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ORIGINAL ARTICLE

Molecular evolution and epidemiological links study of Newcastle disease virus isolates from 1995 to 2016 in Iran

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Abstract In the case of Newcastle disease virus, multiple factors such as host adaptation, immune response evasion, and selective pressures have been suggested to result in evolution of viruses and the emergence of genetic variants. Multiple studies on virus classification and global epidemiological links have yielded consistent data. Here, we have performed a molecular analysis study of circulating Newcastle disease viruses in Iran (1995-2016). According to evolutionary divergences, subgenotype VIg, VIj, VIIj, VIId, XIIIa and XIIId isolates have been circulating in the country during a 21-year period. Based on data analysis, VIg isolates shared highest sequence identity with Russian and Polish isolates of the VIg subgenotype, while VIj subgenotype isolates (2012) were most similar to a virus isolated in 2015 in India. Analysis of the evolutionary divergence of subgenotype VIIj suggests that Chinese and Ukrainian viruses may have played a crucial role in the emergence of VIIj isolates. Evolutionary difference studies also indicated that XIIIa isolates circulating in Iran may have caused the emergence of adapted variants of subgenotype XIIId. Therefore, we propose that the evolutionary and epidemiological study of virulent Newcastle disease viruses could help to provide accurate molecular data about variants circulating in the region, thus aiding in the design of more efficient recombinant vaccines.

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Introduction

Newcastle disease (ND), a destructive infection disease that causes huge economic losses to the poultry industry, is caused by a virus of the family *Paramyxoviridae*, in genus *Avulavirus*, named Newcastle disease virus (NDV) [1–3]. The NDV genome is a single stranded negative-sense RNA molecule encoding six proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA-polymerase (L) [4, 5]. Three major defined pathotypes of ND viruses, velogenic (virulent), mesogenic (intermediate) and lentogenic (avirulent), are determined based on pathogenicity indices, including intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI), and mean death time (MDT) [2, 3, 6, 7]. Another determinant of the pathogenicity of NDV strains is believed to be the cleavage site of the fusion protein, which is proteolytically cleaved to the disulfide-linked polypeptides F_1 and F_2 [8, 9]. In general, the homotrimeric fusion protein is responsible for injection of the NDV genome into the cytoplasm by pore formation [9, 10]. This protein also contributes to fusion of the virus to the cell when coexpressed with the hemagglutinin-neuraminidase protein [9, 10].

Various classification systems based on fusion gene sequences have been recommended for NDVs [11–15]. An earlier method sorts out the viruses into seven lineages and different sublineages based on partial F sequence (374 bp) [11]. Class I and II viruses, which are subdivided into 9 and 18 genotypes, respectively, are classified by another system that was proposed later [12, 13, 15], but a later and more reliable method groups the NDVs into genotypes and subgenotypes based on the evolutionary distances of the complete coding sequence of fusion gene. This results in

class II viruses including 18 genotypes and class I viruses containing only one genotype [14].

Based on NDV taxonomy, the early genotypes I, II (including lentogenic viruses used for making live vaccines), III, IV and IX appeared between the 1930s and 1960s. Genotypes V, VI, VII, VIII and XI, which are known as late genotypes, include virulent isolates of NDV that emerged after 1960s. G genotype V viruses related to the late genotypes from the 1970s were isolated in South, Central and North America from poultry species and cormorants. Doves and pigeons are the most frequent host for genotype VI isolates, while genotype VII viruses involve a wide range of hosts in the Middle East and Asia. The importance of this group of isolates is related to their role as pathogens of poultry. ND outbreaks involving genotype VII isolates occur worldwide. Low-virulent genotype X viruses (isolated from waterfowl and shorebirds) have been reported in North America. Genotype XII isolates of NDV have been found in South America (in poultry) and China (in geese). Genotype XIII includes pathogenic isolates from Russia, Iran and Pakistan from 1995 to 2008. In West and Central Africa, virulent isolates of genotype XIV, were found from 2006 to 2008, whereas viruses of genotype XV have been isolated from chickens and geese in China [14]. Recently, genotypes XVI (Dominican Republic), XVII and XVIII in South and Central Africa have also been identified [16, 17].

In the present study, we focused on the classification and characterization of evolutionary divergence and epidemiological links related to all 121 available Iranian NDV fusion protein sequences published in GenBank.

Materials and methods

Analysis of phylogenetic relationships

We evaluated the divergence of available F gene sequences of virulent ND viruses isolated in Iran from 1995 to 2016. The evolutionary distances among 121 virulent Iranian isolates with fusion gene nucleotide sequences available in the GenBank database as of 09/04/2017 were investigated based on both 374-bp and complete F sequences using the two well-known proposed classification systems [13, 14], and the F sequences of various genotypes and subgenotypes were compared. Some of the F sequences from Iran were submitted in 2 or 3 segments, which were assembled in order to perform a more accurate analysis. Detailed information about virulent NDV isolates from Iran is given in Table 1.

A phylogenetic tree for 189 complete F sequences (1662 bp) including isolates from Iran was constructed based on 500 bootstrap replicates using MEGA6 software. The analysis was performed using the maximum-likelihood (ML) method and the general time-reversible (GTR) model with a discrete gamma distribution (+G). In addition, the first, second, and third codon positions and non-coding regions were used in our study. Fig. 1 shows the phylogenetic relationships of the full F sequences of different NDV isolates including the Iranian isolates under study, which are indicated by red triangles. In addition, a study of 239 partial sequences of F (374 bp) was done using the same criteria as the full sequences analysis described above. A phylogenetic tree based on 374-bp sequences is shown in Fig. 2 (Iranian isolates are indicated by red triangles).

The evolutionary relationship of 69 partial (374 bp) nucleotide sequences of the fusion genes of genotype VI isolate including viruses from Iran originating from pigeons was analyzed using the maximum composite likelihood method (MEGA6 software), using the general time reversible model and a discrete gamma distribution (+G). The included codon positions were 1st+2nd+3rd+Noncoding. Fig. 3 shows Iranian subgenotypes isolates (with black triangles) compared to other genotype VI NDV isolates.

We also generated a phylogenetic tree for a total number of 82 complete fusion protein coding sequences (1662 bp) including genotype VII viruses and the Iranian isolates under study. In this investigation, the maximum-likelihood method was used with the general time-reversible model (MEGA6 software). The codon positions mentioned for genotype VI phylogenetic relationships were candidates. Fig. 4 shows a phylogenetic tree of genotype VII viruses. The genotype VII Iranian isolates in this study are shown in red. In addition, a Chinese isolate of subgenotype VIIj [18] is indicated by a red rectangle. The same criteria were also applied to determine the evolutionary relationships among 46 complete fusion protein sequences (1662 bp) of genotype XIII isolates, including viruses from Iran, which are shown in Fig. 5.

