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小山 傑夫

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杉田　繁夫

Summary

The hemagglutinin (HA) genes of influenza type A (H1N1) viruses isolated from swine were cloned into plasmid vectors and their nucleotide sequences were determined. A phylogenetic tree for the HA genes of swine and human influenza viruses was constructed by the neighbor-joining method. It showed that the divergence between swine and human HA genes might have occurred around 1905. The estimated rates of synonymous (silent) substitutions for swine and human influenza viruses were almost the same. For both viruses, the rate of synonymous substitution was much higher than that of nonsynonymous (amino acid altering) substitution. It is the case even for only the antigenic sites of the HA. This feature is consistent with the neutral theory of molecular evolution. The rate of nonsynonymous substitution for human influenza viruses was three times the rate for swine influenza viruses. In particular, nonsynonymous substitutions at antigenic sites occurred less frequently in swine than in humans. The difference in the rate of nonsynonymous substitution between swine and human influenza viruses can be explained by the different degrees of functional constraint operating on the amino acid sequence of the HA in both hosts.

Introduction

A number of human H1N1 influenza viruses (type A) isolated in the 1930s are related antigenically to swine influenza viruses, but the exact origin of the pandemic viruses is still unknown (Shope 1931; Murphy and Webster 1990). Analysis by oligonucleotide mapping, nucleotide sequencing, and detailed characterization of the hemagglutinin (HA) antigen with monoclonal antibodies suggests that human H1N1 viruses have evolved faster than swine H1N1 viruses (Nerome *et al.* 1985a,b; Raymond *et al.* 1986; Kida *et al.* 1987, 1988). Analysis of the evolutionary pathway for a number of human epidemic H1N1 viruses isolated between 1950 and 1983 indicated that these viruses contained both main-and side-stream mutations, which have led to many different branches (Raymond *et al.* 1986). In addition, antigenic characterization of HA has shown that the antigenic structure of the HA of swine influenza viruses had been well conserved since its first isolation in 1930 (Meier-Ewert *et al.* 1970; Kendal *et al.* 1977; Nerome *et al.* 1983; Hinshaw *et al.* 1984; Sheerar *et al.* 1989).

In early 1976, some 500 military recruits in Fort Dix, New Jersey, were infected with swine (H1N1) influenza virus (Palese and Schulman 1976a; Gold-field *et al.* 1977). H1N1 viruses that were both antigenically and genetically similar to a classical strain of swine influenza virus was isolated from the recruits. In 1988, a pregnant woman 32 years old in the United States died after being infected with a swine influenza virus (Morbidity and Mortality Weekly Report 1988). These incidents show that swine influenza viruses can infect humans. Therefore, swine (H1N1) influenza viruses are still of much concern. An understanding of the future epidemic and genetic behavior of these viruses is not possible without knowledge of the molecular evolution of field isolates. Swine

influenza virus is a useful subject for the understanding and the prediction of the possible appearance of a new pandemic virus in humans.

With the aim of elucidating evolutionary relationships between human and swine influenza viruses, we constructed a phylogenetic tree for these viruses. In particular, we describe a pattern of nucleotide substitutions unique to swine and human influenza viruses.

Materials and Methods

Virus Strains and RNAs.

Four strains of influenza viruses A/sw/Iowa/15/30 (H1N1), A/sw/Illinois/63 (H1N1), A/sw/Hong Kong/1/74 (H1N1), and A/sw/Ehime/1/80 (H1N2) were propagated in fertile hen's eggs 11 days after being laid by intraallantoic inoculation. Virus particles in the allantoic fluid were harvested after incubation at 34°C for 2 days and were purified by a method described previously (Nerome *et al.* 1983). Viral RNA was extracted from the purified viruses by the hot-phenol method (Palese and Schulman 1976b).

Serological Test.

Hemagglutination inhibition (HI) tests with antisera to reference strains were done as described by Nerome *et al.* (1983). Antisera were prepared by intravenous inoculation of rabbits with the purified viruses. Injections were repeated four times at weekly intervals.

Cloning and Nucleotide Sequences.

