minor differences in cluster assignments between chickens and ducks observed in the cluster analysis were not as robust as the dissimilar epidemiology of H5N1 in the two species would have suggested. However, only duck viruses were assigned to cluster 8, the cluster containing those viruses with the greatest genetic distances from the progenitor virus. The average genetic distances for each gene segment seen in Table 4 also indicate that across all eight segments of the H5N1 genetic code the duck viruses isolated in Vietnam had greater amounts of genetic change. While the variation in genetic distances between species is weaker than between years of isolation, it is still significantly different across all eight gene segments and in the summary model. This is further confirmed in the interaction plots, where chicken and duck viruses in the first year of the study period, 2003, have generally similar genetic distance means, but diverge in the following years.

The importance of time in the evolution of H5N1 avian influenza viruses is not surprising, but is strongly supported in this research. Clear temporal patterns in the cluster assignments can be seen, with viruses at low genetic distances from the progenitor virus grouped together in 2003 and 2004, viruses at further genetic distances assigned to clusters in 2005 and the viruses with the greatest genetic distance from the 2002 Hong Kong virus detected in 2007 and assigned to clusters 7 and 8. The strength with which the temporal progression of the clusters mirrors their genetic characteristics, but with much weaker spatial links to cluster patterns, suggests that genetic change in the dataset is primarily a factor of time, not space. The results from the MANOVA and the interaction plots further indicate the differences in genetic characteristics of viruses across the length of the study period. For seven of the eight gene segments, 2007 (represented as 5 years of temporal distance from the progenitor virus) has the highest mean genetic distance among viruses of both species.

The massive vaccination campaign undertaken by the Vietnamese government in 2006, focusing on geographic regions with high levels of H5N1 infection, could potentially influence these findings. Vaccination ideally reduces the rate of mutation by reducing the amount of viral reproduction, shedding and transmission among infected and susceptible birds (3, 7, 34). Vaccination, however, also has the potential to increase viral mutation via selection for vaccine-resistant traits (9). Both chickens and ducks were included in Vietnam's vaccination campaign of 2006, and studies have found comparable rates of protective efficacy for both species in terms of morbidity and mortality with a number of different vaccines (17, 22, 32, 35, 38). Within this study, the amount of genetic change from 2005 (prior to vaccination efforts) to 2007 (following vaccination efforts) increased for both chickens and ducks on seven of eight gene segments (Figure 6). Additionally, the trajectories for both chickens and ducks are similar between these years. Thus, while the vaccination status of viral hosts is unknown in this study, it is unlikely that the higher rate of genetic change observed among duck isolates is caused by differing levels of vaccination among duck and chicken populations.

The differing epidemiology of H5N1 viral infections in chickens and ducks appears to also express itself in the genetic characteristics of the viruses isolated in each of those species. As chickens typically experience higher rates of mortality and faster courses of infection, there is perhaps less time for them to experience co-infection with multiple viral strains or to transmit the virus to other chickens in their flock or other flocks. In contrast, the longer duration of infection in asymptomatic but virus-shedding ducks could allow for greater viral mixing and possibly account for the greater genetic distance seen in duck isolates in Vietnam.

Previous research in Thailand implicated free-ranging duck populations as a driver of H5N1 incidence and suggested that new restrictions on the housing and grazing of ducks would decrease H5N1 outbreaks in that country (13, 33). In Vietnam, the sharing of duck grazing areas among multiple farms was positively associated with H5N1 outbreaks (14). Perhaps free-ranging backyard duck populations in Vietnam are driving not only H5N1 incidence in Vietnam but also viral evolution. Chickens are typically confined to the backyard of households whereas ducks are generally free-ranged and travel outside of the household to fish ponds and rice fields. Thus, duck epidemiological response to influenza infection, coupled with their ecological patterns, could allow for greater viral mixing, including those viruses from wild birds, and for emergence of novel genotypes. Our study suggests that attempts to control influenza in duck populations, particularly via regulation of backyard duck husbandry practices, could also curtail the evolution of H5N1 viruses.

