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ORIGINAL ARTICLE

An evolutionary insight into Newcastle disease viruses isolated in Antarctica

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Abstract The disease caused by Newcastle disease virus (NDV) is a severe threat to the poultry industry worldwide. Recently, NDV has been isolated in the Antarctic region. Detailed studies on the mode of evolution of NDV strains isolated worldwide are relevant for our understanding of the evolutionary history of NDV. For this reason, we have performed Bayesian coalescent analysis of NDV strains isolated in Antarctica to study evolutionary rates, population dynamics, and patterns of evolution. Analysis of F protein cleavage-site sequences of NDV isolates from Antarctica suggested that these strains are lentogenic. Strains isolated in Antarctica and genotype I reference strain Ulster/67 diverged from ancestors that existed around 1958. The time of the most recent common ancestor (MRCA) was established to be around 1883 for all class II viruses. A mean rate of evolution of 1.78×10^{-3} substitutions per site per year (s/s/y) was obtained for the F gene sequences of NDV strains examined in this study. A Bayesian skyline plot indicated a decline in NDV population size in the last 25 years. The results are discussed in terms of the possible role of Antarctica in emerging or re-emerging viruses and the evolution of NDV populations worldwide.

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Introduction

The disease caused by Newcastle disease virus (NDV) is one the most important diseases of poultry, affecting the poultry industry worldwide [1]. NDV belongs to the genus *Avulavirus* of the family *Paramyxoviridae*, and its genome is a non-segmented, single-stranded, negative-sense RNA molecule of approximately 15,186 nucleotides (nt) in length [2].

NDV isolates have been grouped by virulence phenotype, with lentogenic, mesogenic, and velogenic strains, in order of increasing virulence [3]. Lentogenic viruses typically cause subclinical infections or mild respiratory disease. Mesogens are of intermediate virulence, usually resulting in moderate respiratory disease with occasional nervous signs. Velogens are the most virulent viruses and may cause extensive hemorrhagic lesions, particularly in the gastrointestinal tract (viscerotropic), and/or a predominance of nervous signs (neurotropic) [4].

NDV infection is initiated by the action of two envelope glycoproteins. One of these mediates attachment of the virus to a host-cell receptor and is designated HN (hemagglutinin-neuraminidase). The other glycoprotein, designated as the fusion (F) protein, is responsible for virus penetration into the host cell and syncytium formation [5]. The F protein plays a key role in viral virulence and is a major target for the immune response [6]. The NDV F protein is a trimeric type I integral membrane protein that is synthesized as an inactive precursor, F0 (66 kDa), which is posttranslationally cleaved by host-cell proteases into two disulfide-linked subunits, the N-terminal F2 (12.5 kDa) and the C-terminal F1 (55 kDa) [7, 8]. The sequence of the F protein cleavage site is a major determinant of NDV pathogenicity. The cleavage sites of virulent NDV strains usually contain multiple basic residues, whereas avirulent strains have fewer basic residues [9].

The consensus sequence of the F protein cleavage site of velogenic and mesogenic strains is ^{112}(R/K)RQ(R/K)RF^{117}, while the consensus sequence of the lentogenic F cleavage site is ^{112}(G/E)(K/R)Q(G/E)RL^{117} [10]. Most of the recent virulent NDV strains bear the virulence motif ^{112}RRQKRF^{117} at the cleavage site of their F0 protein [11, 12]. Seven neutralizing epitopes have been mapped on the F protein of NDV [5, 13, 14]. Critical amino acids involved in neutralization sites are sites 72, 74, 75, 78, 79 and 343, as well as a stretch of amino acids from residues 157 to 171 [14, 15].

NDV strains are divided into two classes based on genetic analysis: class I strains, which are mainly isolated from wild birds and are generally avirulent, and class II strains, which are isolated from wild and domestic birds can be either virulent or avirulent [16]. Class I viruses comprise a single genotype, while class II viruses are divided into 18 or possibly 19 genotypes (I–XIX) [17–19]. Strains of genotypes V, VI and VII of class II are currently circulating in chickens throughout the world [20].