Double-strand complementary DNA was synthesized from viral genomic RNA with use of a synthetic oligonucleotide primer. Complementary DNA specific for viral RNA was inserted into the plasmid pBR322 or pUC118. The nucleotide sequences of the HA genes of A/sw/Iowa/15/30, A/sw/Illinois/63, A/sw/Hong Kong/l/74, and A/sw/Ehime/1/80 viruses were determined by the dideoxy method (Mizusawa *et al.* 1986).

Evolutionary Analysts of HA Genes.

For the sequences newly determined in this paper and sequences previously published for the HA genes of human and swine influenza viruses, the numbers of synonymous and nonsynonymous substitutions were estimated by the Nei-Gojobori method (Nei and Gojobori 1986). Using the numbers obtained, we constructed phylogenetic trees by the neighbor-joining (N-J) method (Saitou and Nei 1987). The evolutionary rates were computed by the least-squares method with use of the results from the phylogenetic trees.

Results

Sequence Homology at the Nucleotide and Amino Acid Levels

Nucleotide sequences of the HA genes of A/sw/Iowa/15/30 (IOWA30), A/sw/I11inois/63 (ILL63), A/sw/Hong Kong/l/74 (HK74), and A/sw/Ehime/l/80 (EHM80) were determined (data not shown) and deposited in the DNA Data Bank of Japan (DDBJ). The amino acid sequences of the HA from four strains of swine influenza viruses were aligned with other HA sequences available (Fig. 1). Swine influenza viruses are less variable than human influenza viruses at potential antigenic sites, particularly at the Sa and Sb sites (Caton *et al.* 1982).

Unlike in the human influenza viruses, a signal for potential N-linked glycosylation was absent at the Sa and Sb sites on the surface of the HA in swine influenza viruses. In human influenza viruses, new potential glycosylation sites appeared at positions 127 (PR834, A/Puerto Rico/8/34; USSR77, A/USSR/90/77) and 160 (USSR77). Glycosylation at the antigenic sites of influenza viruses seems to be more important in humans than in swine for escaping from the immune system.

A comparison of the amino acid sequences we determined with those already published data showed that the amino acids at the receptor-binding site were well conserved among different subtypes, HI, H2, H3, H5, H7, and Hl0 (Feldmann *et al.* 1988; Weis *et al.* 1988). In the swine influenza viruses, residues 130-135 and 221-225, which were thought to be on the surface of the receptor-binding pocket, were substituted at only two positions. The proportion of amino acid changes on the surface of the receptor-binding pocket of human influenza viruses was higher; it was calculated to be 54.5% (= 6/11).

The pattern of amino acid substitutions in the HA gene was different between human and swine influenza viruses, which was supported by the serological reactivity of a number of field isolates (Fig. 2). Antisera to the classical swine strain, IOWA30, gave a variety of reaction patterns with swine influenza viruses isolated between 1930 and 1982 (NVD82; A/sw/Nevada/82), but antisera to an earlier human strain, PR834, failed to inhibit Hemagglutination of all human influenza viruses isolated from 1947 to 1986.

Phylogenetic Analysis

To examine the evolutionary pathway between human and swine influenza viruses, a phylogenetic tree was constructed by the N-J method using a total of 26 nucleotide sequences of the HA gene (Fig. 3). The value given to each branch of the tree in the figure represents the number of nucleotide substitutions per synonymous site. The branching features of the HA of swine influenza virus isolates were quite different from those of human HA genes, although classical swine virus, IOWA30, has been taken to be a representative of swine and human influenza viruses from 1918 to 1930 (Nakajima *et al.* 1984).

The tree for the HA genes of human influenza viruses isolated between 1933 and 1983 showed much variety in terms of genealogy. Two human influenza viruses from the 1933 epidemic seemed to be on the independent branches. The tree also showed that there were multiple pathways of evolution even during the same period. Of the branches containing epidemic strains prevalent during the A prime era (1946-1956), the branch giving rise to A/Fort Warren/1/50 (FW50) was most closely related to USSR substrains.