Abbreviations

BIC	Bayesian Information Criterion
GIS	Geographical Information System
MANOVA	Multiple Analysis of Variance
NCVD	National Center for Veterinary Diagnostics
RNA	Ribonucleic Acid

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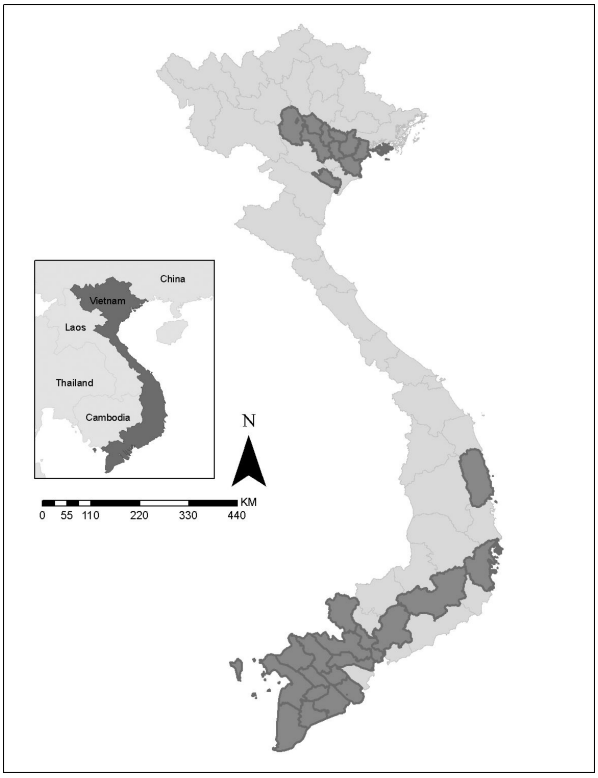

Figure 1.
Distribution of 110 chicken and duck H5N1 viruses in Vietnam. Darkened provinces indicate locations of virus isolation.

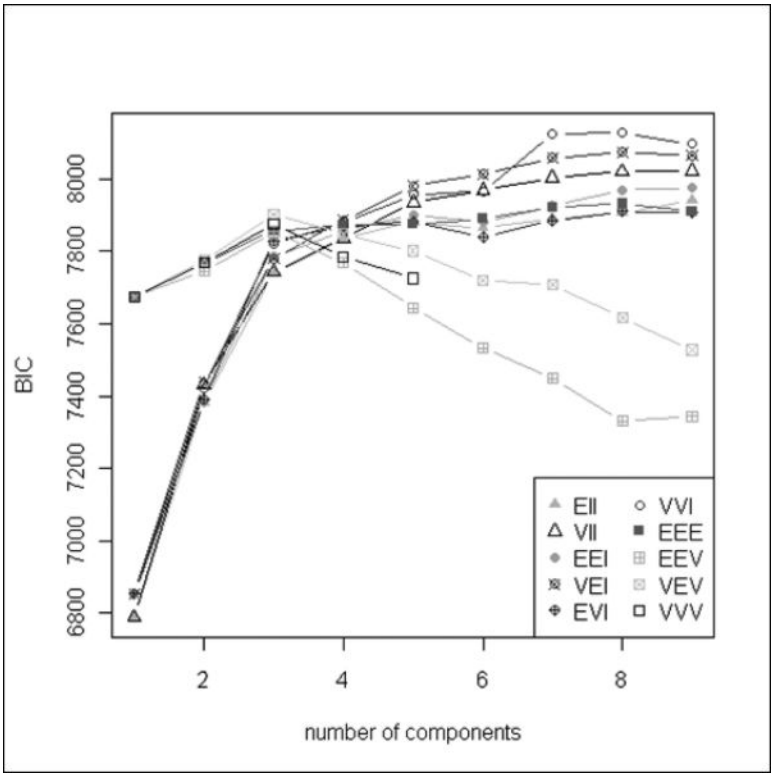

Figure 2.
Bayesian Information Criterion (BIC) scores for ten types of clustering algorithms with variable numbers of clusters. The BIC is calculated given the log-likelihood, the dimensionality of the data and the number of mixture components. BIC scores indicate that a VVI (diagonal varying volume and shape) model with eight clusters, best describes the dataset.

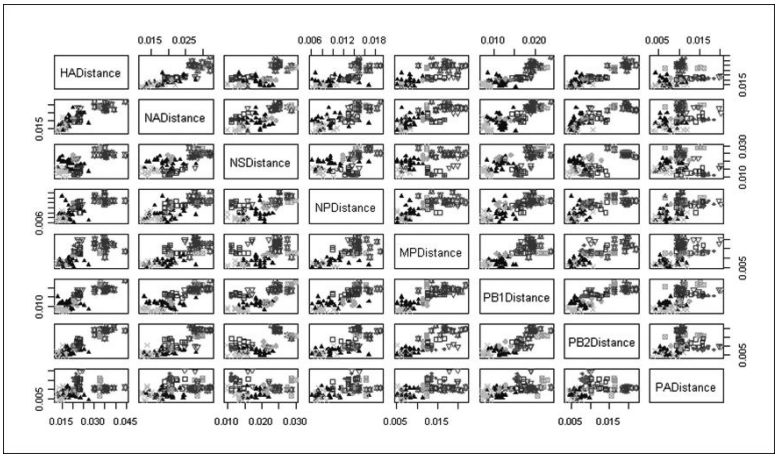

Figure 3.