Since NDV was first reported in poultry in 1926, vaccination has been widely used for prevention and control of the disease caused by NDV [21]. The most commonly used live vaccines are LaSota and Clone-30, which belong to genotype II [22]. Characterization of NDV strains is important to evaluate field changes, anticipate new outbreaks, and develop adequate control measures [23]. Large gaps in our current knowledge in the areas of epidemiology and evolution limit the possibilities for controlling the disease [24, 25].

Three main panzootics have occurred in the last century. The first one (1926 to 1960) was caused by viruses belonging to genotypes II, III and IV, while the second (1960 to 1973) and third (1970–1980) were caused by viruses of genotypes V–VI [14]. Severe outbreaks in Western and Southern Europe [26, 27], South Africa [28] and Taiwan [29] in the 1990s were caused by genotype VII, the currently circulating genotype in Asia, Africa and Europe [14]. A recent outbreak of NDV in South America (Venezuela) has also been attributed to a genotype VII virus, suggesting that viruses of this genotype are spreading worldwide [30, 31].

In 2010, infection by virulent NDV was confirmed in 80 countries, including infections of wild birds in Canada, Germany, Israel, Italy, Kenya, Mongolia and the USA, and infections in domestic poultry in countries of North and South America, Europe, Africa, and Asia [32]. Moreover, recent studies revealed the isolation of NDV in penguins from King George Island in the Antarctic region [33]. Detailed studies on the mode of evolution of these new NDV strains are relevant for inferring the evolutionary history of NDV. In order to gain insight into these matters, Bayesian coalescent studies were performed to investigate the evolutionary rates, population dynamics and patterns of evolution of NDV.

Materials and methods

Sequences

Nucleotide sequences from NDV strains were obtained using ARSA from the DDBJ database (available at: http://arsa.ddbj.nig.ac.jp/). Strain names and accession numbers can be found in Supplementary Material Table 1.

Sequence alignment and *in silico* translation of nucleotide sequence

Sequences were aligned using the MUSCLE program [34]. Nucleotide sequences were translated to amino acids *in silico* using software from the MEGA 5 program [35].

Bayesian coalescent Markov chain Monte Carlo (MCMC) analysis

In order to gain insight into the evolutionary rate and mode of evolution of NDV strains, we used a Bayesian Markov MCMC approach as implemented in the BEAST package v.1.7.5 [36]. For strains included in these analyses, see Supplementary Material Table 1. First, software from the Datamonkey server [37] was used to identify the optimal evolutionary model that best fitted our sequence dataset. Akaike information criteria and the hierarchical likelihood ratio test indicated that the HKY + Γ model was the most accurate. Using this model and 50 million steps of MCMC, different population models were tested (constant population size, exponential population growth, expansion population growth, logistic population growth and Bayesian Skyline). Statistical uncertainty in the data was reflected by the 95 % highest probability density (HPD) values. Results were examined using the TRACER v1.5 program (available from http://beast.bio.ed.ac.uk/Tracer) from the BEAST package. Convergence was assessed with ESS (effective sample size) values after a burn-in of 2 million steps. Models were compared by calculating the Bayes factor (BF) [38] from the posterior output of each of the models using the TRACER v1.5 program as explained on the BEAST website (http://beast.bio.ed.ac.uk/Model comparison). A log BF (natural log units) values greater than 2.3 indicates strong evidence against the null model. The Bayesian skyline model was the best fit to the data. Maximum clade credibility trees were generated using the Tree Annotator program from the BEAST package and the FigTree program v1.4.1 (available at: http://tree.bio.ed.ac.uk) was used for the visualization of the annotated trees.

Bayesian skyline plots (BSPs) were used to infer how the effective population size has changed over time [38, 39].

Results

Mapping of amino acid substitutions found in the fusion proteins of NDV strains isolated in Antarctica

Previous studies have identified NDV strains isolated in Antarctica as class II strains [33]. In order to gain insight into the virulence status of these strains, partial F gene sequences from NDV isolates from Antarctica (positions 4502 to 4995 relative to NDV reference strain LaSota, accession number AF077761) were aligned with the corresponding sequences of members of nine genotypes of class II strains for which complete genome sequences had been determined. For names and accession numbers of NDV strains included in this analysis, see Supplementary Material Table 1. Once aligned, they were translated *in silico* to amino acids using the MEGA 5 program [35], and the results are shown in Figure 1.