Estimation of evolutionary divergence

We performed an analysis to estimate the number of base substitutions per site for 74 partial fusion coding sequences, using the maximum composite likelihood model (500 bootstrap replicates) using MEGA6 software. The included codon positions were 1st+2nd+3rd+Noncoding, with elimination of positions that were covered in fewer than 95% of the sequences. Table 2 shows the evolutionary difference between VI subgenotype isolates, including Iranian NDV isolates.

Investigation of evolutionary distances based on complete F sequences of NDV isolates from Iran belonging to genotype VII and XIII was performed using the maximum composite likelihood model with 500 bootstrap replicates and position elimination where there was less than 95% site coverage. Table 3 shows the evolutionary difference among Iranian NDV isolates of subgenotype VIIj, and the

Table 1 Detailed data about all of the Iranian NDV isolates for which sequences are available

Accession no.	Virus	Cleavage site	ICPI	MDT	Genotype	Host	Date	Location
JQ267585.1	Iran-EMM/1/2008	RRQKRF	1.74	51	XIIId	Chicken	2008	-
JQ267584.1	Iran-EMM/2/2008	RRQKRF	1.65	62	XIIId	Chicken	2008	-
JQ267583.1	Iran-EMM/3/2009	RRQKRF	1.81	49	XIIId	Chicken	2009	-
JQ267582.1	Iran-EMM/4/2010	RRQKRF	1.63	56	XIIId	Chicken	2010	-
JQ267581.1	Iran-EMM/5/2011	RRQKRF	1.72	53	XIIId	Chicken	2011	-
JQ267580.1	Iran-EMM/6/2011	RRQKRF	1.49	54	XIIId	Chicken	2011	-
JQ267579.1	Iran-EMM/7/2011	RRQKRF	1.86	48	XIIId	Chicken	2011	-
KJ176996.1	IRI 1392k	RRQKRF	-	-	VIIj	Poultry	2013	Semnan
KP771863.1	IL-1	RRQKRF	-	-	VIIj	Chicken	2014	Ilam
KX268351.1	Beh15	RRQKRF	1.8	72	VIIj	Chicken	2015	Behshahr
KY205742.1	Maz15	RRQKRF	1.8	72	VIIj	Chicken	2015	Mazandaran
KY205741.1	Isf16	RRQKRF	1.8	48	VIIj	Chicken	2016	Isfahan
KC161990.1	NR-29	RRQKRF	-	-	VIIj	Chicken	1999	-
JX129800.1	NR-86	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129801.1	NR-87	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129802.1	NR-88	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129803.1	NR-90	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129804.1	NR-91	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129805.1	NR-92	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129806.1	NR-94	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129807.1	NR-95	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129808.1	NR-97	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
JX129809.1	NR-98	RRQKRF	-	-	VIIj	Chicken	2010-2011	Shiraz
AY928934	KND/20	RRQRRF	1.9	39.6	XIIIa	Chicken	1998	Qazvin
AY928935	KND/30	RRQRRF	1.96	41.4	XIIIa	Chicken	1998	Kermanshah
AY928936	KND/35	RRQRRF	1.97	49	XIIIa	Chicken	1999	Ahvaz
AY928937	KND/40	RRQRRF	1.92	40.6	XIIIa	Chicken	2004	Karaj
AY928938	KND/45	RRQRRF	1.91	44.2	XIIIa	Chicken	1995	Nishabour
AY928933	MK13/75	RRQRRF	1.97	47.4	XIIIa	Chicken	1996	Torbat-e Jam
HM460889.1	NRO-231	-	-	-	XIIIa	Ostrich	2001	-
HM460890.1	NRO-268	-	-	-	XIIIa	Ostrich	2002	-
HM460891.1	NRO-289	-	-	-	XIIIa	Ostrich	2002	-
HM460892.1	NRO-350	-	-	-	XIIIa	Ostrich	2002	-
KC161979.1	NR-2	RRQRRF	-	48.3	XIIIa	Chicken	2010	Northwest
KC161980.1	NR-3	RRQRRF	-	44.3	XIIIa	Chicken	2010	Northwest
GU228494.1	NR-5	RRQRRF	-	-	XIIIa	Chicken	1999	-
KC161981.1	NR-6	RRQRRF	-	52.7	XIIIa	Chicken	2010	Northwest
KC161982.1	NR-7	RRQRRF	-	43.6	XIIIa	Chicken	2010	Northwest
KC161983.1	NR-9	RRQRRF	-	54.2	XIIIa	Chicken	2010	Northwest
KC161984.1	NR-10	RRQRRF	-	45.9	XIIIa	Chicken	2014	Northwest
KC161985.1	NR-11	RRQRRF	-	51	XIIIa	Chicken	2010	Northwest
GU228496.1	NR-12	RRQRRF	-	-	XIIIa	Chicken	2000	-
KC161986.1	NR-13	RRQRRF	-	44.6	XIIIa	Chicken	2010	Northwest
KC161987.1	NR-14	RRQRRF	-	57.9	XIIIa	Chicken	2010	Northwest
KC161988.1	NR-15	RRQRRF	-	47.1	XIIIa	Chicken	2010	Northwest
GU228498.1	NR-17	RRQRRF	-	-	XIIIa	Chicken	1999	-
GU228499.1	NR-18	RRQRRF	-	-	XIIIa	Chicken	1999	-
GU228500.1	NR-20	RRQRRF	-	-	XIIIa	Chicken	1999	-
JN001191.1	NR-24	RRQRRF	-	-	XIIIa	Chicken	1999	-
GU228501.1	NR-36	RRQRRF	-	-	XIIIa	Chicken	1999	-

Table 1 (continued)