Eight genetic distance variables with individual viral cluster assignments shown. The chart is symmetrical along the diagonal, showing the cluster divisions for pairs of gene segments (e.g. HA versus NA). The clean division of viruses into eight clusters across eight distance variables is seen in the tight groupings of symbols.

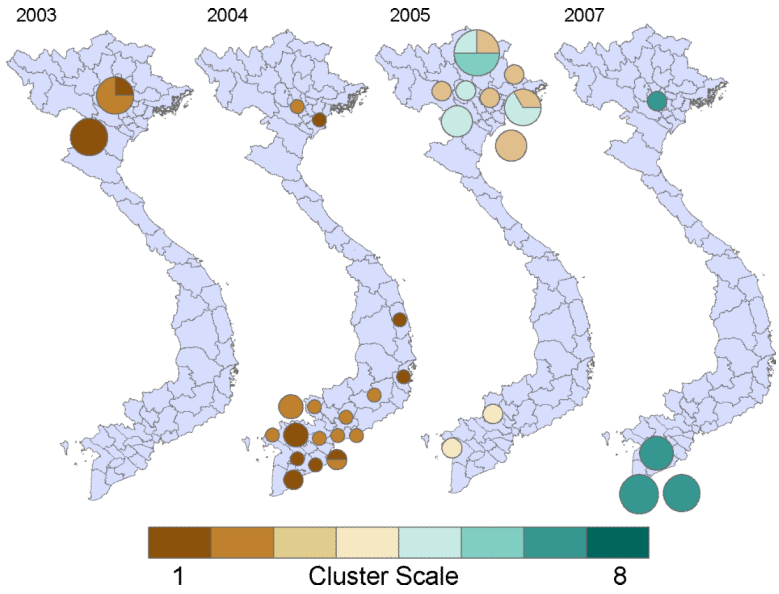

Figure 4.
H5N1 viruses isolated in chickens, according to cluster assignment and province of isolation. Color indicates which cluster each virus was assigned, size of pie chart indicates the number of viruses located in that province.

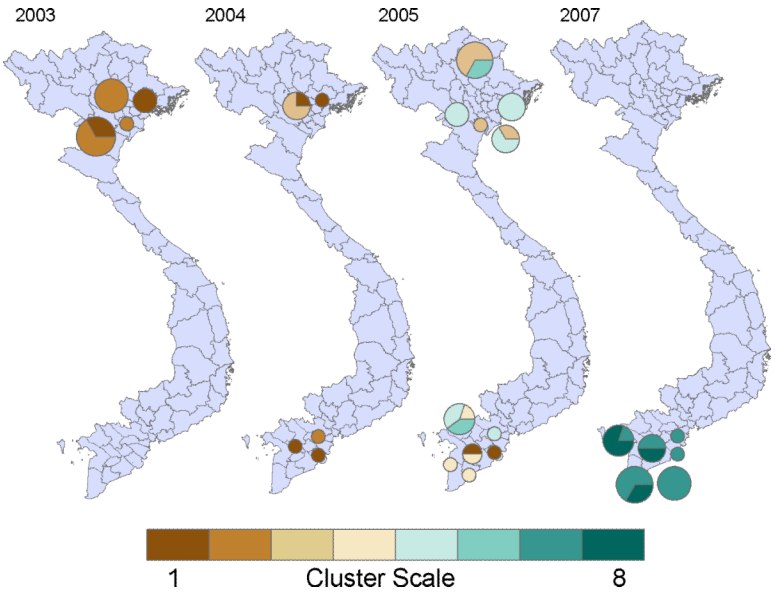

Figure 5.
H5N1 viruses isolated in ducks, according to cluster assignment and province of isolation. Color indicates which cluster each virus was assigned, size of pie chart indicates the number of viruses located in that province.