As already reported, USSR77 seems to have re-emerged in humans in I 977 after a 25-year absence (Nakajima *et al.* 1978; Hayashida *et al.* 1985; Buonagurio *et al.* I 986; Saitou and Nei I 986). As shown in the evolutionary tree, USSR77 and FW50 viruses seemed to be genetically very similar with each other. A/Brazil/I 1/78 (BRZ78), A/Texas/29/82 (TX2982), A/Dunedin/6/83 (DUN683), and A/Hong Kong/32/ 83 (HK83) appeared to be representatives of four evolutionary pathways arising in or after 1977. Viruses isolated from many parts of the world in 1983 showed multiple pathways of evolution.

To estimate the year of divergence and the rate of synonymous substitution, the numbers of nucleotide substitutions were calculated for all of the HA sequences of human and swine influenza viruses. The numbers of synonymous substitutions for human and swine influenza viruses from each putative origin were respectively plotted against the year of isolation. As shown in Fig. 4, linear regression analysis indicated that, with the exception of USSR substrains, the number of synonymous substitutions for swine and human influenza viruses was proportional to the year of isolation. The result obtained was consistent with the previous report that nucleotide substitutions in human influenza virus genes can be used as a good molecular clock (Hayashida *et al.* 1985). However, the

USSR substrains were on a regression ,line different from other human strains.

Table 1 shows the rates of nucleotide substitutions for swine and human virus HA genes. The rates of synonymous substitution in swine and human influenza viruses were about the same. The results are consistent with the value obtained for human H3 HA gene reported by Hayashida *et al.* (1985). The rates of substitution at the third position of a codon were 0.00565 and 0.00717 for swine and humans, respectively. Because most of the nucleotide substitutions at the third position of a codon do not cause amino acid changes, the substitution rate at the third position of a codon for the HA gene of the swine influenza viruses was about the same as that of the human influenza viruses. The estimation of the synonymous substitution rate allowed us to calculate the time of divergence of the root leading to the two different genealogies for swine and human influenza viruses; it was around 1905.

The rates of nucleotide substitution at the first and second positions of a codon for the genes of the swine influenza viruses were estimated to be 0.0006 and 0.00017 per site per year. These values were about one-third and one-fifth, respectively, of those for human influenza viruses. These results are consistent with our observation that the rate of nonsynonymous substitution of swine influenza viruses was about three times lower than that of human influenza viruses. For both human and swine influenza viruses, the rate of synonymous substitution was much higher than that of nonsynonymous substitution (Table 1). This feature is consistent with the neutral theory of molecular evolution (Kimura 1968, 1983), contrary to the general belief that positive selection dominates evolutionary changes in human influenza A viruses (Air *et al.* 1990).

Discussion

A series of H1 HA genes of swine and human influenza viruses was analyzed in detail from both the evolutionary and the phylogenetic points of view. Raymond *et al.* (1986) classified the evolutionary pathways of the human influenza viruses into main-and side-stream mutations by phylogenetic tree. However, we did not follow their classifications because our tree showed that there was no main stream in evolutionary pathways for human influenza viruses.

Many of nucleotide sequences have been published by several authors (Raymond *et al.* 1986; Kida *et al.* 1987, 1988). They showed that the number of nucleotide substitutions for the HA genes of animal influenza viruses was lower than for the HA genes of human influenza viruses. However, our phylogenetic tree indicated that the rates of synonymous substitutions for swine and human influenza viruses were about the same. Influenza A virus has the highest rate of all the animal viruses examined so far. We estimated the rate of synonymous substitution to be 0.0080-0.011 per site per year, which was 10^6 times the rate for mammalian genes (Hayashida *et al.* 1985). This may be due to a higher error rate of the RNA polymerase, a lack of a repair mechanism for the RNA genome, and a shorter generation time for the influenza A viruses. For the swine influenza viruses, the rate of nonsynonymous substitution of the HA gene does not seem to be proportionally related to that of synonymous substitution at antigenic sites (Fig. 5). This is different from the evolutionary pattern of the human viral genes, which had a proportional relationship between rates of nonsynonymous and synonymous substitution at antigenic sites. This is explained by the fact that for swine influenza viruses, the rate of synonymous substitution is extremely small. However, it was not clear whether the slower rate of

nonsynonymous substitution was due to absence of antigenic selection for swine influenza viruses.