Accession no.	Virus	Cleavage site	ICPI	MDT	Genotype	Host	Date	Location
GU228502.1	NR-37	RRQRRF	-	-	XIIIa	Chicken	1999	-
KC161992.1	NR-43	RRQRRF	-	-	XIIIa	Chicken	1999	-
GU228503.1	NR-46	RRQRRF	-	-	XIIIa	Chicken	1999	-
GU228504.1	NR-47	RRQRRF	-	-	XIIIa	Chicken	1999	-
JN001190.2	NR-19	RRQRRF	-	-	XIIIa	Chicken	1999	-
EU049543.1	NR-21	RRQRRF	-	-	XIIIa	Chicken	-	-
KC161991.1	NR-31	RRQRRF	-	-	XIIIa	Chicken	1999	-
JN001193.1	NR-41	RRQRRF	-	-	XIIIa	Chicken	1999	-
EU049539.1	NR-16	RRQRRF	-	-	XIIIa	Chicken	-	-
KC161993.1	NR-44	RRQRRF	-	-	XIIIa	Chicken	1999	-
JN001194.1	NR-45	RRQRRF	-	-	XIIIa	Chicken	1999	-
AY036960.1	MK7	RRQRRF	1.8	56	XIIIa	Indigenous	1995	Mashhad
AY036961.1	MK12	RRQRRF	1.91	44.2	XIIIa	Chicken	1996	Nishabour
AY036963.1	MK14	RRQRRF	1.96	45.6	XIIIa	Indigenous	1996	Mashhad
AY036965.1	Kasra97	RRQRRF	1.96	41.4	XIIIa	Chicken	1997	Kermanshah
AY036966.1	GH77	RRQRRF	1.9	39.6	XIIIa	Chicken	1998	Qazvin
AY036967.1	KH2/78	RRQRRF	1.97	49	XIIIa	Chicken	1999	Khuzestan
AY036964.1	Krd76	RRQRRF	1.92	40.6	XIIIa	Chicken	1997	Karaj
DQ334274.1	45-IR-05	-	-	-	XIIIa	-	-	-
AY036968.1	ES1/99	RRQRRF	1.93	47.4	XIIIa	Chicken	1999	Isfahan
AF548617.1	MK13	RRQRRF	1.77	47.4	XIIIa	Chicken	1996	Torbat-e Jam
LC055515.1	PPMV1-52CT	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055514.1	PPMV1-51B	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055512.1	PPMV1-21TS	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055513.1	PPMV1-21CS	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055510.1	PPMV1-18TS	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055511.1	PPMV1-18CS	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055508.1	PPMV1-16T	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055509.1	PPMV1-16CT	KRQKRF	-	-	VIg	Pigeon	2014	Ahvaz
LC055507.1	PPMV1-14B	KRQKRF	-	-	VIg	Pigeon	2013	Ahvaz
LC055506.1	PPMV1-8TS	KRQKRF	-	-	VIg	Pigeon	2013	Ahvaz
LC055505.1	PPMV1-7TS	KRQKRF	-	-	VIg	Pigeon	2013	Ahvaz
LC055504.1	PPMV1-2CN	KRQKRF	-	-	VIg	Pigeon	2013	Ahvaz
KT343662.1	NDPR2	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343678.1	NDP95	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343677.1	NDP93	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343676.1	NDP92	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343675.1	NDP83	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343674.1	NDP79	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343673.1	NDP64	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343672.1	NDP61	KRQKRF	-	-	VIg	Pigeon	2013	-
KT343671.1	NDP27	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343670.1	NDP25	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343669.1	NDP22	RRQKRF	-	-	VIj	Pigeon	2012	-
KT343668.1	NDP21	RRQKRF	-	-	VIj	Pigeon	2012	-
KT343667.1	NDP20	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343666.1	NDP19	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343665.1	NDP17	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343663.1	NDP14	KRQKRF	-	-	VIg	Pigeon	2012	-
KT343664.1	NDP11	KRQKRF	-	-	VIg	Pigeon	2012	-

Table 1 (continued)

Accession no.	Virus	Cleavage site	ICPI	MDT	Genotype	Host	Date	Location
JQ344315.1	Isolate 1	RRQKRF	-	-	VIIj	Chicken	2011	-
JQ344316.1	Isolate 2	RRQKRF	-	-	VIIj	Chicken	2011	-
JQ344317.1	Isolate 3	RRQKRF	-	-	VIIj	Chicken	2011	-
JQ344318.1	Isolate 4	RRQKRF	-	-	VIIj	Chicken	2011	-
JQ344319.1	Isolate 5	RRQKRF	-	-	VIIj	Chicken	2011	-
JQ344320.1	Isolate 6	RRQKRF	-	-	VIIj	Chicken	2011	-
JX131350.1	IR-Ostrich-HGT.2012	RRQKRF	-	-	VIIj	Ostrich	2012	Tehran
JX131360	IR-HGT2012.4	RRQKRF	-	-	VIIj	Chicken	2012	Mazandaran
JX131359	IR-HGT2012.3	RRQKRF	-	-	VIIj	Chicken	2012	Azarbaijan
JX131358	IR-HGT2012.2	RRQKRF	-	-	VIIj	Chicken	2012	Isfahan
JX131357	IR-HGT2012.1	RRQKRF	-	-	VIIj	Chicken	2012	Alborz
JX131356	IR-HGT2011.2	RRQKRF	-	-	VIIj	Chicken	2011	Mazandaran
JX131355	IR-HGT2011.1	RRQKRF	-	-	VIIj	Chicken	2011	Mazandaran
JX131354	IR-HGT2010.3	RRQKRF	-	-	VIId	Chicken	2010	Azarbaijan
JX131353	IR-HGT2010.2	RRQKRF	-	-	VIIj	Chicken	2010	Kerman
JX131352	IR-HGT2010.1	RRQKRF	-	-	VIIj	Chicken	2010	Gilan
JX131351.1	PCRLAB/HG2010	RRQKRF	-	-	VIIj	Chicken	2010	-
KP347437.1	BJ2013 clone NDa	RRQKRF	-	-	VIIj	Chicken	2012-2013	Ahvaz
KP347438.1	BJ2013 clone NDb	RRQKRF	-	-	VIIj	Chicken	2012-2013	Ahvaz
KP347439.1	BJ2013 clone NDd	RRQKRF	-	-	VIIj	Chicken	2012-2013	Ahvaz
JF820295.1	Isolate 1390	RRQKRF	-	-	VIIj	Chicken	2010	-

evolutionary divergence of subgenotype VII isolates (82 nucleotide sequences) is shown in Table 4.

An estimate of evolutionary divergence among seven XIIId subgenotype isolates is given in Table 5, and Table shows the percentage of sequence variation of 49 nucleotide sequences of genotype XIII.

