From sporadic to global: The changing face of H5N8

Andrew Dalby

The H5N8 influenza subtype is a relatively new highly pathogenic avian influenza. Until the recent Korean outbreak of H5N8 the viral subtype had only been detected sporadically. This paper looks at the multiple reassortment events between H5 hemagglutinin and N8 neuraminidase segments that explain the breaks in the history of the H5N8 subtype, especially in the United States and that show that the recent reassortment has produced an outbreak that has very different characteristics to previous H5N8 outbreaks.

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Introduction

Recently H5N8 has emerged as a widely circulating highly pathogenic avian influenza (HPAI). The H5N8 influenza A subtype was first isolated from a turkey in Ireland in 1983 (Murphy 1986). After that initial outbreak there was not another case of H5N8 until 2001 when a case was identified during environmental monitoring in a wild bird in New Jersey. There were then a few more sporadic detections in the United States and Thailand until the first sustained outbreak of H5N8 that began in Korea in 2013 (Lee et al. 2014).

The Korean outbreak was preceded by cases in Eastern China in 2010 that are distinct from the American and Irish virus. Although the Korean outbreak strains are of the same H5N8 subtype as the earlier Asian cases, the current outbreak seems to have been the product of another reassortment event, where another H5 containing subtype such as H5N1 or H5N5 has undergone reassortment with a N8 containing subtype (Lee et al. 2014; Zhao et al. 2013).

The 2013 Korean outbreak has been subdivided into two lineages one of which is closely related to the Chinese sequences and that has only been identified in a small number of samples in Gochang and a second that contains all the other cases and that was originally identified in Buan (Fan et al. 2014; Jeong et al. 2014). In the winter of 2014 the virus also spread to Europe and the US via migratory birds (Bouwstra et al. 2015; Hanna et al. 2015; Lee et al. 2015). There were only a limited number of European and US cases, but there has been widespread infection of domestic geese in Taiwan.

There are a large number of gaps in the history of H5N8 subtype and multiple years when it has not been isolated. These breaks in detection could be explained by inadequate sampling. The virus might have been present in wild birds, but because it is often asymptomatic it was not reported. However, some of the H5N8 cases have been detected as part of a systematic environmental study of bird diseases in the Delaware Bay, the Southeastern Cooperative Wildlife Disease Study, and if the virus had been present then it is likely that it would have been sampled (Brown et al. 2007).

Another possibility is that the H5N8 virus occurs sporadically as a result of multiple reassortment events, but that it does not circulate widely as it is not competitive with other avian influenza subtypes. The aim of this paper is to demonstrate that this second explanation is the actual cause for the sporadic nature of the detection of H5N8 sequences, by showing that reassortment has played a major role in the history of the H5N8 subtype.

Materials and Methods

All of the available H5N8 hemagglutinin and neuraminidase nucleotide sequences were downloaded from the NCBI Influenza Virus Resource on the 29th of June 2015. Both sets of sequences were aligned with Muscle v3.8.31 (Edgar 2004) within Mega6.06.

Manual inspection and editing of the sequences was carried out using Mega6.06 (Tamura et al. 2013). During manual editing the 5' end of the sequence was edited to remove the un-translated region. All sequences were trimmed to the start codon and stop codons. Sequences with missing nucleotides were removed (table 1).

The test for the appropriate evolutionary model was carried out in Mega6.06 and showed that the general time reversible with gamma correction and invariant sites model was optimal (GTR+G+I) (Posada & Crandall 1998; Tavaré 1986). Alignment was carried out using Muscle within Mega6.06 (Edgar 2004). Maximum likelihood phylogenetic trees for the H5N8 hemagglutinin and neuraminidase sequences were calculated using the GTR+G+I evolutionary model with 1000 bootstrap repetitions using Mega6.06. These trees provide the reference for further comparisons.

Trees for the H5N8 subtype were also created using alignment with MAFFT v6.864b using the default settings and then FastTree2.1 was used to create a maximum likelihood tree using the GTR + gamma evolutionary model (Katoh & Standley 2013; Price et al. 2010). The command for the tree generation are given below:

Fasttree –nt –gtr –gamma –quote filename.fas > filename.tree

All of the available H5 hemagglutinin subunits (4007 sequences) and N8 neuraminidase subunits (1840 sequences) were downloaded from the NCBI Influenza Virus Resource on the 27th of June 2015 (Bao et al. 2008). The search was restricted to full-length sequences from any host. This data was used to carry out a complete phylogenetic analysis for both the H5 hemagglutinin and N8 neuraminidase segments. The sequences were aligned using the default settings within MAFFT v6.864b (Katoh & Standley 2013). FastTree2.1 was used to create a maximum likelihood tree for all of the sequences using the GTR + gamma evolutionary model (Price et al. 2010). Given the large number of taxons it is computationally challenging to calculate realistic boot-strapped trees, instead FastTree calculates a local support values of each of the splits within the tree using the Shimodaira-Hasegawa (SH) log likelihood test (Shimodaira & Hasegawa 1999). This has been shown to have a high correlation to conventional bootstrap values (Price et al. 2010).

The resulting trees were edited, visualised and annotated with FigTree 1.4.2 (Rambaut 2007). All trees were displayed as cladograms to aid clarity. The full name, chronological and geographical information was included in the trees because these are essential for the interpretation of the results. Nodes were labelled with the support values calculated by FastTree, which are a Log Likelihood Ratio.

Supplementary data-files for the phylogenetic analysis of the H5 hemagglutinin are available from http://dx.doi.org/10.5281/zenodo.20653 and for the N8 neuraminidase from http://dx.doi.org/10.5281/zenodo.20655.

Results and Discussion

The Mega maximum likelihood phylogenetic tree for the H5N8 hemagglutinin is shown in figure 1. Very low bootstrap values are often a result of sequence identity. Sequence degeneracy is frequent in viral data and cannot be resolved using maximum-likelihood or distance based measures, but it is important in Bayesian and coalescent analysis, where tip ages can be used. For this reason identical sequences were not removed. This tree shows that the US H5N8 sequences prior to the current outbreak (2001-2011) form the most distinctive clade. This is then followed by the sequences from the Irish outbreak in 1983. There are then a series of singleton clades before the Gochang clade and the final clade contains the rest of the sequences from the current outbreak. There is some evidence for sub-clades within the current outbreak and appears to have broken into 3 or four different sub-lineages, one of which contains the 2014 US sequences.

The Mega maximum phylogenetic tree for the H5N8 neuraminidase dataset (figure 2) contains fewer features when compared to the hemagglutinin trees but the overall topology is the same. For the Irish cluster there is only a single full-length sequence available. There is also less clade structure in the recent outbreak, although there is still a distinct sub-lineage for the US 2014 sequences. Apart from this clade there is very little recent evolutionary change in the neuraminidase sequences.

These trees are in good agreement with the much more detailed and rigorous coalescent analysis carried out previously (Dalby & Iqbal 2015). They show that the maximum likelihood trees using the general time reversible model is appropriate for creating phylogenetic trees oh H5N8.

These trees provide a reference set for the approximate maximum likelihood trees produced by FastTree. This allows a comparison to be made of the SH local branch support values to the bootstrap values for the same tree. The trees for hemagglutinin and neuraminidase are shown in figures 3 and 4 respectively. It can be seen that there is a very strong correlation between the FastTree local support values and the calculated bootstrap values in the Mega maximum likelihood tree. There is almost complete agreement in the most important nodes which divide the US and Ireland clades from the Korean outbreak.

These trees give an overview of the pattern of H5N8 evolution. However they do not contain information about the reassortment of the hemagglutinin and neuraminidase genes. To discover reassortment events phylogenetic trees for all of the H5 hemagglutinin and N8 neuraminidases have to be constructed.

The phylogenetic trees for all of the N8 neuraminidase segments and all of the H5 hemagglutinin segments are very large (supplementary data files 1 and 2) and so they have been edited in order to focus on only the clusters that contain the H5N8 sequences (figures 5 to 13). These clusters show good agreement in the geographical location and chronology of the possible reassortments in both the hemagglutinin and neuraminidase trees. This consistency of trees between independent genes is strong evidence that the phylogenetic analysis is valid.

These trees show that the apparently simple H5N8 phylogenetic trees for the two envelope segments (figures 1-4) are actually more complex and that multiple reassortment events have occurred resulting in the creation of novel H5N8 subtype lineages. These events cannot be seen in the structure of the H5N8 only trees but they need to be taken into account if the phylogenetic trees are going to be calculated correctly, especially if coalescent methods are going to be used.

Out of the 7 hemagglutinin and neuraminidase clusters 5 of them are singletons, New Jersey 2001, California 2011, Thailand 2012, Quang Ninh 2013 and California 2014 (quail). Of these the most surprising are the US sequences as it was expected that these would be from a single lineage, and that the gaps in time between collected sequences reflect a lack of sampling.

However it is clear from the trees presented here that the sporadic appearance on H5N8 has arisen from multiple reassortment events. This is made especially clear in figure 5, which shows three reassortment events that have produced the H5N8 subtype in Colorado in 2006 and in California in 2011 and 2014. These three H5N8 reassortments had a hemagglutinin that originated in the H5N2, H5N1 and H5N5 subtypes.

The first cases of H5N8 avian influenza were in turkeys in Ireland in 1983 (figure 6). From the clusters of sequences for the neuraminidase and hemagglutinin, the neuraminidase is most closely related to those found in H3N8 infected ducks in the Ukraine in 1963 and the hemagglutinin is most closely related to H5N2 found in an Italian turkey in 1982. Considering

the geographical spread and the gaps in the timeline it is impossible to state for sure that this is a reassortment of the H5N2 and H3N8. There are also German sequences from 1984 and 1985 with a similar hemagglutinin from the H5N6 and H5N2 subtypes respectively. This lends further support to believing that the H5N2 subtype is the most likely source of the H5 hemagglutinin.

The New Jersey 2001 H5N8 is derived from either an H5N7 or H5N2 hemagglutinin in shorebirds in New Jersey/Delaware Bay (figure 7). The next closest hemagglutinin sequences are for Japanese H5N3 ducks in 2002 but these are geographically very distant and it is difficult to imagine a migratory connection without further evidence of widespread dispersal. This hemagglutinin most likely combined with an H6N8 or possibly an H11N8, which were also circulating in Delaware Bay from 1993. The absence of H6N8 sequence data from Delaware Bay between 1993 and the occurrence of H5N8 in 2001 is of some concern. This location is a focus of the South-eastern Cooperative Wildlife Disease Study that carries out regular sampling. If the virus was present during this period it would be expected that it would be sampled more frequently.

The California 2011 H5N8 case is less ambiguous and it is clearly a reassortment of an H5N1 viral subtype with and H3N8 subtypes in mallards in California (figure 8). The H5N1 and H3N8 sequences seem to have been the dominant subtypes in this location and might the reason for there not being a wider distribution of H5N8.