To examine the mechanism by which the antigenic sites in HA of swine influenza viruses are conserved, the ratio of the number of nonsynonymous substitutions to that of synonymous substitutions (N/S) at antigenic sites was calculated using PR8 as a reference strain (Caton et al., 1982). We assumed that when the N/S ratio was greater than 1, the amino acid change at the antigenic site of the HA would occur through the mechanism of positive selection. However, the N/S ratio at antigenic sites in the swine virus HA was always 1 or less, suggesting no positive selection.

On the contrary, some of the N/S ratios at the antigenic sites of human influenza viruses were more than 1. In particular, 10 of the 14 values between WSN33 and PR834 (called the A0 group) and other human influenza viruses (the A1 group) were more than 1. Thus, antigenic drift between the A0 and A1 groups might have occurred by positive selection of the antibodies. However, the N/S values for most of pairs of substitutions at antigenic sites were nearly 1 or less.

Our data suggest that the stronger conservation of amino acids at the antigenic sites in swine influenza viruses might be due to more severe functional constraints, assuming the antigenic sites of swine influenza viruses are present at the same positions as human PR8 strains.

One of the aims of this study was to conduct a phylogenetic analysis of swine and human influenza viruses in relation to the origin of these viruses. We found that the H1N1 viruses from these two hosts had diverged from a common ancestor in around 1905, long before the 1918-1919 outbreak of influenza viruses in humans. Many variants of swine influenza viruses continue to circulate in many parts of the world. Our

study suggests that their stable conservation in the swine population would be closely related to an unusual fashion of evolution explained above.

Sequence data available for analysis in this study was limited to only a few sequences, which may not be representative of swine influenza viruses. This was due to difficulties in collecting many wild swine strains. Considering high variability of RNA virus genome, it might be also problematic to sequence viruses after propagation in laboratory cells and eggs but not directly the circulating viruses in nature. Further effort should be waste to minimize these problems.

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Table 1. The rates of nucleotide substitutions (per site per year) of swine and human virus genes*

Types and positions of substitutions	Swine (H1) (4-1062)†	Human (Old H1) (4-1062)†	Human (H3HA)‡
Synonymous	0.01106	0.00796	0.0141
First	0.00060	0.00184	
Second	0.00017	0.00084	
Third	0.00565	0.00717	
Nonsynonymous	0.00041	0.00125	0.0029

* The rates of nucleotide substitutions per site per year were calculated from the phylogenetic tree presented in Fig. 3

† Estimates obtained present study. Accumulations of synonymous substitutions in swine and human are shown in Fig. 4

‡ After the report of Hayashida *et al.*

Legends for figures

Fig. 1 Comparison of the amino acid sequences of the HAs between swine and human influenza viruses. For comparative analysis of amino acid substitutions, the HA gene of IOWA30 was used as the reference sequence. S.P. represents N-terminal signal sequences. Potential glycosylation sites are boxed. Antigenic regions characterized in the PR834 (Mt. Sinai) strain are marked as follows: a, Sa; b, Sb; c, Cb; 1, Ca_1; and 2, Ca_2 (Caton *et al.* 1982). Residues corresponding to receptor binding site are indicated by R (Weis *et al.* 1988). It is probable that a change at residues 153 and 155 would alter host specificity of the virus. Theses residues are indicated by S (Both *et al.* 1983; Kilbourne *et al.* 1988a, b).

Fig. 2. Comparison of the antibody response among field isolates of swine and human influenza viruses. Antisera to IOWA30 and PR834 were treated with receptor destroyed enzymes (RDE). Nonspecific HA in the sera was removed by addition of washed chicken red blood cells. The following strains were used in the HI tests: IOWA30, 197631 (A/sw/1976/31), ILL63, NJ76, EHM80, NVD82 (A/sw/Nevada/82), NWS33 (A/NWS/33), PR834, FM47 (A/FM/1/47), KJY52 (A/Kojiya/4/52), OMC53, (A/Ohmachi/1/53), SJK56 (A/Shinjuku/1/56), and YMG86 (A/Yamagata/120/86).