Identification of epidemiological links

In order to obtain accurate data on the epidemiology of NDV isolates circulating in Iran, we performed a temporal and geographic study of the isolates under study assembled a database including the collection date, host, and province in which the Iranian viruses were isolated between 1995 and 2016 (Table 1), and compared them to the circulating NDV strains worldwide.

Results

According to data obtained from a study of the phylogenetic relationships of complete and partial F coding sequences, viruses of genotypes VI, VII, and XIII have circulated in Iran since 1995 (Figs. 1 and 2).

The results confirmed the presence of 27 virulent VIg isolates in Iran from 2012 to 2014, while only two NDV isolates from Iran belonged to subgenotype VIj (Fig. 3). Moreover, we recently reported a novel VII subgenotype named VIIj [19]. However, in a study published in 2016, Xue et al. simultaneously introduced a subgenotype named VIIj, including 103 isolates for which the accession numbers were not reported as well as one isolate recovered in China in 2015 under the accession number KU200253 [18]. Based on those results, we found circulation of subgenotype VIIj isolates in Iran on the basis of phylogenetic tree construction (Fig. 4) in the same cluster that we introduced previously, while phylogenetic analysis showed that the KU200253 isolate belonged to subgenotype VIIb (instead of VIIj) (Fig. 4). According to our analysis, one of 121 Iranian isolates under study was grouped as VIId. In addition, the NDV isolates of genotype XIII in Iran were clustered in sub-genotype XIIIa and the novel subgenotype XIIId (Fig. 5).

A study of evolutionary divergence revealed the presence of virulent genotype VI viruses (isolated in 2012 to 2014) in Iran, which were classified into clusters VIg and VIj (Table 2) and differed by 3 to 10% within their own groups (data not shown). Thirty-six virulent subgenotype VIIj viruses (the same cluster as in our previous report) were also circulating in the region during 1999 to 2016 with a divergence of less than 2.1% within group VIIj (Table 3) and 3.9%-10% from subgenotypes VIIa-VIIi (Table 4). Moreover, evolutionary divergence analysis of the available documented cases in Iran indicated the circulation of 48 virulent subgenotype XIIIa isolates between 1995 and 2014. We

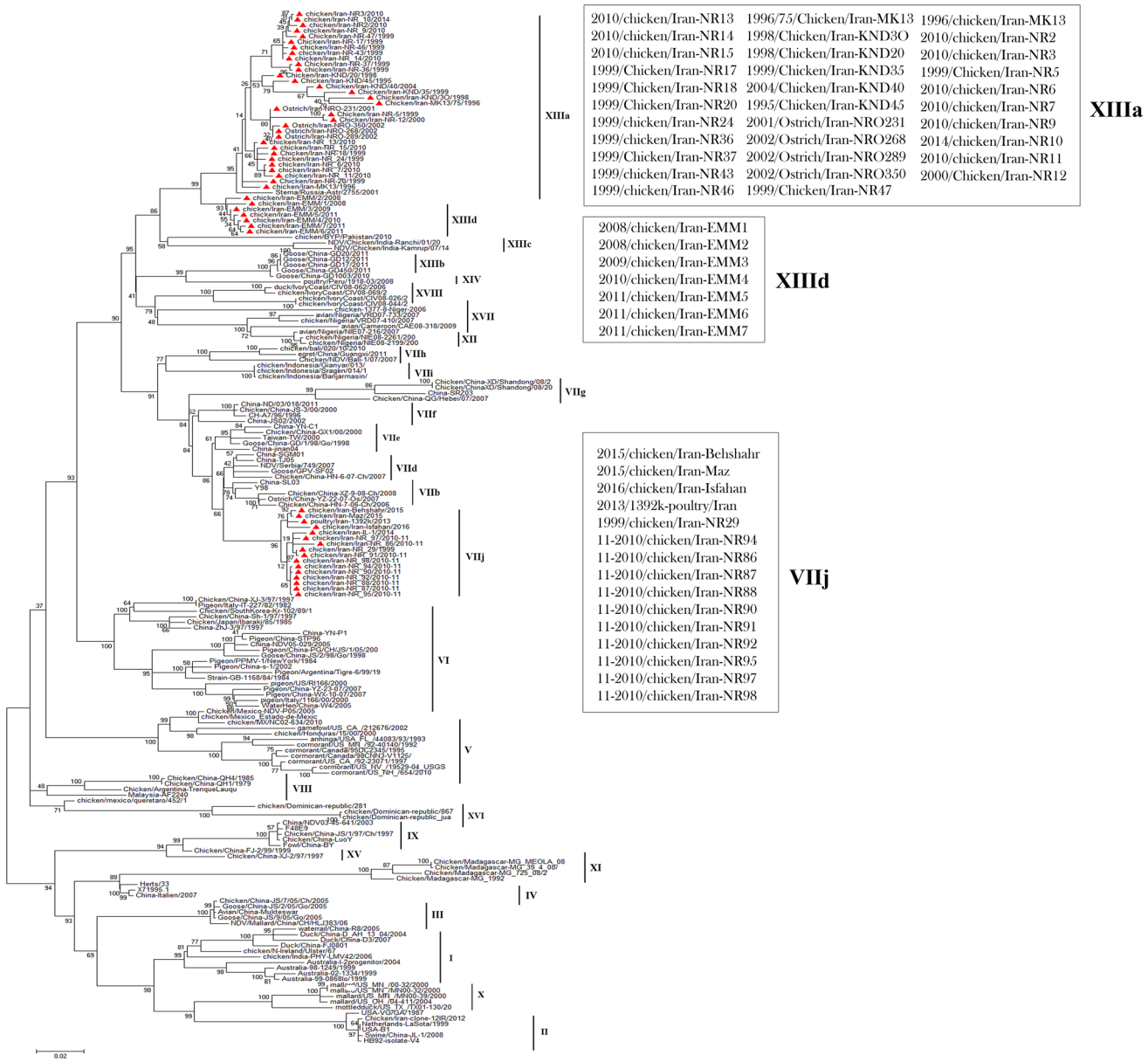

Fig. 1 Molecular phylogenetic analysis of NDV isolates from Iran based on the complete F gene sequence. The evolutionary history was inferred by using the maximum-likelihood method based on the general time-reversible model. The tree with the highest log likelihood (-28101.0646) is shown. The percentage of trees in which t0he associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with the highest log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+*G*, parameter = 0.7030]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 31.2922% of sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 185 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The Iranian isolates under study are indicated by red triangles

also detected the emergence of a novel subgenotype named XIIId, which included seven virulent Iranian viruses (containing cleavage sites characteristic of pathogenic strains) isolated from 2008 to 2011, with 3 to 1.4% sequence divergence within the cluster (Table 5) and more than 3.8% divergence from other subgenotypes of XIII (Table 6).