175 The Thailand 2012 reassortment is more complex (figure 9). The sequence is part of a H5N2
176 cluster in wild birds in Xianghai but there are also some H5N3 cases. The neuraminidase clusters
177 with a group of H3N8 sequences mostly from ducks in Eastern China. This group also includes a
178 Vietnamese duck sequence. From 2013 onwards this cluster is dominated by H10N8 sequences
179 and these could have replaced H5N8. This would fit with the reassortment event having taken
180 place in the Xianghai region.

181

182 Quang Ninh is a coastal region of North Vietnam that border with China. The Quang Ninh
183 cluster for hemagglutinin is mostly H5N1 subtype sequences from Vietnamese Muscovy ducks
184 (figure 10). The neuraminidase cluster is from a mixed group that also includes H3
185 hemagglutinins in ducks from Jiangxi and Vietnam. This is consistent with the reassortment
186 having occurred in Vietnamese wild ducks between the H5N1 and H3N8 subtypes.

187

188 The California 2014 reassortment is also unambiguous. Almost all of the sequences in the
189 hemagglutinin cluster are of the H5N5 subtype from mallards in California (figure 11). The
190 neuraminidase cluster is similarly almost homogeneous for H3N8 sequences also from mallard in
191 California and so it seems clear that the reassortment took place in Californian wild ducks that
192 then spread the virus to quail. This reassortment event complicated the monitoring of H5N8 and
193 the spread of the Korean H5N8 lineage by wild birds. When multiple lineages coincide
194 geographically this can make a coherent response to the spread of the virus more difficult as
195 there is uncertainty about how the virus is spreading.

196

In the past H5N8 outbreaks have been short lived and localised, but in the recent outbreak the subtype has persisted through two breeding seasons and spread over three continents (figures 12 and 13). This new outbreak contains the Guangdong H5 hemagglutinin that has become the predominant form of the H5 hemagglutinin in China and the H5N8 sequences are part of the 2.3.4.4 H5 clade (Donis & Smith 2015). While the current outbreak came to prominence in Korea there were earlier cases in China. The tree structure shows the presence of at least two distinct lineages. One lineage probably originated around Jiangsu in 2010 from a possible reassortment of H5N1 and H3N8 (Kang et al. 2015). This has been named the Gochang lineage after the region in Korea where the most recent sequence was detected (Jeong et al. 2014). This lineage forms a distinctive cluster (figures 1-4 and 12-13, shown in red) in all of the neuraminidase and hemagglutinin trees. The Shandong 2013 neuraminidase sequence is also particularly distinct to other members of the current outbreak.

A second Korean lineage has been named the Buan lineage (Jeong et al. 2014) from the Buan region of Korea where it was isolated. From the phylogenetic trees the hemagglutinin from this lineage appears to be splitting into two sub-lineages in Korea (figures 1,3 and 12 shown in magenta and blue) and a third in North America (figures 1-4 and 12-13, shown in green). The presence of sub-clades or sub-lineages has also been proposed in recent work by Hill *et al.* in a study that combines ecological data with the phylogenetic data (Hill et al. 2015). They propose that the lineages are dependent on geographical location, but they only used the H5 hemagglutinin in the analysis.

Even more intriguing is that there has been a reassortment in North America to produce a new H5N2 virus, which contains the Guangdong H5 lineage hemagglutinin. It is possible that this hemagglutinin might undergo further reassortment allowing the Guangdong H5 hemagglutinin to displace the existing US H5 hemagglutinin in other influenza subtypes (Verhagen et al. 2015).

Conclusions

The H5N8 subtype is made up of at least 7 distinct lineages that have each been produced by a distinct reassortment event. The presence of these recombination events affects the phylogenetic analysis and has to be accounted for in the H5N8 phylogenetic trees. This is likely to be a more general observation when constructing phylogenetic trees of influenza sub-types where reassortment will mean that segments can have a very different evolutionary history. A recent study of Dengue virus has called into question to idea of serotype in that virus because there is greater variation in antigen response within serotypes than between them, indicating a pattern of reassortment affecting antigenicity (Katzelnick et al. 2015).

Reassortment events need to be accounted for before any reliable phylogenetic analysis can be carried out. These events can have a significant effect on coalescent analysis and should be taken into account in building evolutionary hypotheses. Analysis that does not explicitly account for reassortments is likely to be unreliable, especially if it is used for calculating varying mutation rates along different branches.

The results here have shown an unexpected degree of reassortment, especially amongst the sequences from the United States. These events happen very rapidly. Mostly reassortment does not produce a persistent new subtype as shown by the presence of a high proportion of singleton sequences. For H5N8 the most recent reassortment has finally generated a viable subtype that will continue to circulate but it is still possible that it might die out once again and that H5N8 will only return sporadically.

These results show that breaks in the historical record of a viral strain can be the product of extinction and then subsequent regeneration via reassortment rather than the effect of limited sampling. Sampling still remains an issue and we need to make influenza monitoring in the wild bird populations more systematic, but in this case it does not appear to have been the main reason for the absence of the virus.

Acknowledgments

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Tables

Segment	Deleted Sequence Identifiers.
HA	KR233690, AY296086, KR233674, KR232364, KR233682, KJ413855, KJ413856, EF607893.
NA	CY054463, GU052855, GU052862, KP101006, KR233692, JQ973696, KR233676, KR232366, KR233684,

Table 1: The sequences that were removed from the phylogenetic analysis because of truncation.