Fig. 3 Phylogenetic tree for the H1 HA gene of swine and human influenza viruses. The tree was constructed by the N-J method (Saitou and Nei 1987), using the number of synonymous substitutions (Nei and Gojobori 1986). The following viruses were used in the phylogenetic tree: IOWA30, ILL63, HK74, NJ76, EHM80, WSN33, PR834, FW50,

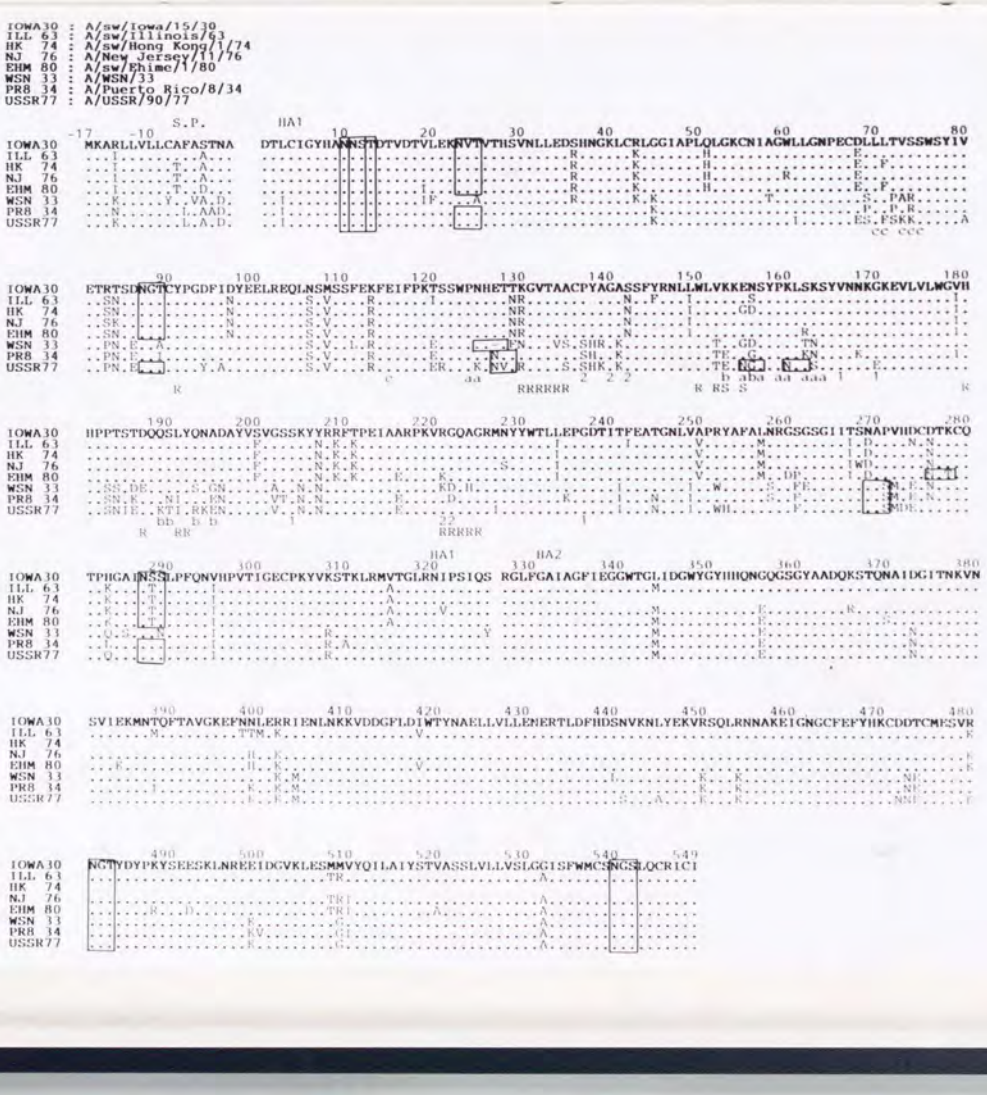
ENG51 (A/England/1/51), FLW52 (A/Fort Leonard Wood /1/52), QSL54 (A/Queensland/34/54), DEN57 (A/Denver/1/57), USSR77, LACK78 (A/Lackland/3/78), BRZ78, ENG80 (A/England/333/80), IND80 (A/India/6263/80), TX1282 (A/Texas/12/82), TX2982, GA7983 (A/Georgia/79/83), GA11483 (A/Georgia/114/83), HK83, CHL83 (A/Chile/1/83), DUN683, DUN2783 (A/Dunedin/27/83), VIC83 (A/Victoria/7/83) (Winter *et al.* 1981; Both *et al.* 1983; Concannon *et al.* 1984; Jabber *et al.* 1985). The putative root between the swine and human groups was inferred from the relationship between the rate and the year of isolation.