Based on epidemiological findings, subgenotype VIg isolates were recovered from the southwest of the country (Ahvaz) from 2012 to 2014, while VIj subgenotype isolates were first reported by Razi Vaccine and Serum Research Institute (Karaj, Iran). Fig. 6a shows the geographic distribution of VIg subgenotype viruses in Iran. Thirty-seven

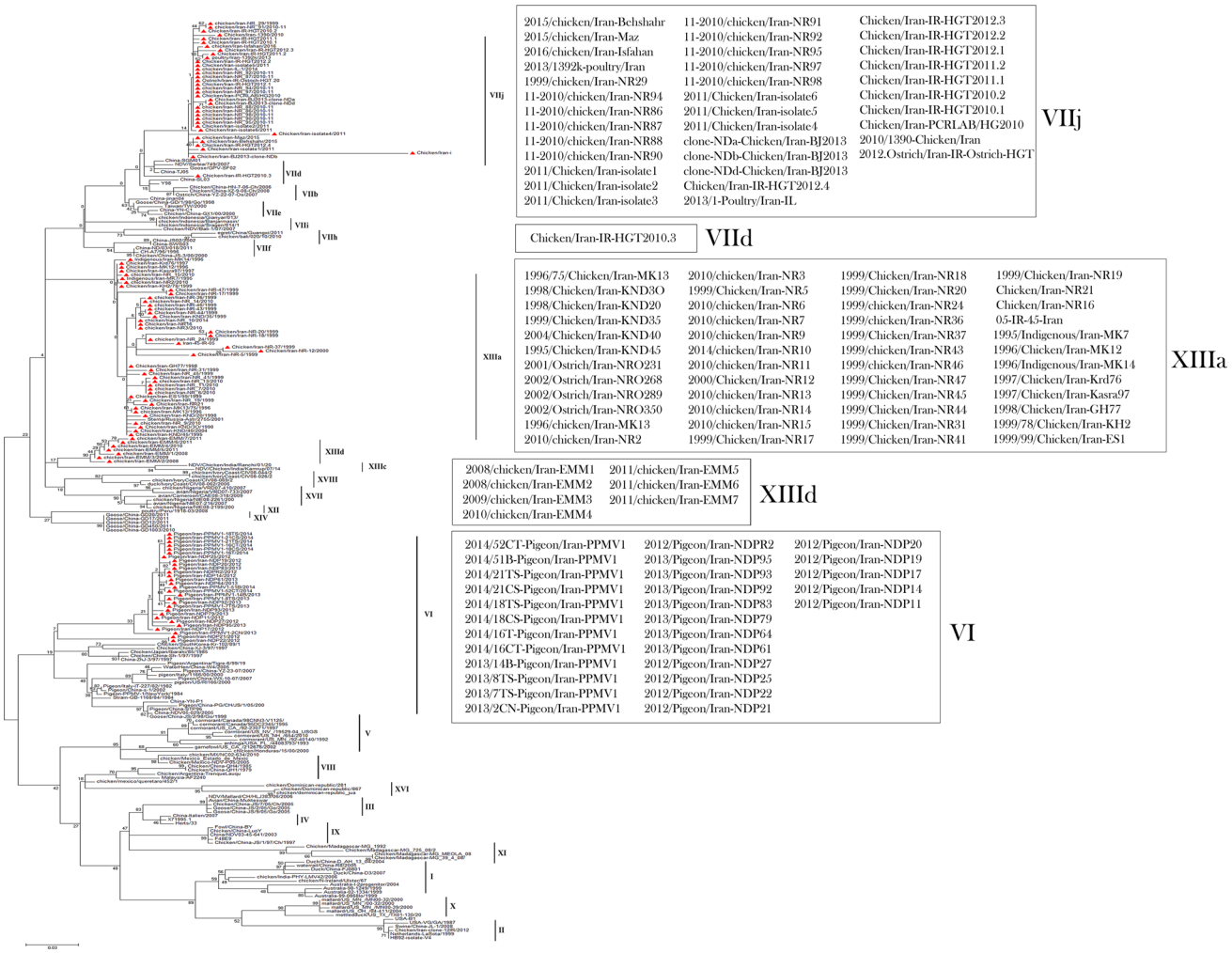

Fig. 2 Molecular phylogenetic analysis of Iranian NDV isolates based on a 374-bp fragment of the F gene sequence. The evolutionary history on the basis of a 374-bp sequence of the fusion protein gene was inferred by using the maximum-likelihood method based on the general time-reversible model. The tree with the highest log likelihood (-6780.1504) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with the highest log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+*G*, parameter = 0.7520]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 12.5316% of sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 239 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 374 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The Iranian isolates used in this study are indicated by red triangles

virulent NDV isolates [20–24] classified into genotype VII cluster (36 isolates, VIIj, 1 isolate: VIId) were recovered from Azarbaijan (northwest), Gilan, Mazandaran (north), Alborz, Tehran, Semnan, Isfahan (center), Ilam (west), Ahvaz (southwest), Shiraz and Kerman (south) provinces in Iran. Genotype VII isolates were found in a a broad geographic region of the country, as shown in Fig. 6b. Previous investigations revealed that 44 genotype XIIIa virulent NDV isolates of Iran with ICPI values of 1.77 to 1.97 [25–28] were isolated between 1995 and 2014 from poultry, whereas, only four isolated viruses of genotype XIIIa were recovered from ostrich (2001-2002). Based on geographic analysis, genotype XIIIa ND viruses have circulated in Qazvin, Karaj, Isfahan, Kermanshah, Ahvaz, Mashhad, Nishabour and Torbat-e Jam (northwestern, central, western, southwestern and eastern provinces of Iran) since 1995. Fig. 6c shows the distribution of genotype XIIIa ND outbreaks in Iran. Furthermore, novel virulent subgenotype XIIId isolates (ICPI