The F protein cleavage-site sequence of NDV isolated in Antarctica is ^{112}GKQGRLI^{118}, suggesting that the NDV strains isolated in that region of the world and included in these studies are lentogenic strains. Nevertheless, more studies will be needed to address this issue. Moreover, no amino acid substitutions were found at positions 72, 74, 75, 78 and 79 of the F2 protein, which were previously shown to be involved in neutralization [15]. An N-linked glycosylation acceptor site (N-X-S/T, where X corresponds to any amino acid except aspartic acid or proline) at position 85-87 of the F2 protein is also conserved [9, 40], as are the cysteine residues at positions 25 and 76 of the F2 protein [41].

Bayesian coalescent analysis of NDV strains isolated in Antarctica

In order to determine the evolutionary rate and mode of evolution of the NDV population, we used a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST package [36]. In this case, the same F gene sequences from NDV strains isolated in Antarctica were aligned with corresponding sequences from 74 NDV strains, representing class I and genotypes I to XIX of class II strains. Names and accession numbers of NDV strains included in these analyses can be found in Supplementary Material Table 1. After performing the alignment and determining that the optimal evolutionary model is HKY + Γ, different population dynamic models were

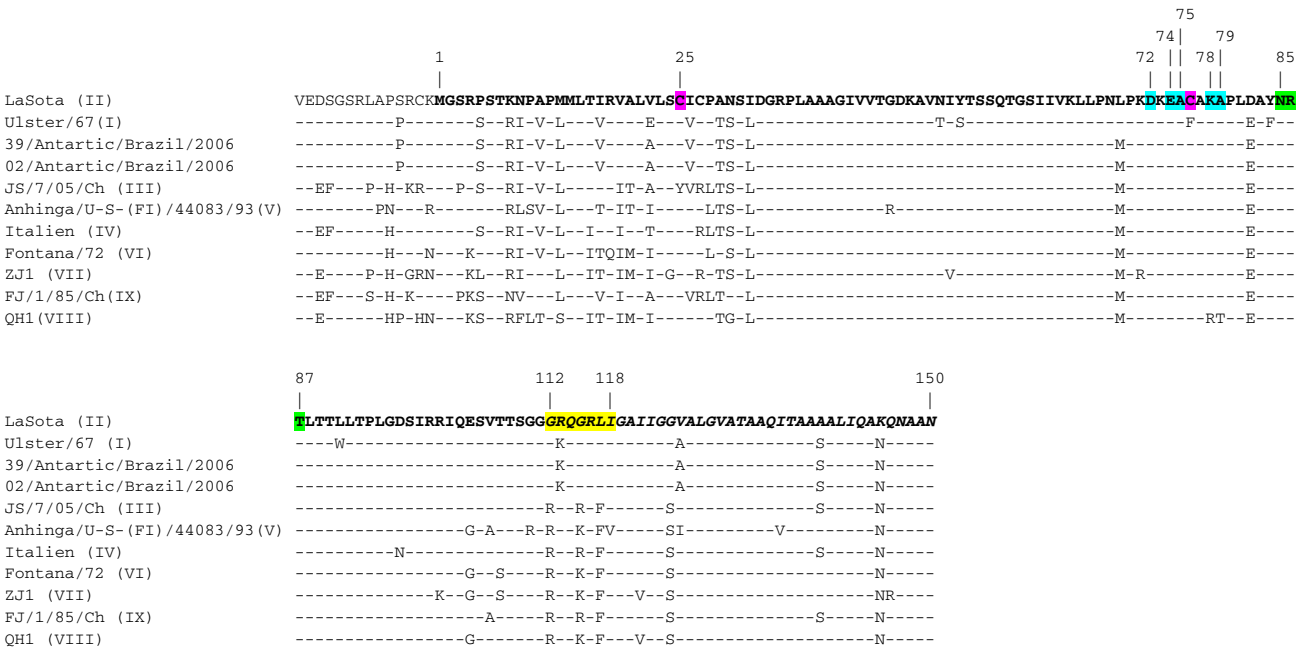

Fig. 1 Alignment of F amino acid sequences of NDV strains. Strain names are shown at the left side of the figure, and their class II genotype is indicated in parentheses. Identity to the LaSota strain (genotype II) is indicated by a dash. F2 sequences are shown in bold, and F1 sequences are shown in bold and italics. Numbers above the alignment indicate amino acid positions. The F protein cleavage site is highlighted in yellow. Amino acid substitutions detected in antigenic sites in neutralization escape mutants are indicated in turquoise [5, 6, 13]. A potential acceptor site for N-linked glycosylation at residues 85-87 is highlighted in green [8]. Cysteine residues at positions 26 and 76, which are conserved among most NDV isolates, are highlighted in fuchsia [22]

tested. The results for 50 million steps of MCMC analysis, using the HKY + Γ model, a relaxed clock and the Bayesian skyline model [42] are shown in Table 1. A mean rate of 1.78×10^{-3} substitutions per site per year (s/s/y) was obtained for the F gene sequences of NDV strains used in these studies. A maximum clade credibility tree revealed that all class II genotype strains have evolved from ancestors that existed around 1883 (130 years before the most recent isolates included in these studies, see Fig. 2). Both classes of NDV strains evolved from ancestors that existed around 1819 (Table 1). Strains isolated in Antarctica and genotype I reference strain Ulster/67 diverged from ancestors around 1958 (Fig. 2). BSPs suggested that a constant effective population size was maintained until the late 1980s (Fig. 3), where a decline in the population is observed.