Figures

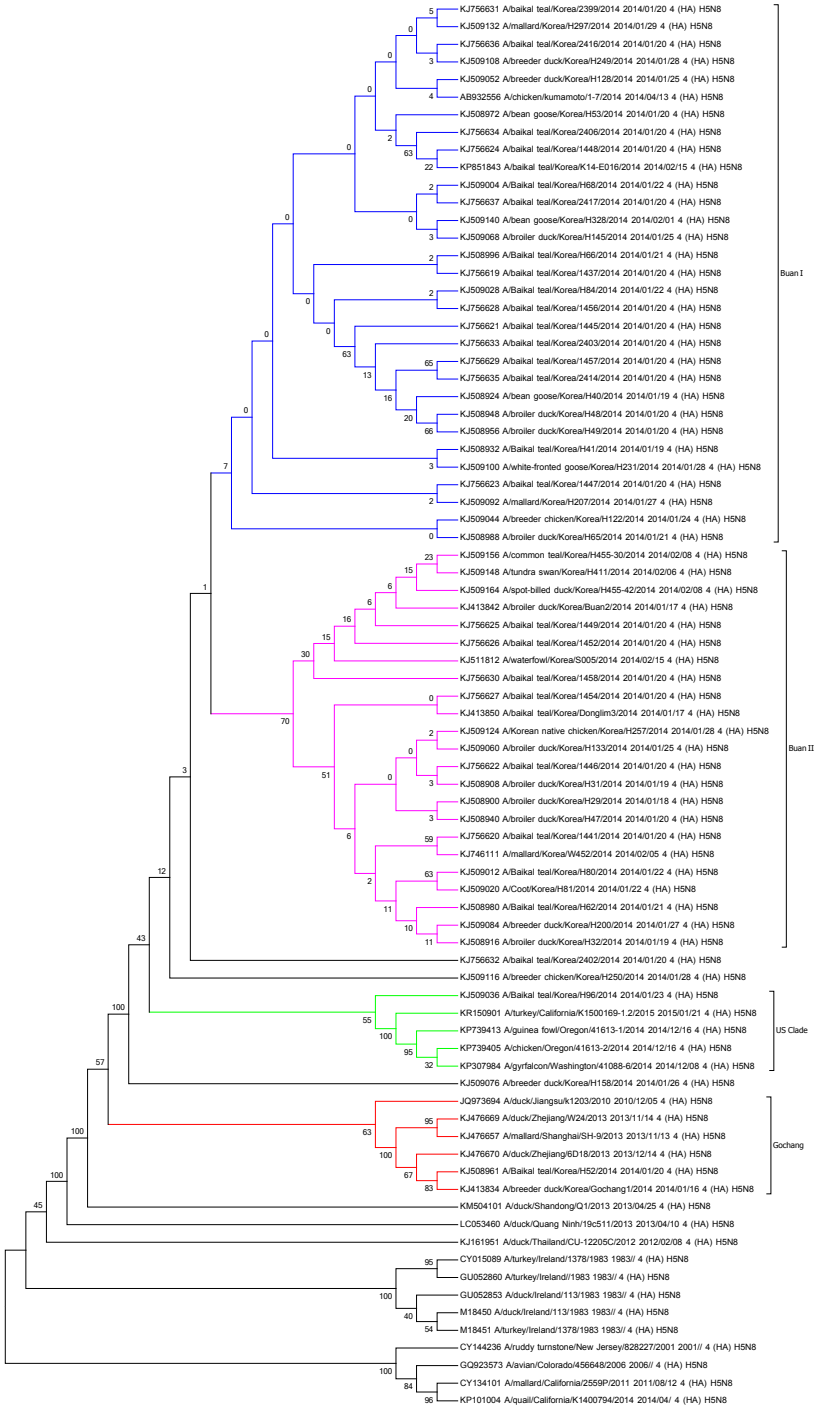
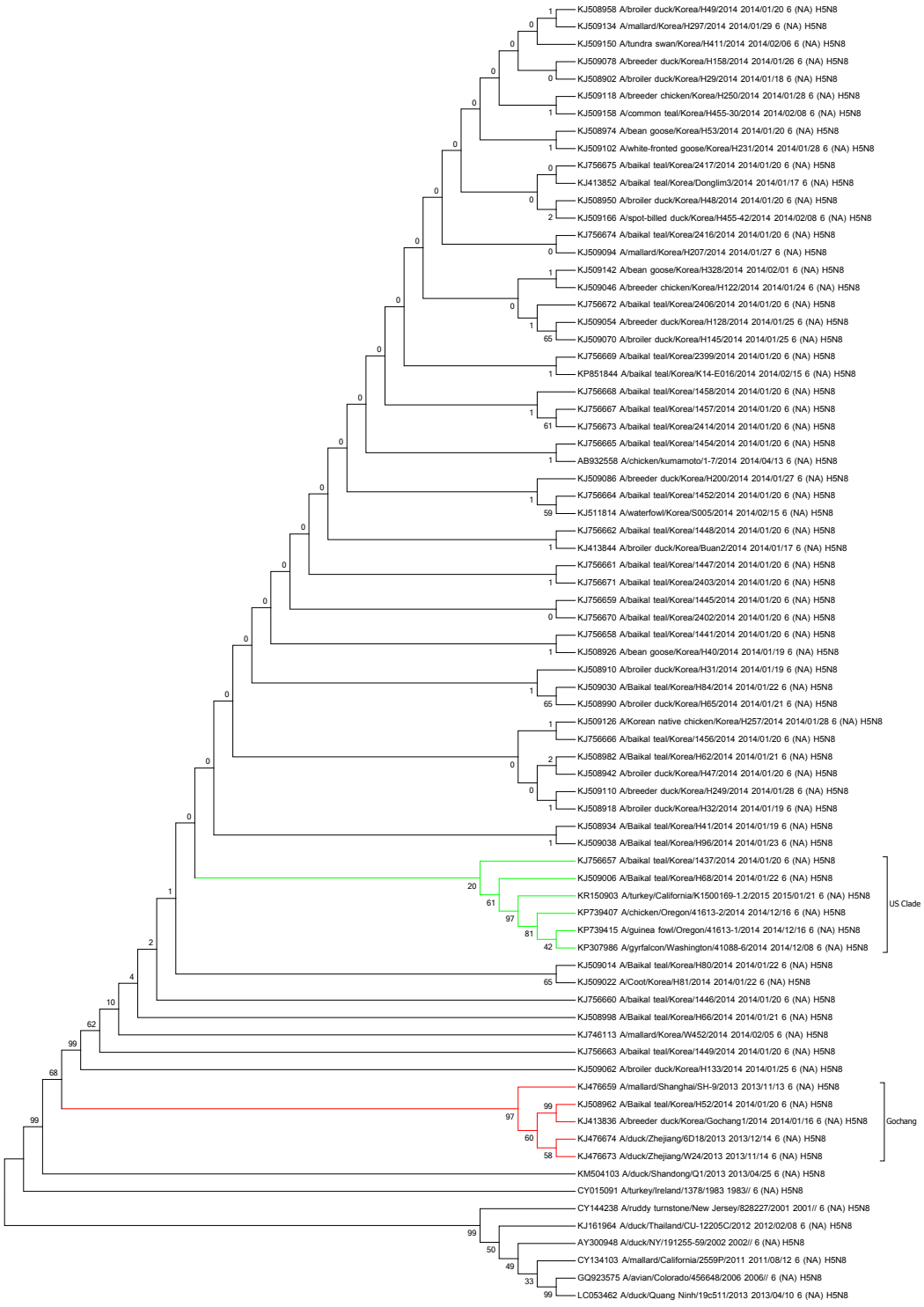
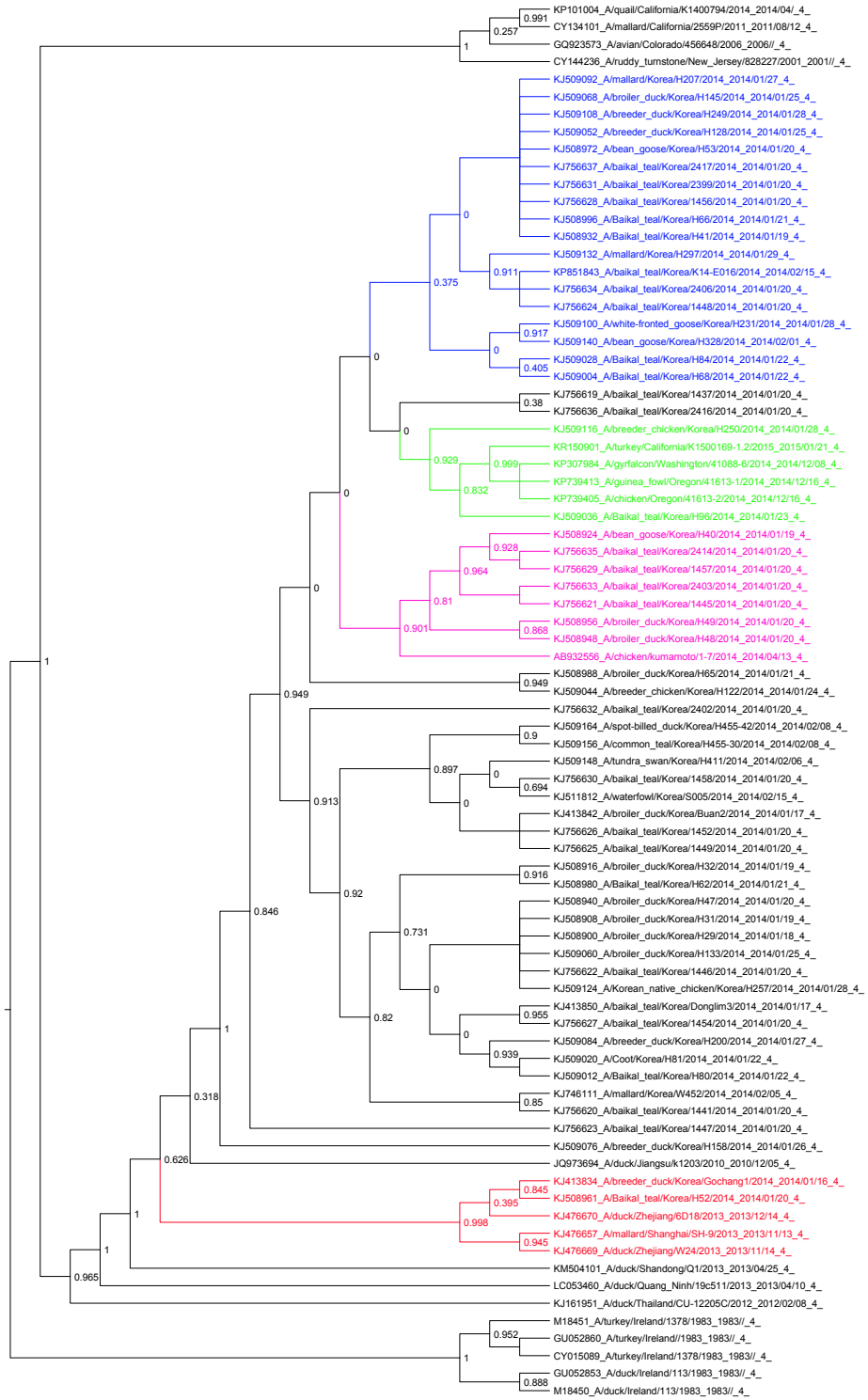


Figure 1: The Mega Maximum Likelihood H5N8 hemagglutinin phylogenetic tree. Internal nodes are labelled with the bootstrap values. The Buan Clades are in blue and magenta and the Gochang clade is in red. The US clade is in green.



330

331 Figure 2: The Mega Maximum Likelihood H5N8 neuraminidase phylogenetic tree. Internal
332 nodes are labelled with the bootstrap values. The Gochang clade is in red and the US clade is in
333 Green.



334

335 Figure 3: The FastTree Maximum Likelihood H5N8 hemagglutinin phylogenetic tree. Internal
336 nodes are labelled with the node support values. The Buan Clades are in blue and magenta and
337 the Gochang clade is in red. The US clade is in green. The nodes are labelled with the Sh local
338 support values.

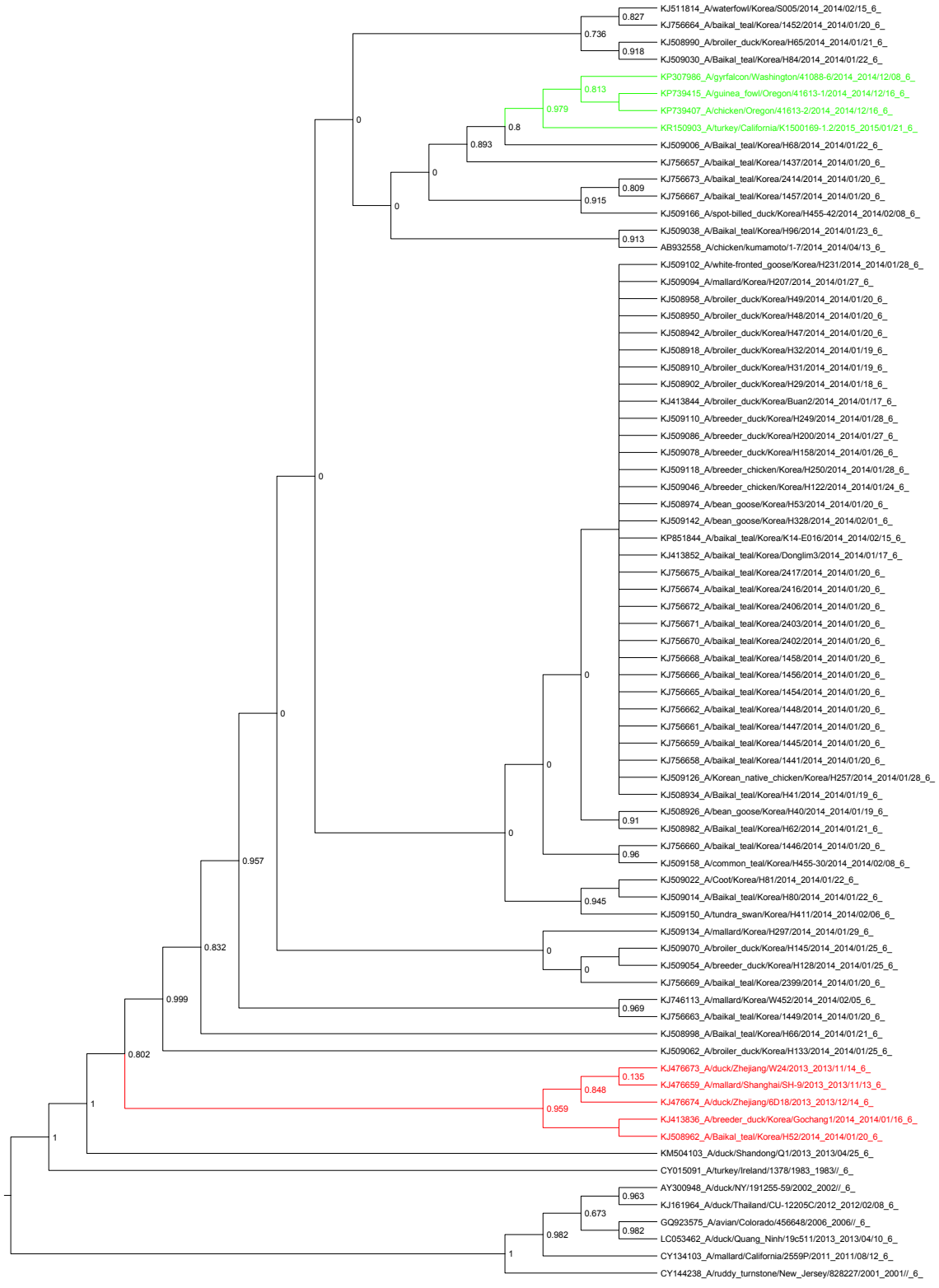


Figure 4: The FastTree Maximum Likelihood H5N8 neuraminidase phylogenetic tree. Internal nodes are labelled with the node support values. The Gochang clade is in red and the US clade is in Green. The nodes are labelled with the SH local support values.