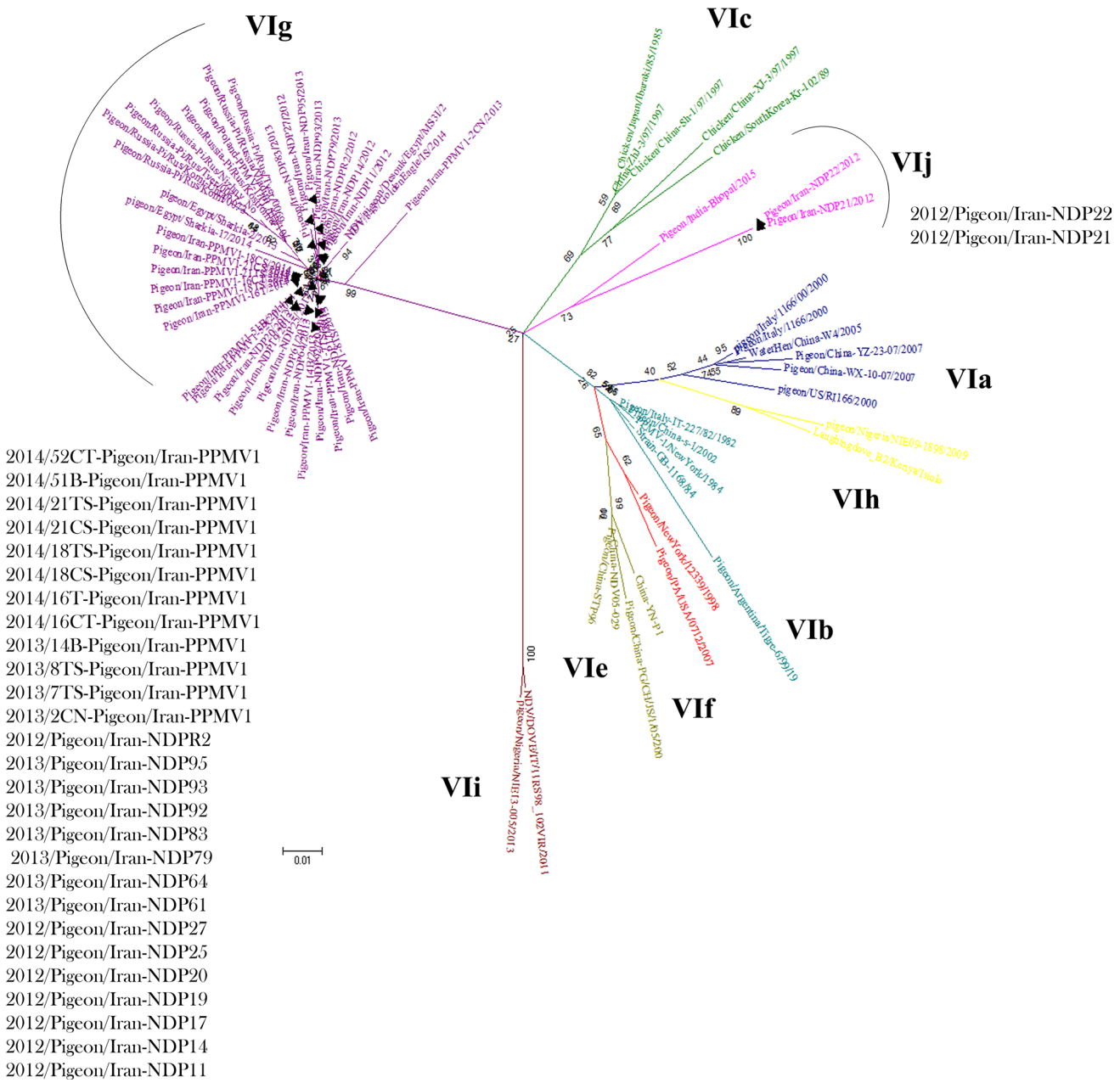

Fig. 3 Molecular phylogenetic analysis of VI subgenotypes including Iranian isolates. The evolutionary history was inferred by using the maximum-likelihood method based on the general time reversible model. The tree with the highest log likelihood (-2089.7291) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with the highest log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+*G*, parameter = 0.9321]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30.6664% of sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 69 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 374 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The Iranian VIg and VIj subgenotypes indicated by black triangles

1.49-1.86) were isolated from chickens (2008-2011) and reported by the Razi Vaccine and Serum Research Institute (Karaj, Iran) [29]. Fig. 6d shows the flyways by which NDV could be introduced into Iran. The timeline of NDV circulation over 21 years in Iran is shown in Fig. 7.

Discussion

A lack of whole-genome sequence data for all of the published NDV isolates has prevented detailed documentation of evolutionary events worldwide [30]. Newcastle disease