Discussion

NDV strains isolated from penguins in Antarctica were assigned to genotype I of class II (Fig. 2), in agreement with previous reports [33] and with antigenic studies of NDV isolated from penguins from Antarctica that showed a reaction against a monoclonal antibody raised against NDV Ulster/67 strain (genotype I) [43]. Viruses of this genotype have been associated with outbreaks in Australia that occurred between 1998 and 2000 [44]. Genotype I viruses from these same outbreaks were found to be velogenic, and previous reports have shown that the origin of these viruses can be traced back to low-virulence NDV strains circulating in waterfowl just prior to the outbreak [45].

NDV strains circulating in one particular avian species may have the ability to cause disease in other avian species. For example, NDV strains from pigeons have been reported to be responsible for outbreaks in chickens [46–48]. Moreover, virtually all domestic and wild bird species are susceptible to infection with NDV [49]. Therefore, although the possibility of direct contact between penguins and chickens seems unlikely, other wild birds may act as carriers of different NDV strains through transmission routes that are not yet fully understood [19].

The presence of NDV strains in Antarctica, where other avian species live, indicates the importance of NDV strain characterization in all regions of the world. Genotypes V, VI, and VII of class II are currently circulating worldwide in chickens [20]. The role of Antarctica in maintaining other NDV genotypes not circulating at the moment also reinforces the relevance of in-depth NDV surveillance studies.

The F protein cleavage-site sequence has been shown to be a major determinant of NDV virulence [50]. The F protein cleavage sites of NDV strains isolated in Antarctica were found to have the consensus cleavage site of avirulent strains (Fig. 1). These cleavage sequences are insensitive to intracellular proteases and depend on extracellular secreted proteases for cleavage, limiting the replication of avirulent strains to the respiratory and enteric tracts [8–10]. More studies will be needed in order to confirm the avirulent (lentogenic) phenotype of NDV isolated from penguins in Antarctica.

Bayesian coalescent analysis revealed a rate of evolution of 1.78×10^{-3} s/s/y for NDV strains (see Table 1). This evolutionary rate is slightly higher than the rate estimated in a recent study for full-length NDV F gene sequences (1.35×10^{-3} s/s/y), although it lies within the confidence intervals of these estimations (0.71 - 1.98×10^{-3} s/s/y) [21]. This evolutionary rate is comparable to rates previously estimated for other fast-evolving RNA viruses such as human immunodeficiency virus type 1 (gp160env; 2.4×10^{-3} s/s/y) [51], human respiratory syncytial virus (G; 1.9×10^{-3} s/s/y) [52] and hepatitis C virus (E2; 3.4×10^{-3} s/s/y) [53].