Fig. 4 Linearity of the number of synonymous substitutions with time for the HA gene of swine and human viruses. Nucleotides 4-1062 in HA were used for linear regression analysis. The branch length was estimated from the trees and was plotted against the year of isolation.

Fig. 5. Relationship between the number of nonsynonymous substitutions and that of synonymous substitutions at antigenic sites (also see Fig. I). Closed squares represent swine viruses and open circles represent human viruses. The mean values of N/S for human and swine influenza viruses were 0.927 and 0.296, respectively. The N/S value between WSN33 and IOWA30 is given with a closed triangle.

Key words

Influenza virus - Swine virus - Hemagglutinin gene - Molecular evolution - Neutral theory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WSN 33 : A/WSN/33
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USSR77 : A/USSR/90/77
S.P.
HA1
HA1
HA2

Fig. 1

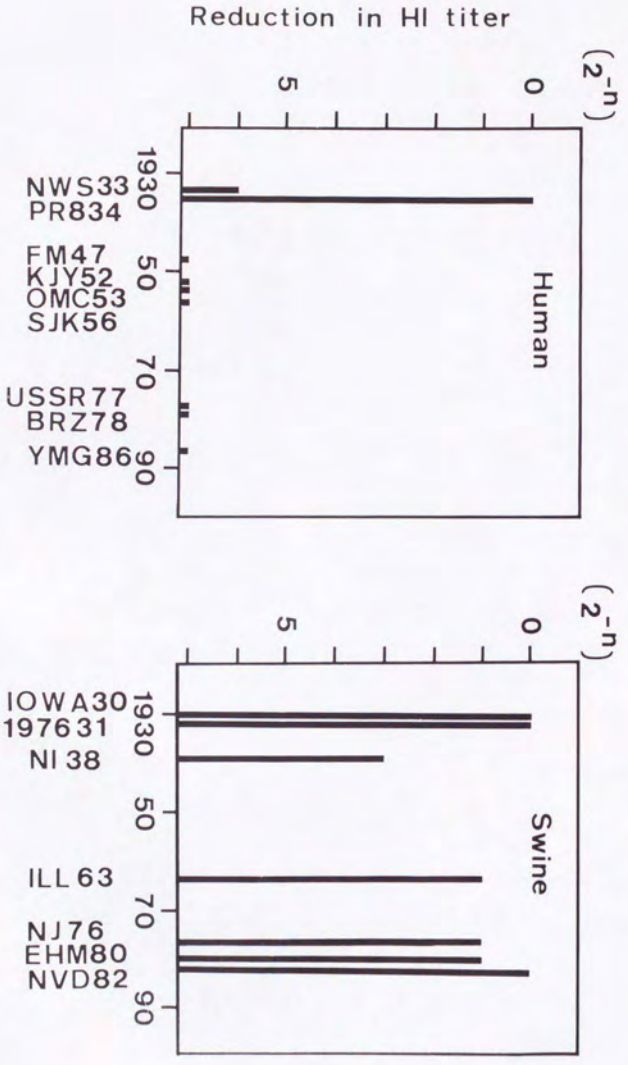
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Fig. 2