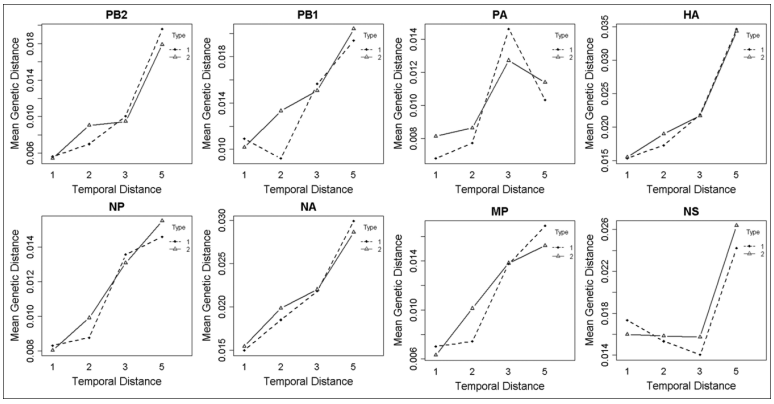

Figure 6.
Interaction plots for mean genetic distance according to temporal distance and species type. Type 1 viruses are chicken isolates (dashed line), type 2 viruses are duck isolates (solid line).

Table 1

Average genetic distances for the viruses assigned to each cluster.

Cluster	PB2 Dist	PB1 Dist	PA Dist	HA Dist	NP Dist	NA Dist	MP Dist	NS Dist	Total
1	0.0067	0.0115	0.0078	0.0182	0.0096	0.0192	0.0077	0.0182	0.0988
2	0.0059	0.0081	0.0071	0.0151	0.0077	0.0158	0.0065	0.0135	0.0798
3	0.0119	0.0163	0.0125	0.0224	0.0124	0.0210	0.0136	0.0130	0.1230
4	0.0101	0.0121	0.0100	0.0206	0.0129	0.0206	0.0117	0.0219	0.1199
5	0.0086	0.0157	0.0160	0.0213	0.0139	0.0206	0.0137	0.0114	0.1212
6	0.0101	0.0183	0.0160	0.0230	0.0136	0.0269	0.0192	0.0189	0.1460
7	0.0190	0.0195	0.0105	0.0352	0.0154	0.0289	0.0168	0.0249	0.1703
8	0.0162	0.0218	0.0129	0.0326	0.0152	0.0288	0.0124	0.0286	0.1684

The sum of the eight average genetic distances indicates which clusters are groupings of viruses with low genetic distance (e.g. Clusters 1 & 2) versus those that are clusters of viruses with high genetic distance (Clusters 7 & 8).

Table 2

Summary of the MANOVA using eight genetic distance measures as the dependent variable and species, temporal distance and geographic distance as explanatory variables.

	df	Wilks λ	approx F	Pr(>F)	
Species	1	0.345	22.52	< 2.2E-16	****
Temporal Distance	1	0.078	139.432	< 2.2E-16	****
Geographic Distance	1	0.477	13.037	1.59E-12	****
Species:Temporal Distance	1	0.827	2.484	0.01715	.
Species:Geographic Distance	1	0.909	1.186	0.31557	
Temporal Distance: Geographic Distance	1	0.386	18.915	< 2.2E-16	****
Species:Temporal Distance:Geographic Distance	1	0.934	0.841	0.56861	
Residuals	102				

Significance values: .0001 ‘**’ .01 ‘*’

**** 0

05

Table 3

Summary of F statistics for eight individual ANOVA models.

Model	PB2 Distance	PB1 Distance	PA Distance	HA Distance	NP Distance	NA Distance	MP Distance	NS Distance
Species	57.3278	78.8278	3.652	91.2428	35.5424	41.6047	22.756	28.1479
Temporal Distance	491.564	288.7553	25.4699	627.6387	210.4701	344.6868	137.6578	96.5648
Geographic Distance	2.7497	46.2216	35.2827	0.2566	21.2953	1.7582	17.1428	22.7102
Species:Temporal Distance	0.1829	1.0706	0.6516	0.0424	0.1126	2.7419	2.2531	4.5904
Species:Geographic Distance	2.5844	1.5705	0.5217	0.8907	0.0009	2.0448	0.6665	1.7636
Temporal Distance:Geographic Distance	24.1043	3.3532	15.2456	26.5468	2.3255	0.8508	3.6359	47.4919
Species:Temporal Distance:Geographic Distance	0.038	0.8617	0.0376	0.1031	0.5557	0.0466	3.3536	1.178

Shading represents statistical significance. Dark shading equals p-values of <.01, light shading equals p-values of <.1, no shading indicates a lack of statistical significance.

Table 4

Average genetic distance on each gene segment stratified by species, with total average genetic distance among chicken and duck H5N1 viruses.

	PB2 Dist	PB1 Dist	PA Dist	HA Dist	NP Dist	NA Dist	MP Dist	NS Dist	Total
Chicken	0.06231	0.08903	0.07414	0.15683	0.07288	0.13373	0.07669	0.09722	0.76284
Duck	0.08734	0.12440	0.09004	0.18726	0.09993	0.18056	0.10223	0.15207	1.02384