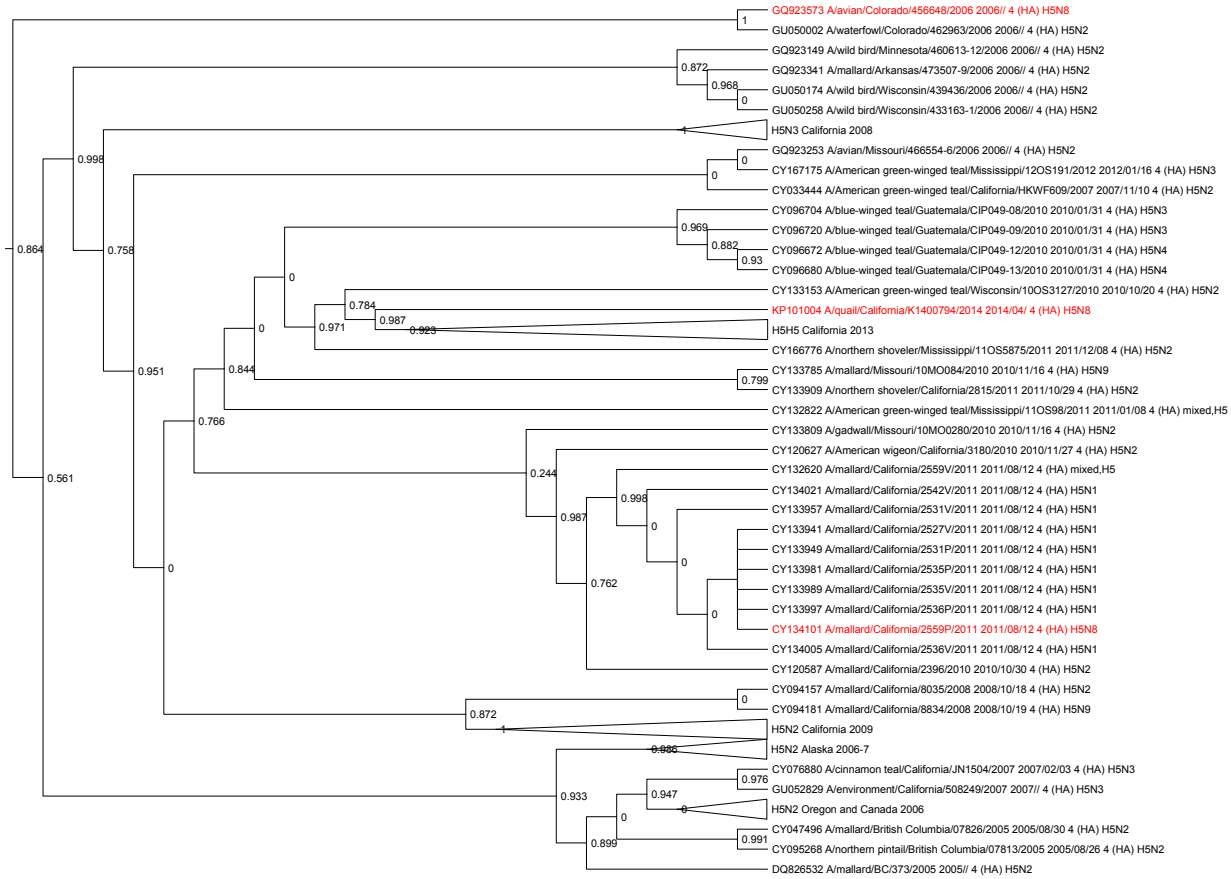


Figure 5: The three reassortment events in the H5 tree that produce the Colorado 2006, California 2011 and California 2014 quail sequences. The nodes are labelled with the SH local support values.

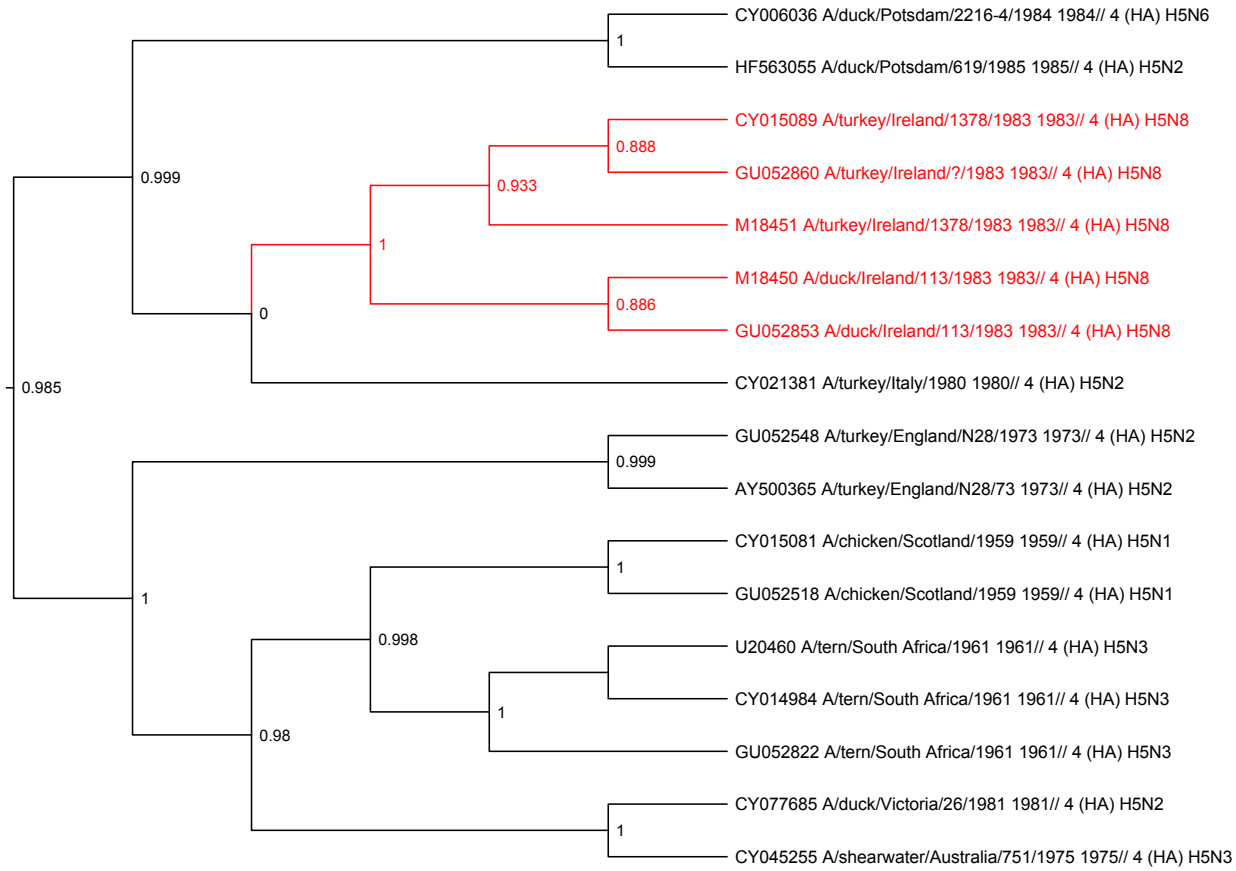
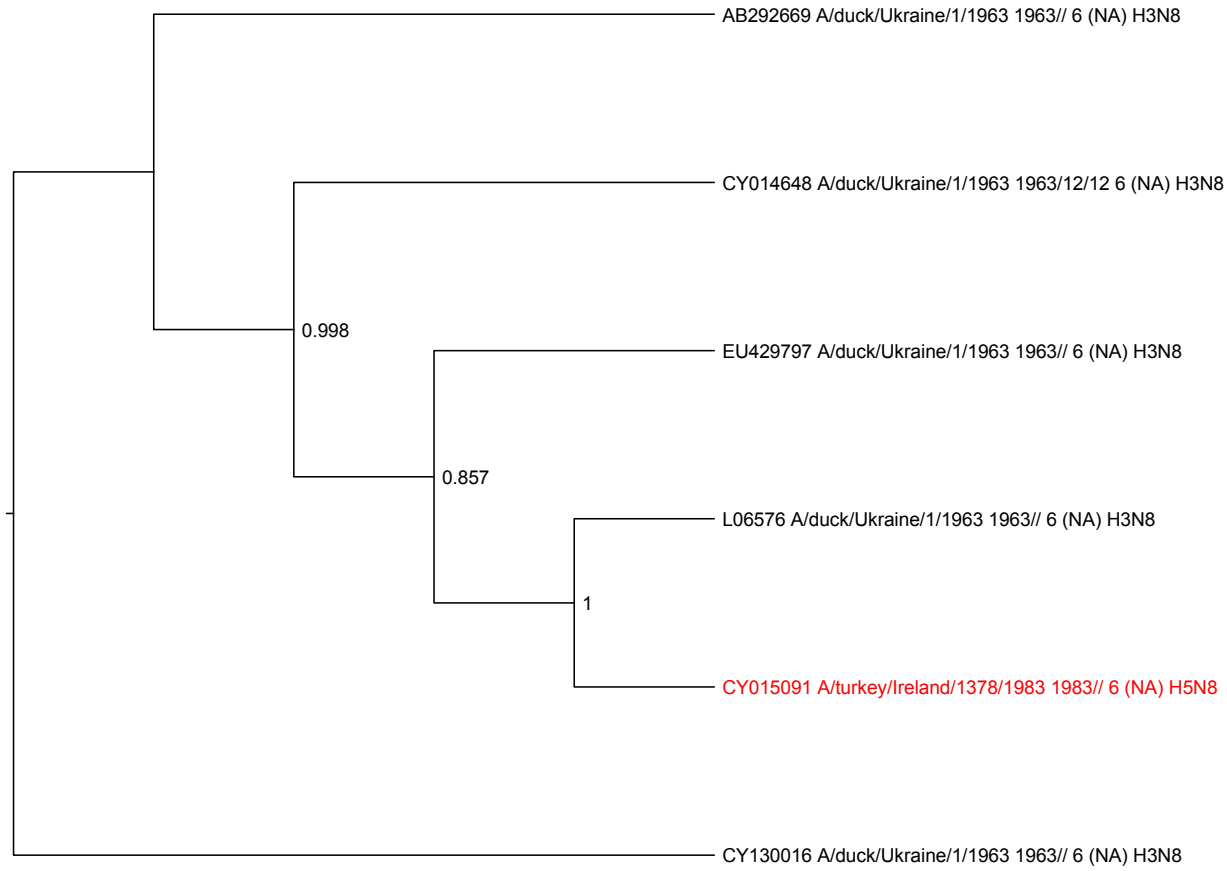


Figure 6A: The hemagglutinin clade for Ireland in 1983. The nodes are labelled with the SH local support values.