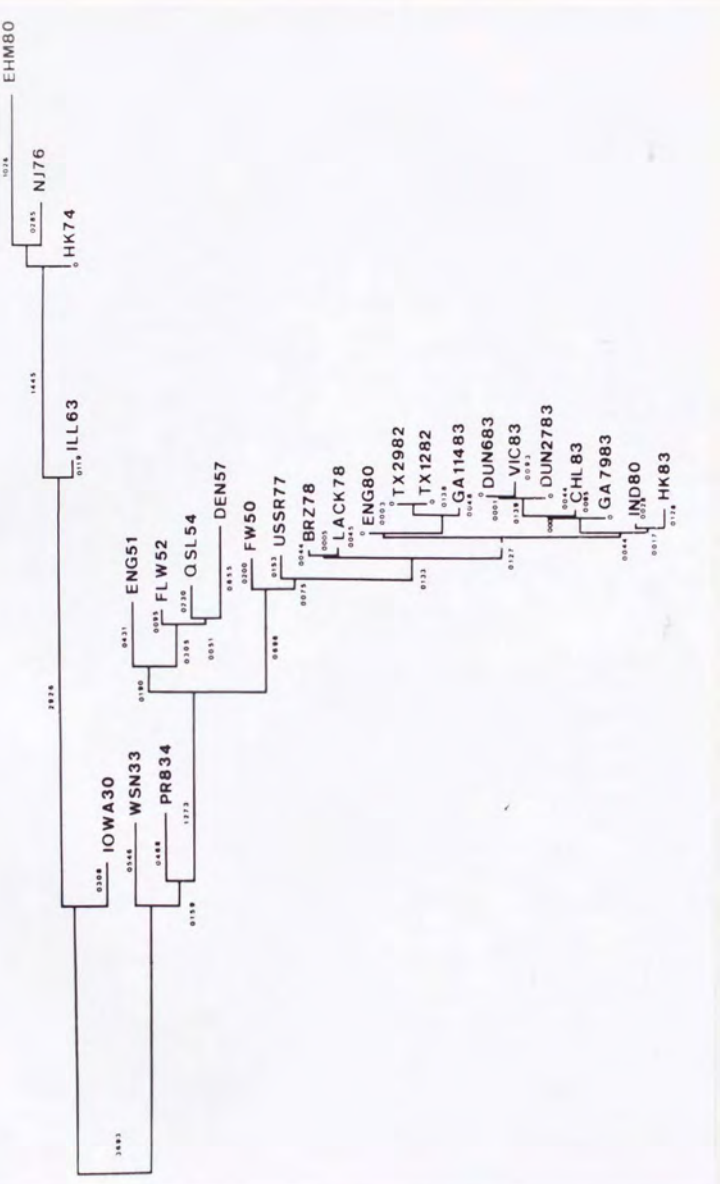
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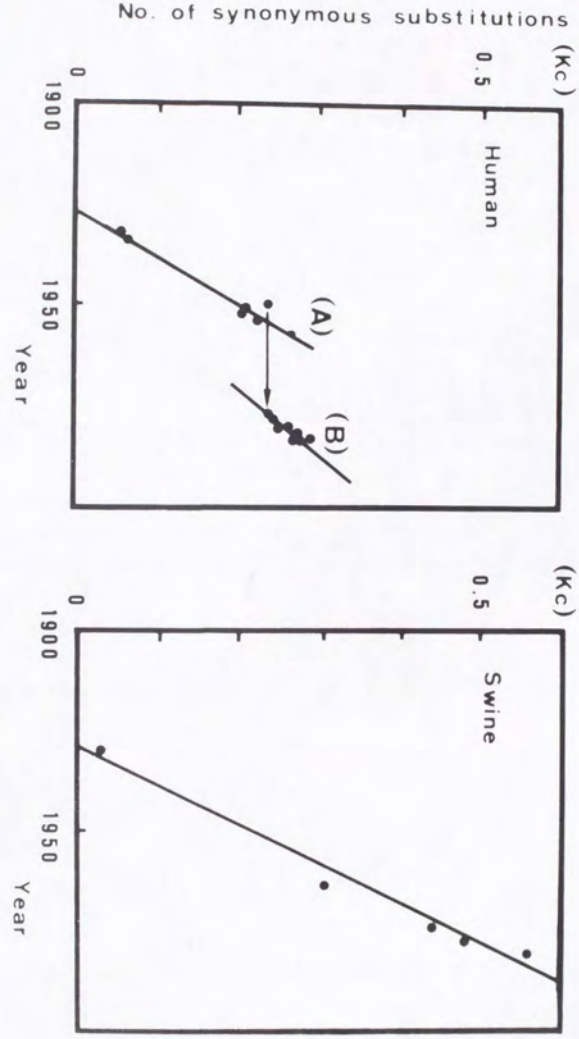
Fig. 3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