The time of the most recent common ancestor (MRCA) was established to be around 1883 for all class II viruses (Fig. 2). This estimate is in agreement with previous reports that established the time of the MRCA for class II NDV strains to be around 1885 [21]. This finding is also in line with studies done by Macpherson in 1956, which suggest that a disease outbreak in domestic birds in

Table 1 Bayesian coalescent inference of Newcastle disease viruses

Group[a]	Parameter	Value[b]	HPD[c]	ESS[d]
F gene sequences	Log likelihood	-5576	-5595 to -5558	4010
	Posterior	-9101	-9055 to -9150	402
	Prior	-3525	-3573 to -3483	287
	Mean rate[e]	1.78×10^{-3}	9.22×10^{-4} to 2.56×10^{-3}	228
	Root age (years)	194	104 to 308	221
	MRCA[f]	1819	1705 to 1909	

[a] See Supplementary Material Table 1 for strains included in this analysis. [b] In all cases, mean values are shown. [c] High probability density values. [d] Effective sample size. [e] Mean rate was calculated in substitutions/site/year. [f] Year of the most common recent ancestor

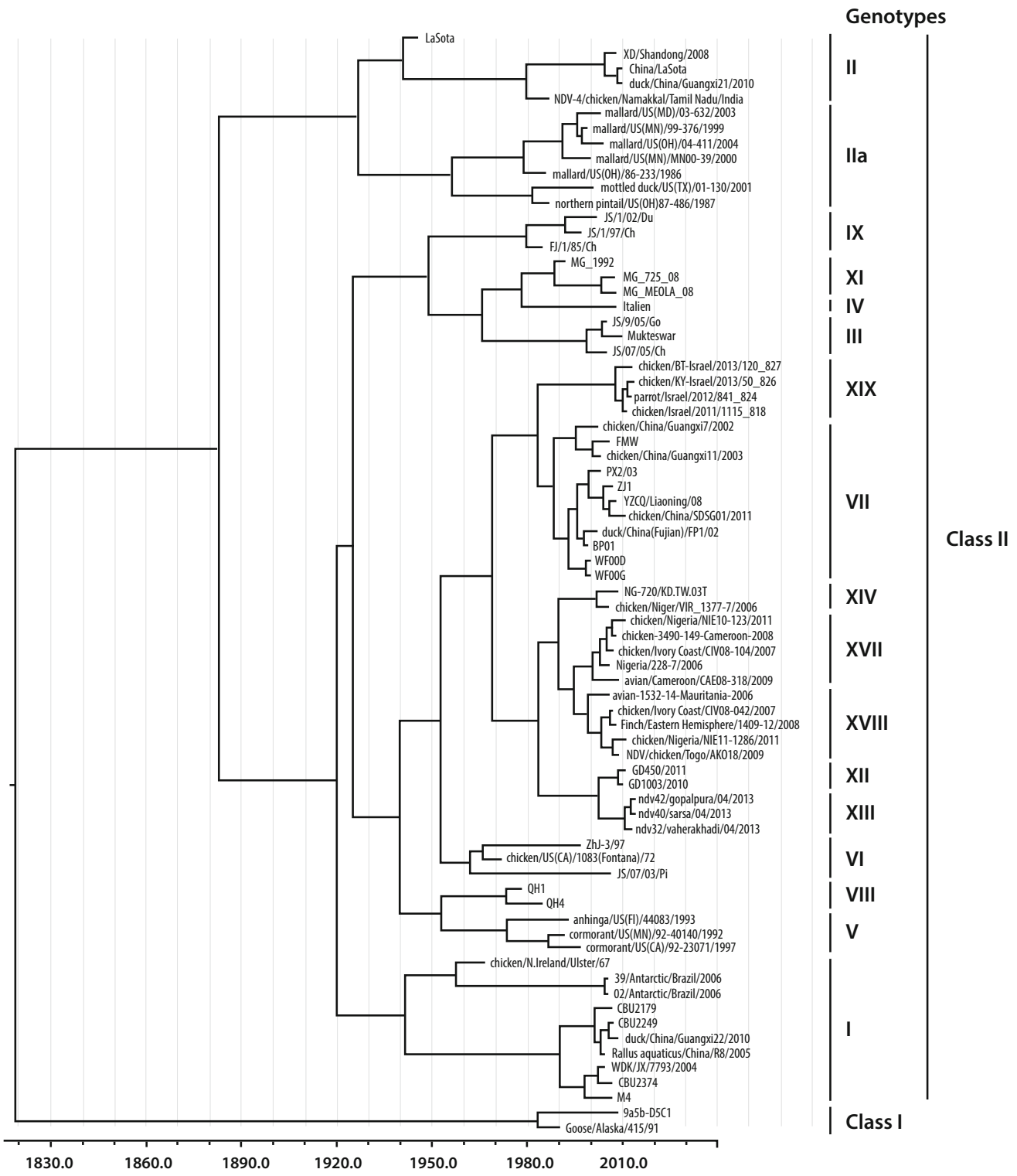

Fig. 2 Bayesian MCMC phylogenetic tree analysis of F genes of NDV strains. A maximum-credibility clade obtained using the HKY + Γ model, the Bayesian Skyline model and a relaxed clock (uncorrelated exponential) is shown. The tree is rooted to the MCRA. Years are indicated on the *x*-axis. Strains are shown by name and their genotypes are indicated on the right side of the figure. Strains isolated in Antarctica are shown by black arrows

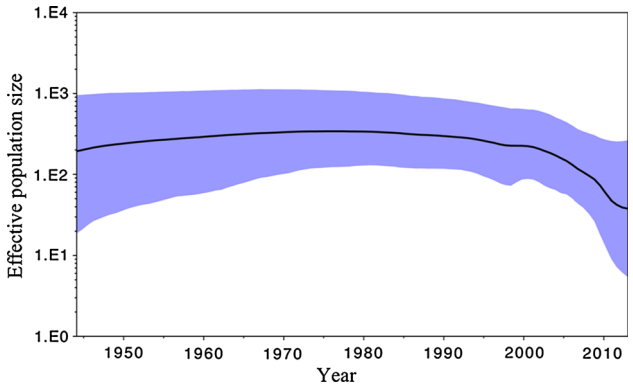

Fig. 3 Bayesian skyline plot depicting the population history of NDV strains. The *x*-axis indicates the year and the *y*-axis shows the product of effective population size and the generation length in years. The thick solid black line is the median estimate, and the blue area shows the 95 % highest probability density (HPD) values [38]