352

353

354 Figure 6B: The neuraminidase clade for Ireland in 1983. The nodes are labelled with the SH
355 local support values.

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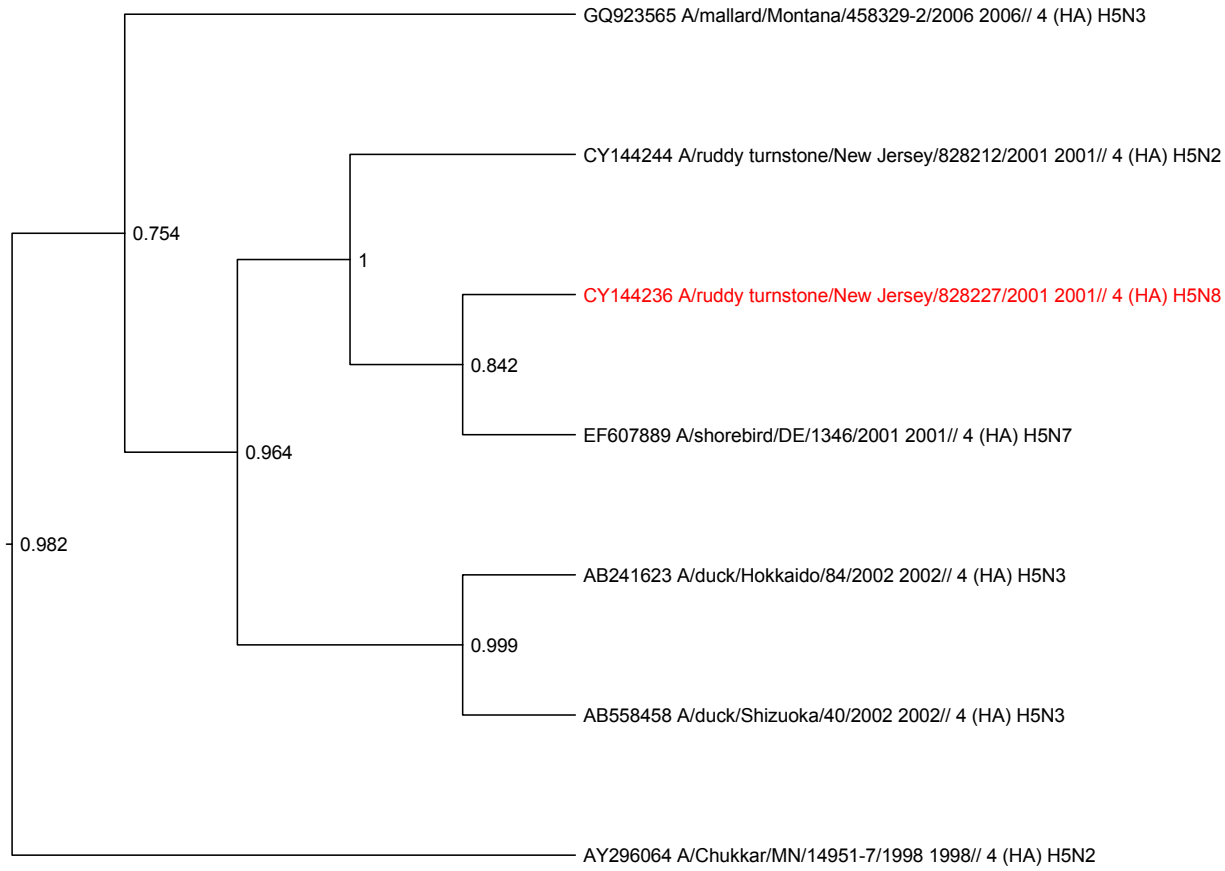
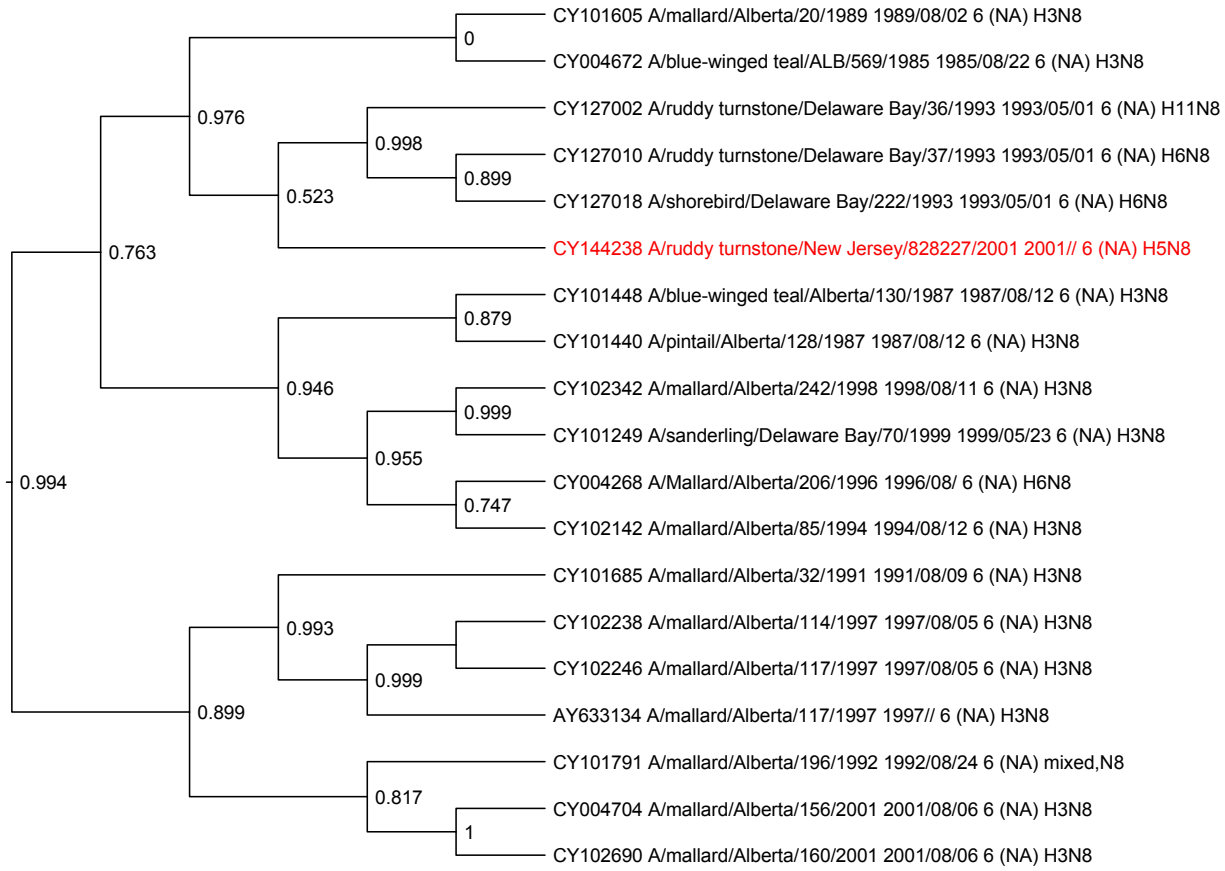


357

358 Figure 7A: The hemagglutinin clade for New Jersey in 2001. The nodes are labelled with the SH
359 local support values.

360



361

362 Figure 7B: The neuraminidase clade for New Jersey in 2001. The nodes are labelled with the SH
363 local support values.

364

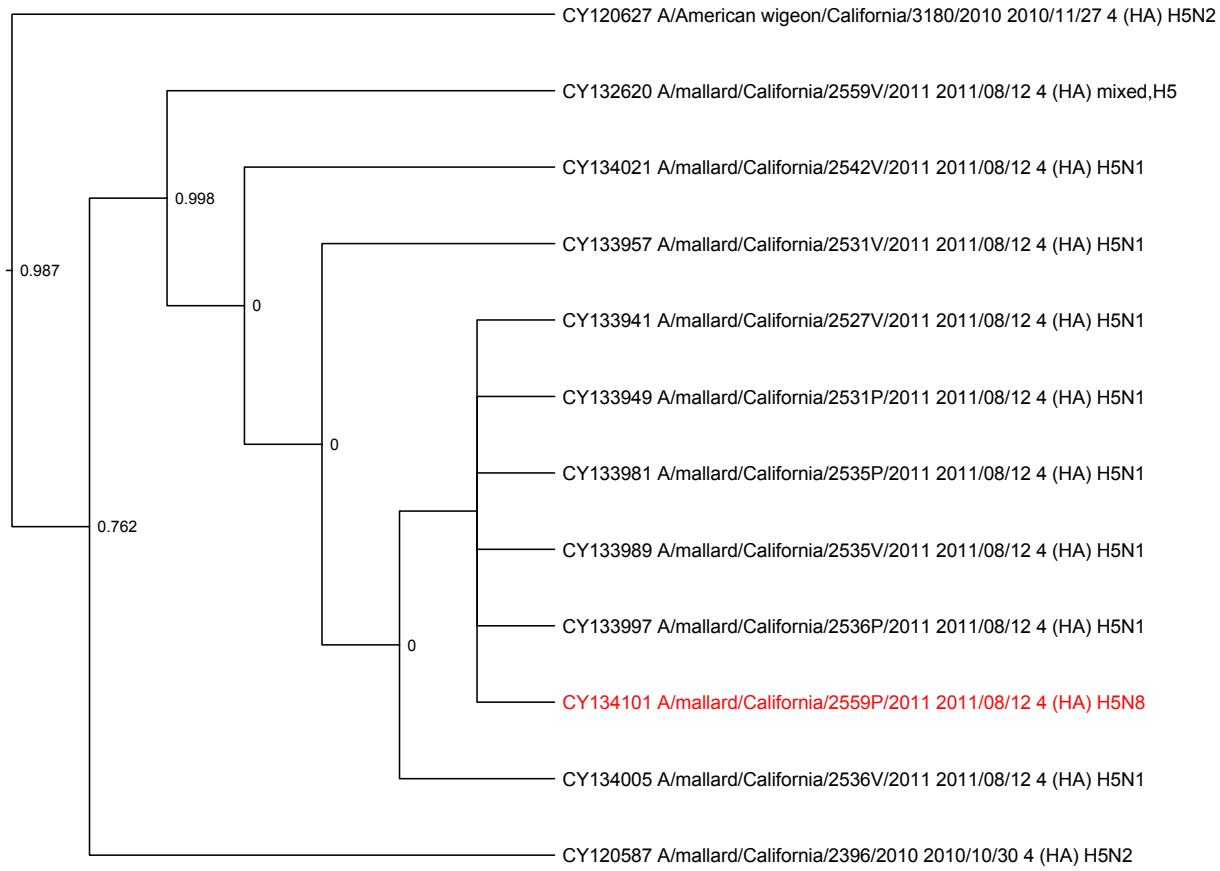
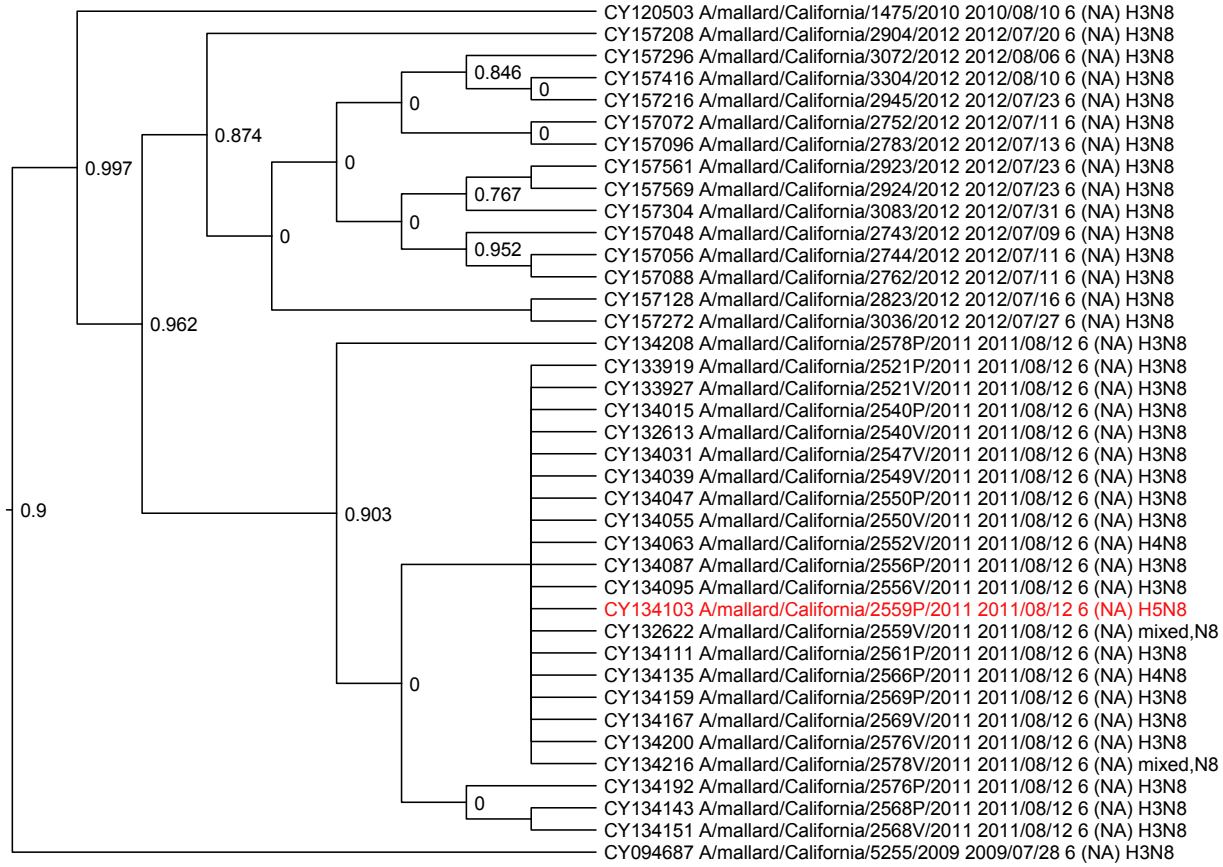