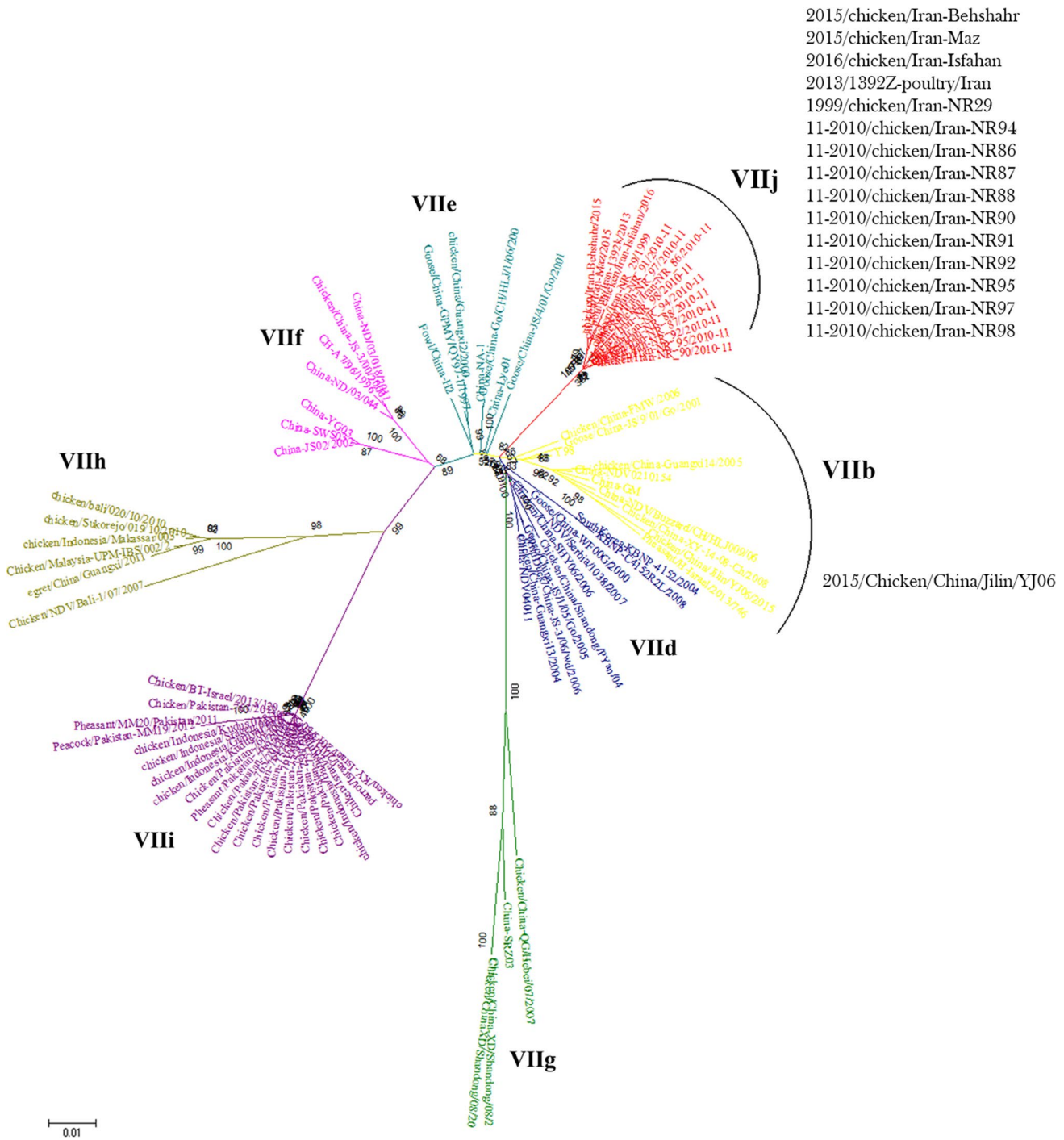

Fig. 4 Molecular Phylogenetic analysis of VIIj subgenotype isolates. The evolutionary history was inferred by using the maximum-likelihood method based on the general time reversible model. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories). The rate variation model allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 82 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The Iranian VIIj isolates in this study are shown in red. The Chinese isolate identified previously as VIIj is also indicated in the figure (VIIb cluster)

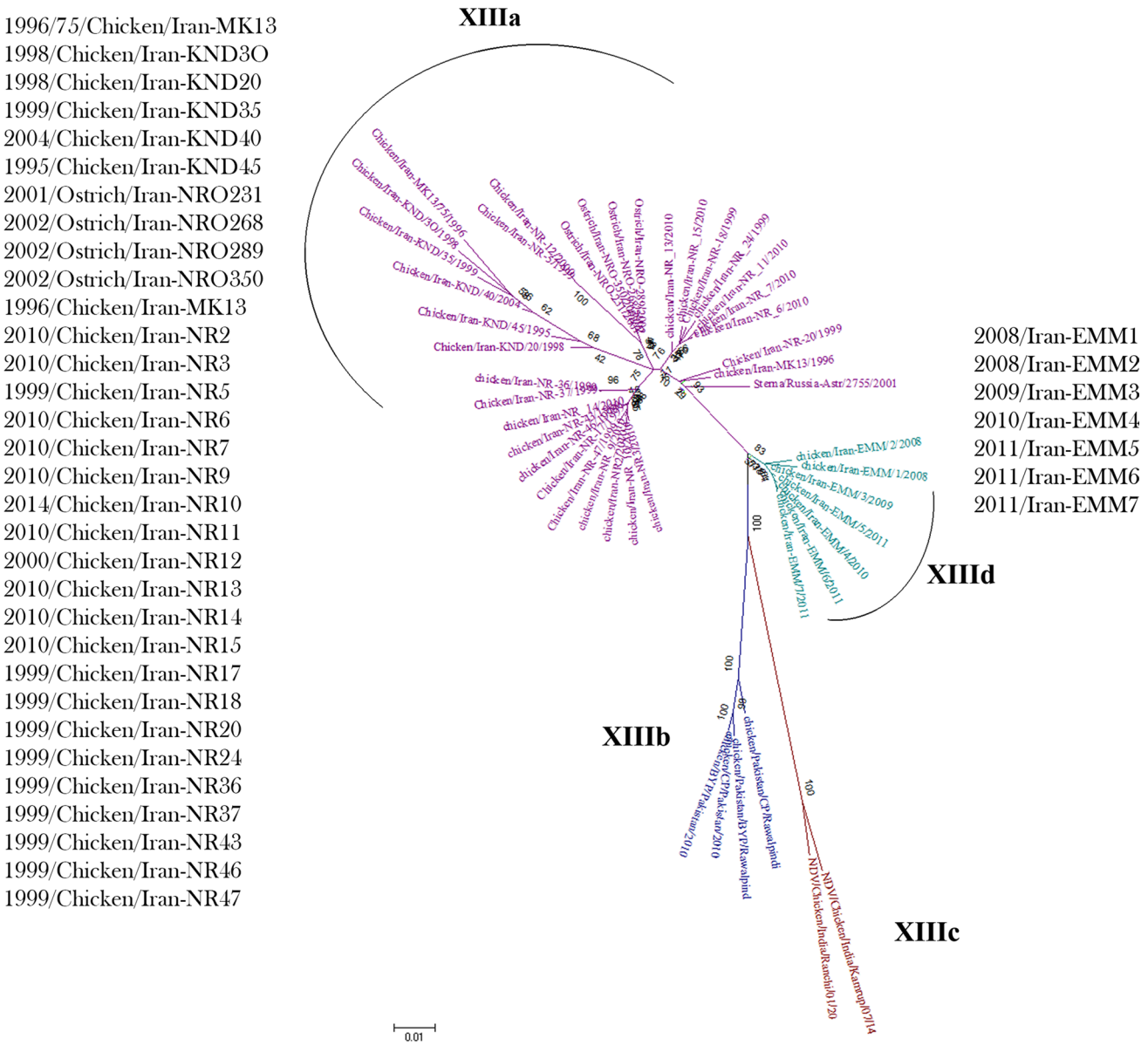

Fig. 5 Molecular phylogenetic analysis of subgenotype XIII ND viruses. The evolutionary history of the complete fusion protein genes of subgenotype XIII isolates was inferred by using the maximum-likelihood method based on the general time-reversible model. The tree with the highest log likelihood (-6034.9781) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with highest log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+*G*, parameter = 0.5812]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 26.1407% of sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included 46 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The Iranian isolates of subgenotype XIIId are shown in green

viruses, like other viruses containing an RNA genome are believed to mutate frequently [31]. Here, we have investigated and evaluated the divergence of the fusion protein coding sequences of all 121 virulent viruses isolated in Iran during 21 years for which sequences are available to obtain an understanding of the NDV strains circulating in the region. Overall, high levels of genetic variability of genotype VI NDV isolates throughout the world has led to division of this group of viruses into subgenotypes VIa to VIj [32, 33]. In addition, genotype VII isolates of NDV are known to form a large and genetically diverse cluster whose members have spread worldwide [14]. Moreover, in case of genotype XIII, the existence of subgenotypes XIIIa, b and c has been reported [32, 34]. On the basis of sequence comparisons and