northwest Scotland between 1897 and 1898 was due to NDV [54].

In recent studies, Chong *et al.* have investigated the demographic history of NDV class II genotypes I-VII through Bayesian coalescent approaches, suggesting the maintenance of a constant effective population size until the late 1990s, when an abrupt decline with a posterior recovery (around 2000) was observed [21]. Roughly similar results were suggested by the analyses performed in the present study, which are summarized in a BSP supported by a narrow 95 % HPD (Fig. 3). Interestingly, the population dynamics observed in the last years of our analysis suggest a different behavior compared to what was reported previously, since a persistent continuous decrease in the effective population size was observed. This behavior can be explained by the larger number of class II genotypes considered in the present analysis (I-XIX), as distinct genotypes have been reported previously to exhibit different population dynamics [21]. Although the reasons for the observed decline are currently unknown, both climate change and avian influenza control measures have been suggested previously as possible factors [21]. More studies should be conducted in order to address these issues.

Considering that NDV seems to evolve rapidly towards higher virulence [55] and that several studies have reported not only increased pathogenicity but also outbreaks in vaccinated animals and increased host range [56, 57], it is becoming clear that it is important to conduct in-depth characterization of new strains isolated during the course of outbreaks worldwide to determine how these viruses are evolving. Additionally, studying viruses isolated from different wild birds and environments might contribute to our understanding of how NDV evolves and spreads around the world.

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