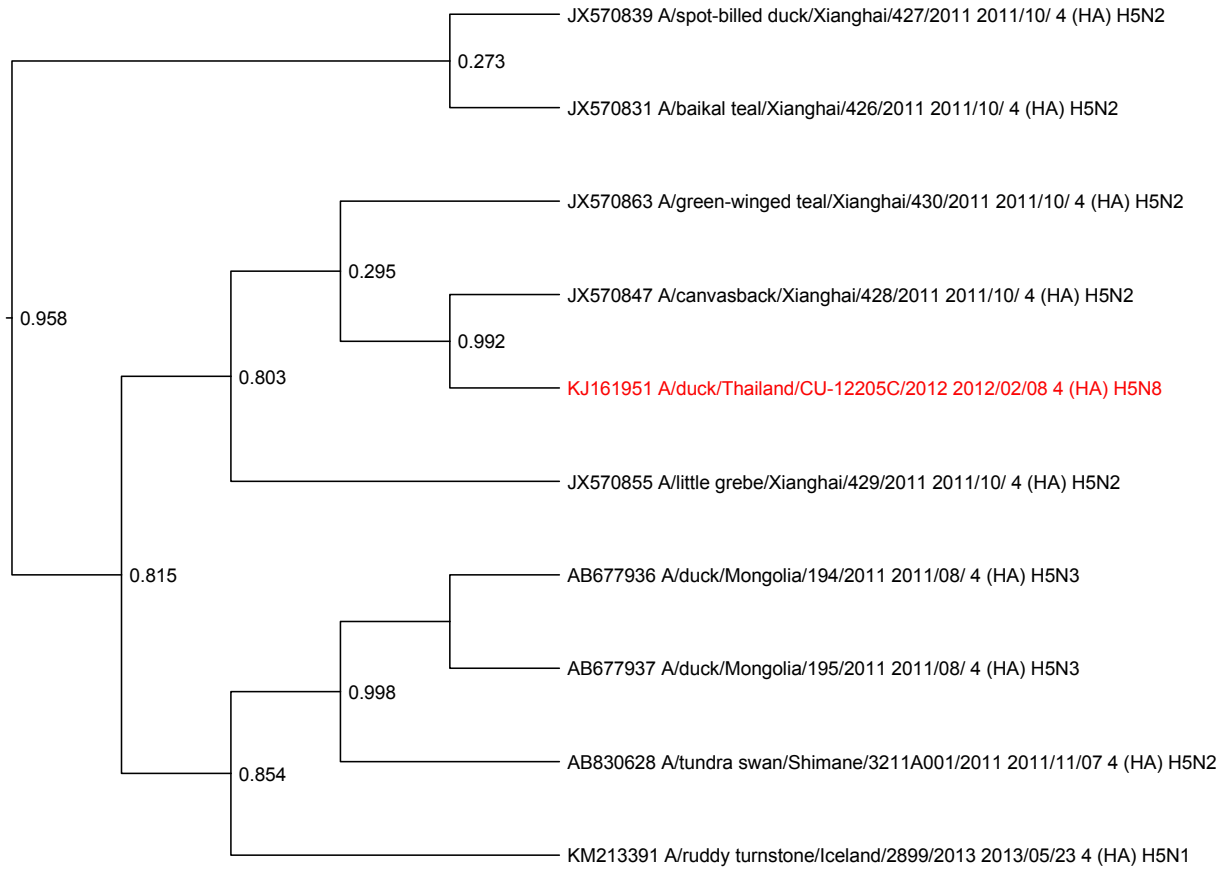
Figure 8A: The hemagglutinin clade for California in 2011. The nodes are labelled with the SH local support values.



370

371 Figure 8B: The neuraminidase clade for California in 2011. The nodes are labelled with the SH
372 local support values.

373



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375

376 Figure 9A: The hemagglutinin clade for Thailand in 2012. The nodes are labelled with the SH
377 local support values.

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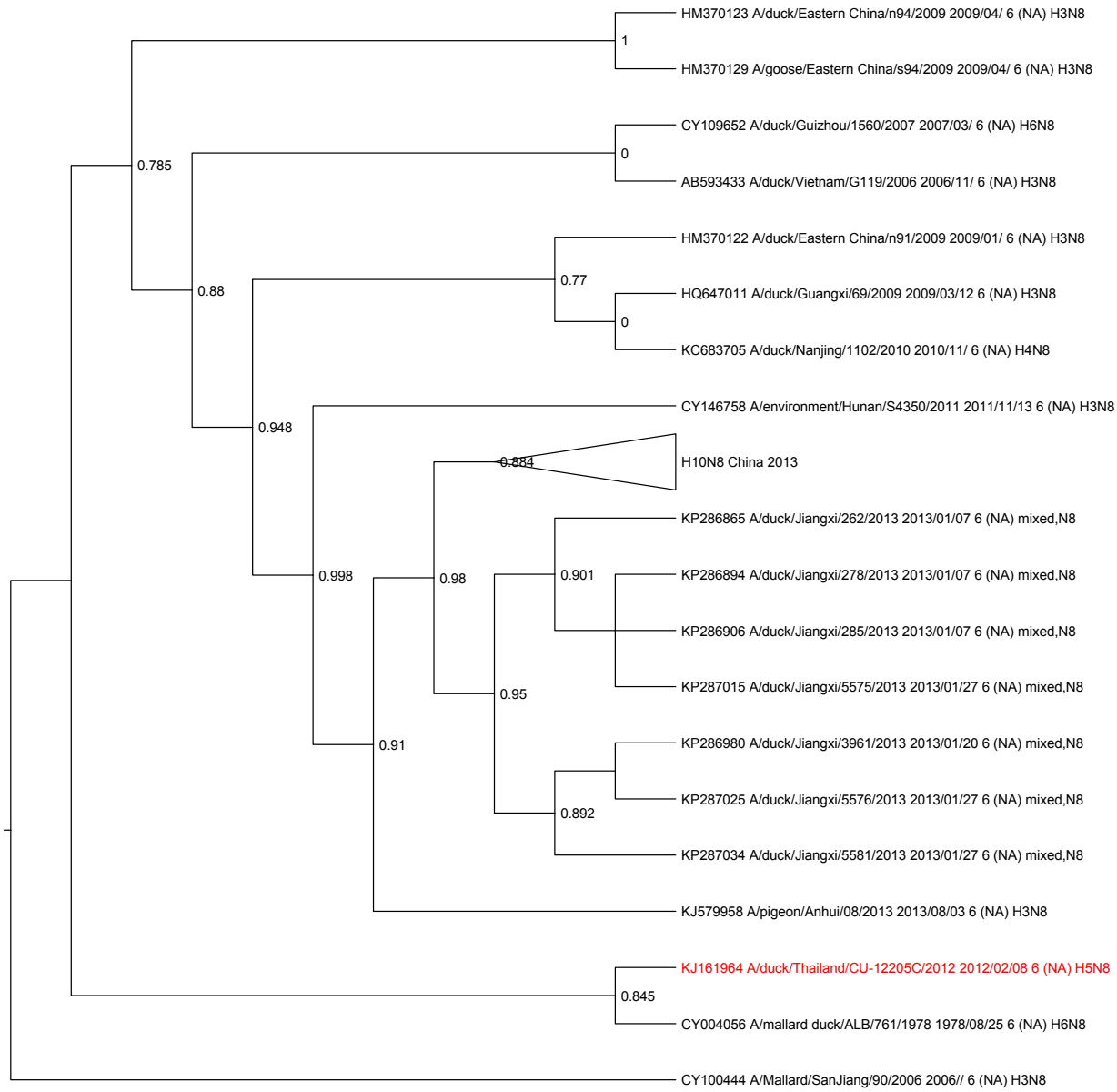
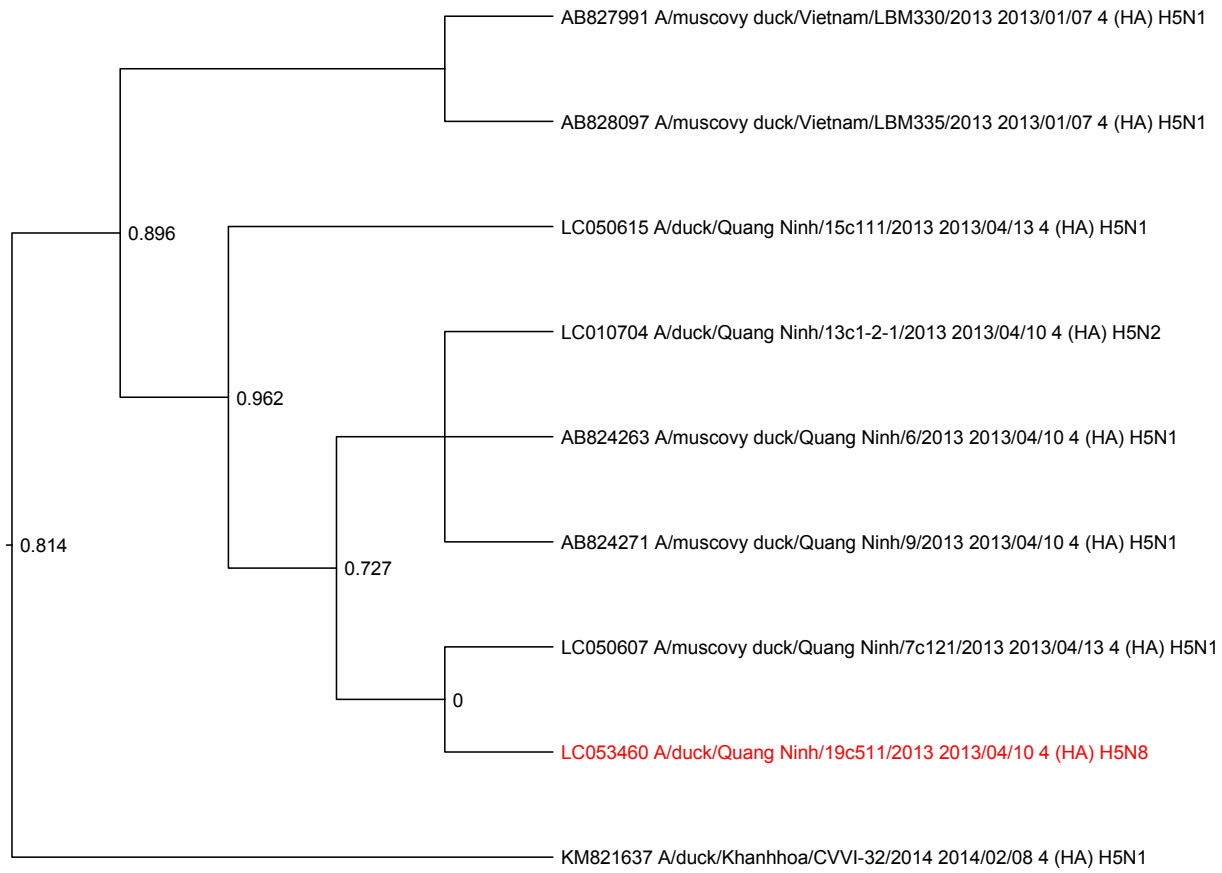