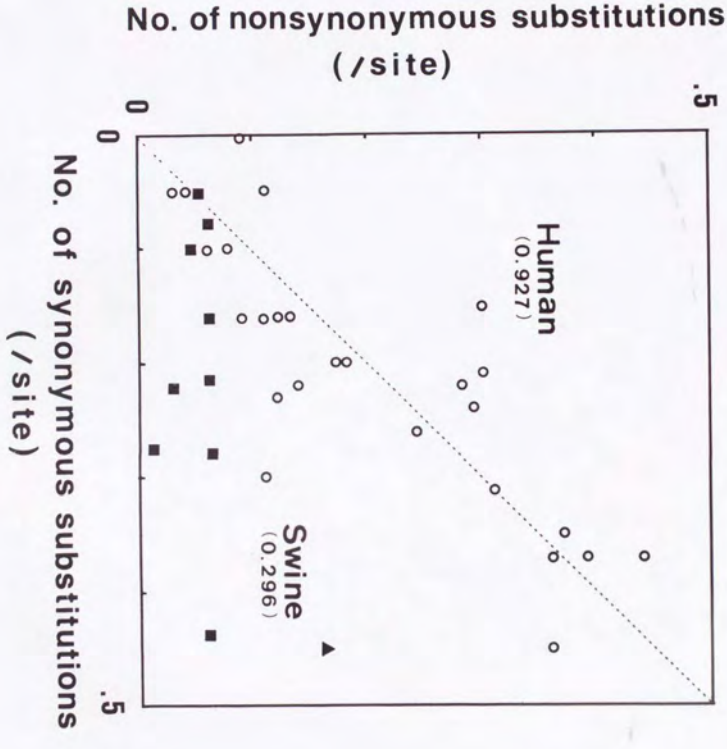
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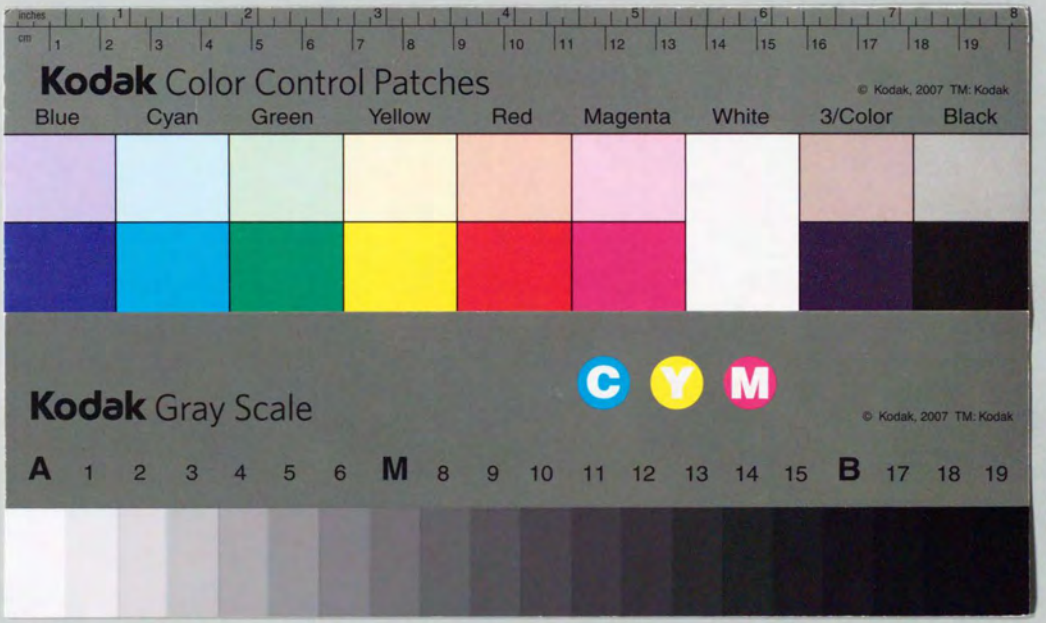
Fig. 5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