Figure 9B: The neuraminidase clade for Thailand in 2012. The nodes are labelled with the SH local support values.



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386 Figure 10A: The hemagglutinin clade for Quang Ninh in 2013. The nodes are labelled with the
387 SH local support values.

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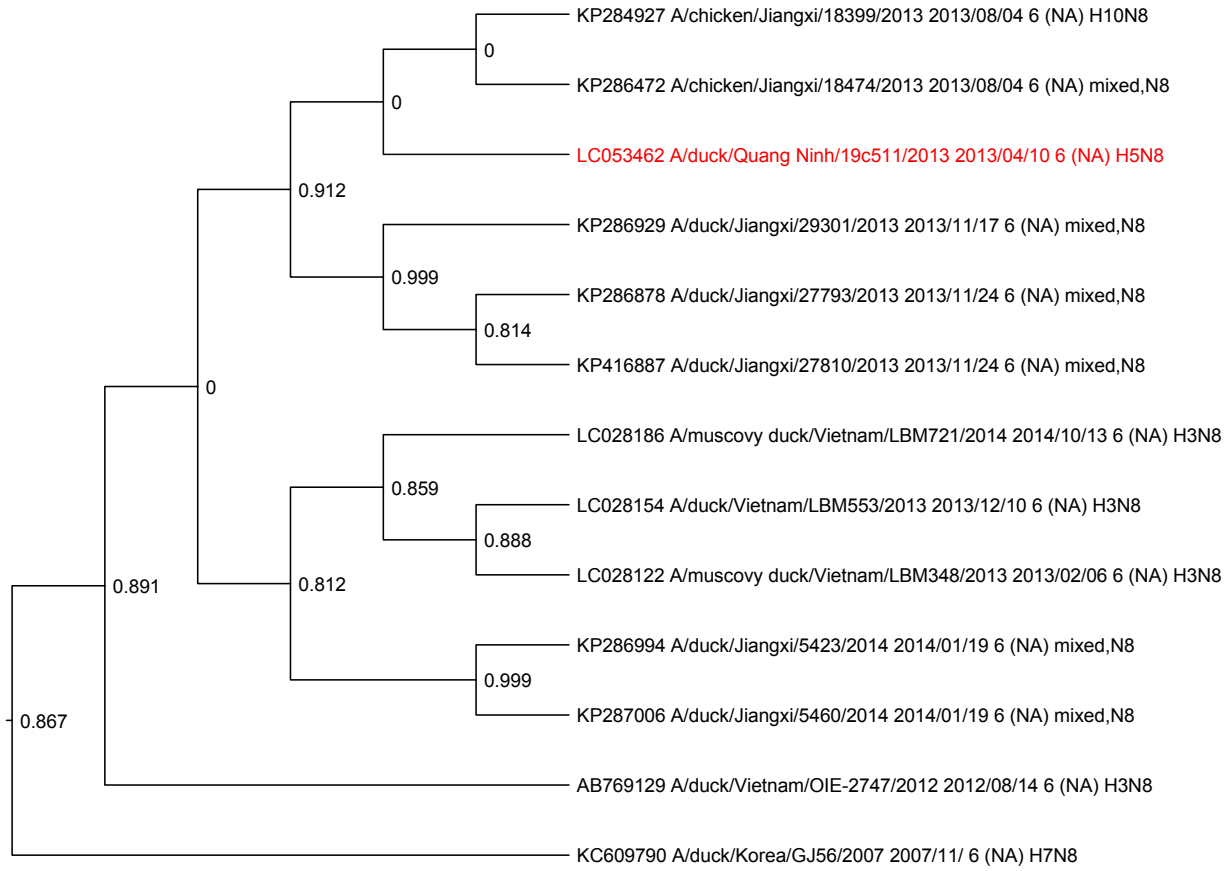
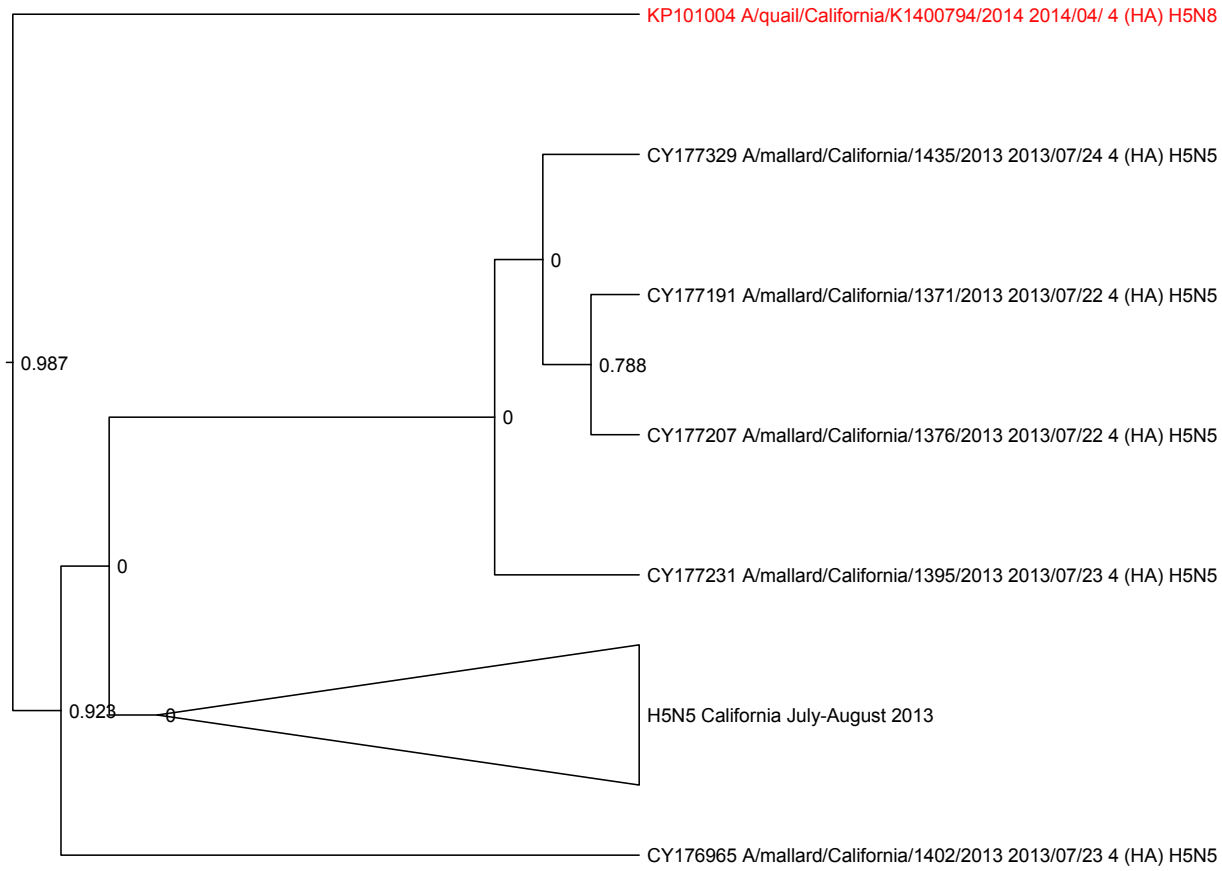


Figure 10B: The neuraminidase clade for Quang Ninh in 2013. The nodes are labelled with the SH local support values.



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397 Figure 11A: The hemagglutinin clade for California in 2014. The nodes are labelled with the SH
398 local support values.

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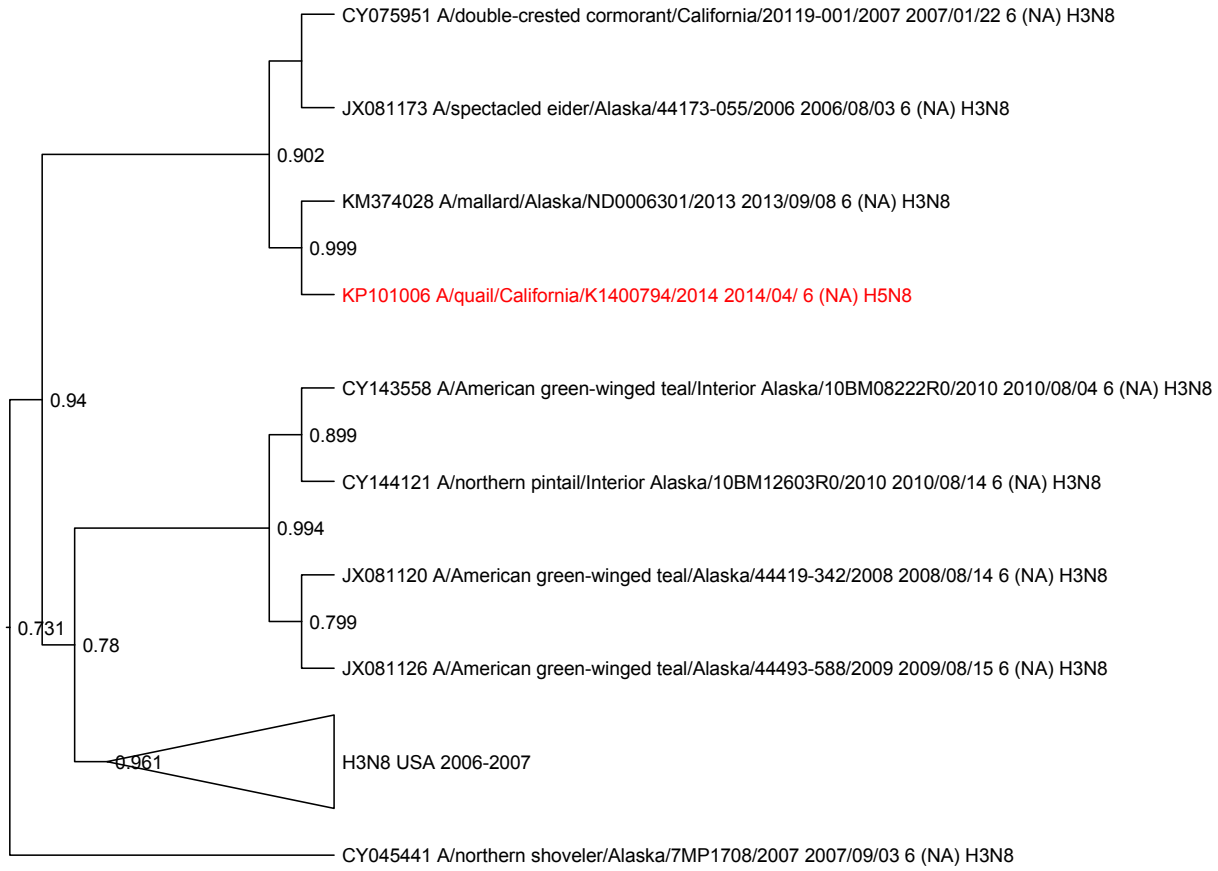
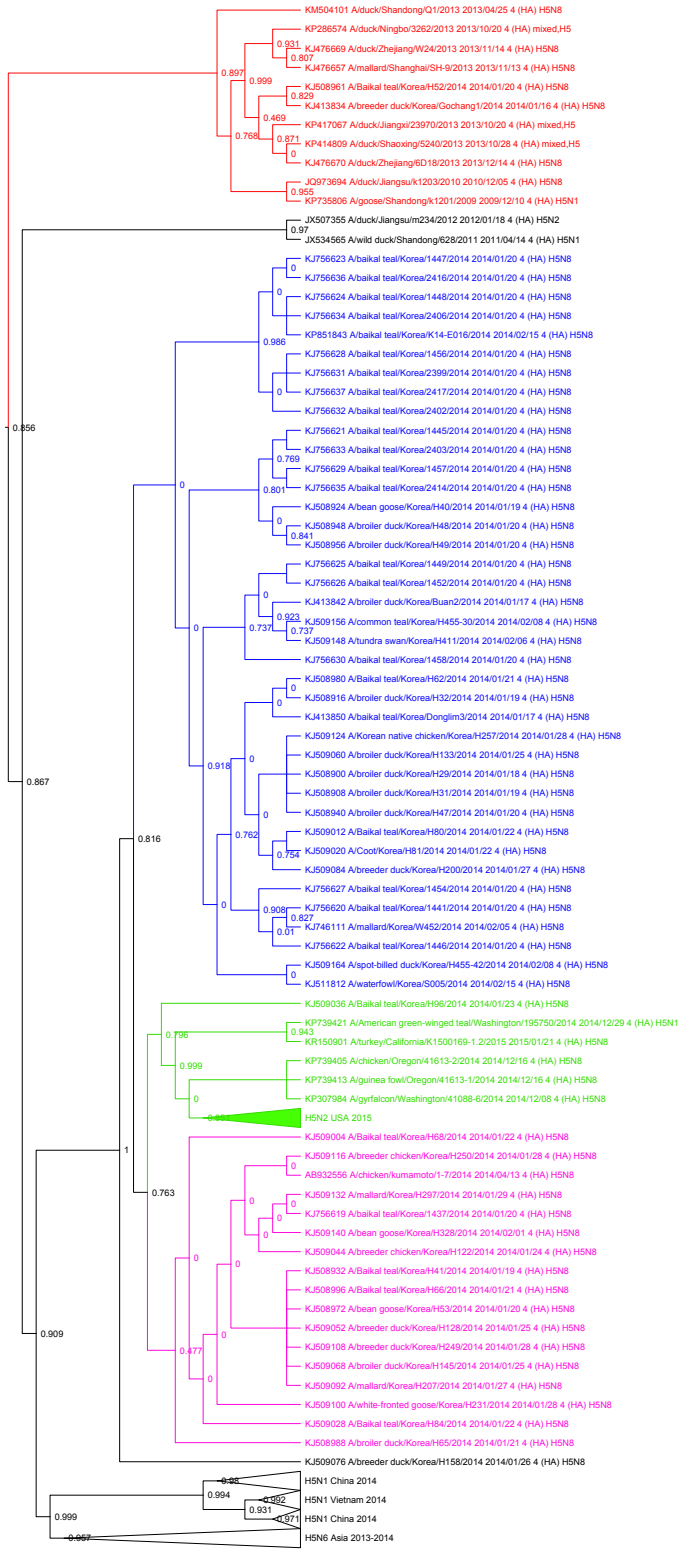


Figure 11B: The neuraminidase clade for California in 2014. The nodes are labelled with the SH local support values.



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407 Figure 12: The hemagglutinin clade for the current outbreak of H5N8. The nodes are labelled
408 with the SH local support values. The Buan Korean clades are coloured in blue (I) and magenta
409 (II), the Gochang clade is coloured in red and the US clade is coloured in green.

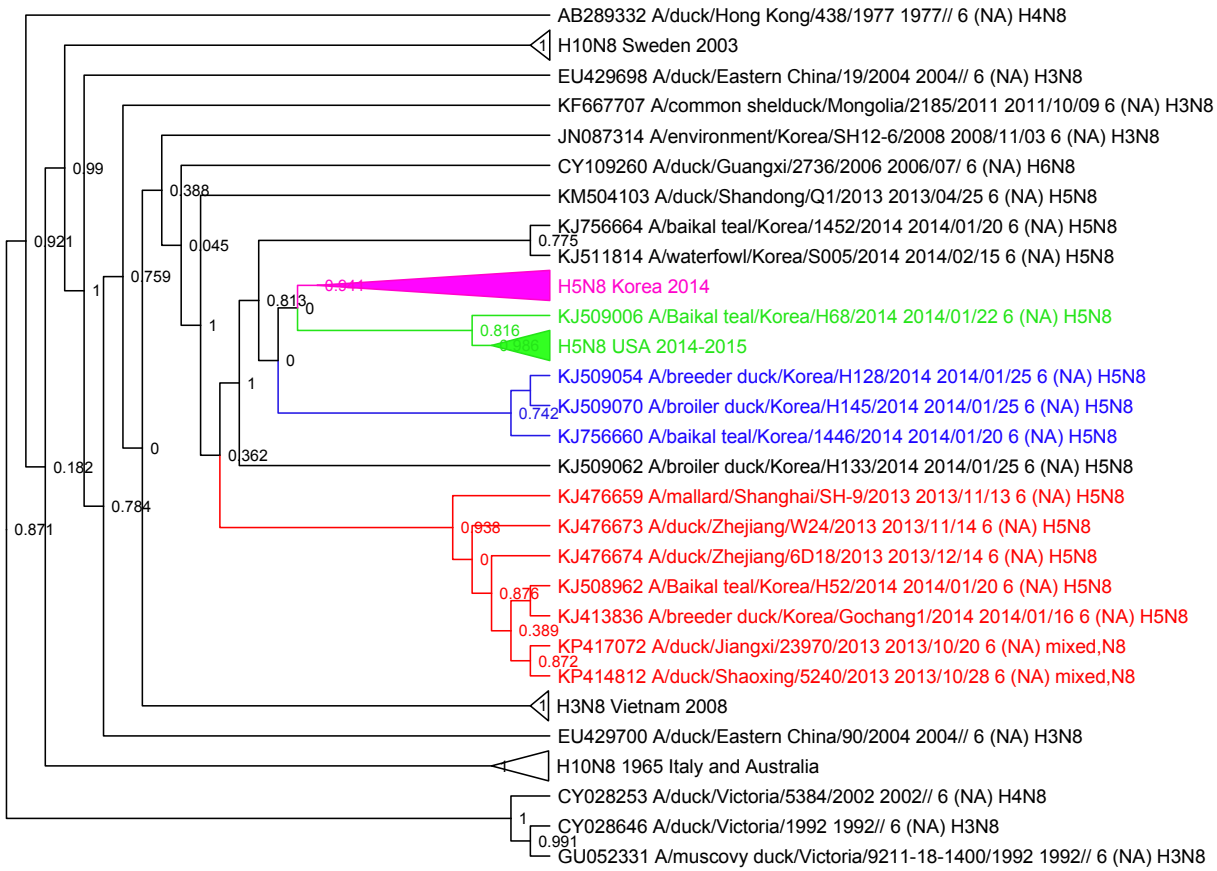

Figure 13: The neuraminidase clade for the current outbreak of H5N8. The nodes are labelled with the SH local support values. The Gochang clade is coloured in red, and the US clade is coloured in green.