Table 2 Estimates of evolutionary divergence between subgenotypes of genotype VI

	VIa	VIb	VIc	VIe	VIf	VIg	VIh	VIi	VIj
VIa		0.012	0.020	0.016	0.013	0.020	0.013	0.027	0.019
VIb	0.058		0.015	0.013	0.011	0.019	0.015	0.024	0.017
VIc	0.107	0.082		0.021	0.018	0.018	0.023	0.024	0.018
VIe	0.078	0.058	0.114		0.011	0.023	0.016	0.028	0.021
VIf	0.063	0.050	0.096	0.046		0.020	0.016	0.028	0.018
VIg	0.101	0.095	0.095	0.119	0.102		0.023	0.027	0.021
VIh	0.064	0.075	0.128	0.080	0.076	0.117		0.030	0.021
VIi	0.139	0.113	0.125	0.139	0.137	0.133	0.156		0.027
VIj	0.098	0.086	0.095	0.110	0.089	0.104	0.108	0.132	

The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). The analysis was conducted using the maximum composite likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included 68 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 374 positions in the final dataset. Evolutionary analysis was conducted in MEGA6

Table 3 Estimate of pairwise evolutionary divergence among VIIj sub-genotype isolates

	IR-Maz	IR-Beh	IR-Isf	1392k	NR-29	NR-86	NR-87	NR-88	NR-90	NR-91	NR-92	NR-94	NR-95	NR-97	NR-98
IR-Maz		0.001	0.003	0.002	0.003	0.004	0.002	0.002	0.002	0.003	0.002	0.003	0.002	0.003	0.002
IR-Beh	0.002		0.003	0.003	0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.003	0.002	0.004	0.002
IR-Isf	0.011	0.013		0.003	0.004	0.005	0.003	0.003	0.003	0.004	0.003	0.004	0.003	0.004	0.003
1392k	0.007	0.008	0.011		0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.003	0.002	0.004	0.002
NR-29	0.009	0.010	0.015	0.011		0.003	0.002	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002
NR-86	0.015	0.015	0.021	0.015	0.010		0.003	0.003	0.004	0.003	0.003	0.004	0.004	0.003	0.003
NR-87	0.008	0.008	0.013	0.010	0.006	0.011		0.000	0.001	0.004	0.000	0.001	0.001	0.002	0.001
NR-88	0.006	0.007	0.012	0.008	0.006	0.011	0.000		0.001	0.003	0.000	0.001	0.001	0.002	0.001
NR-90	0.007	0.008	0.013	0.009	0.006	0.012	0.001	0.001		0.003	0.001	0.001	0.001	0.003	0.002
NR-91	0.010	0.011	0.016	0.011	0.001	0.011	0.016	0.006	0.006		0.003	0.003	0.003	0.002	0.002
NR-92	0.006	0.007	0.012	0.008	0.006	0.011	0.000	0.000	0.001	0.006		0.001	0.001	0.002	0.001
NR-94	0.010	0.010	0.015	0.012	0.006	0.012	0.001	0.001	0.001	0.007	0.001		0.001	0.003	0.002
NR-95	0.007	0.008	0.013	0.008	0.006	0.012	0.001	0.001	0.001	0.007	0.001	0.001		0.003	0.002
NR-97	0.013	0.014	0.019	0.015	0.004	0.011	0.007	0.007	0.007	0.005	0.007	0.008	0.008		0.002
NR-98	0.006	0.007	0.012	0.008	0.005	0.010	0.003	0.003	0.004	0.006	0.003	0.004	0.004	0.006	

The number of base substitutions per site between sequences is shown. Standard error estimates are indicated above the diagonal and were obtained by a bootstrap procedure (500 replicates). The analysis was performed using the maximum composite likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included 15 nucleotide sequences. The codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. Eleven Iranian isolates were compared to the four VIIj viruses (Maz, Beh, Isf and 1392k)

phylogenetic analysis, we have characterized the circulating NDV genotypes in Iran as VIg, VIj, VIId, VIIj, XIIIa, XIIId, of which VIIj and XIIIa subgenotype isolates are the most predominant in the country and have circulated for over 17 years.

For adequate biosecurity and vaccination programs, a detailed and precise understanding of the global epidemiology of the recent circulating viruses is needed [35]. The geographic distribution of NDV isolates of genotypes I, II, VI and VII of class II, and also of viruses of class I, is widespread, while genotypes XI, XIII, XIV, XVI, XVII, and XVIII are limited to specific regions [32]. Multiple records related to genotype VI isolates of NDV report wild and domestic pigeons and doves as their hosts, while some isolates have been recovered from poultry [36]. Abolnik et al. reported the isolation of genotype VI viruses from kestrels, falcons, cockatoos, budgerigars, pheasants, swans and a robin [37]. Genotype VI isolates have been isolated

Table 4 Estimates of evolutionary divergence between subgenotypes in genotype VII

	VIIb	VIId	VIIe	VIIf	VIIg	VIIh	VIIi	VIIj
VIIb		0.007	0.006	0.010	0.021	0.017	0.017	0.009
VIId	0.036		0.006	0.009	0.019	0.016	0.017	0.007
VIIe	0.035	0.031		0.007	0.020	0.015	0.015	0.007
VIIf	0.052	0.047	0.037		0.021	0.014	0.014	0.010
VIIg	0.109	0.098	0.104	0.108		0.027	0.028	0.019
VIIh	0.094	0.089	0.079	0.075	0.147		0.017	0.015
VIIi	0.092	0.087	0.079	0.075	0.147	0.088		0.017
VIIj	0.046	0.039	0.040	0.051	0.102	0.086	0.094	

The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Standard error estimates are shown above the diagonal. The analysis was conducted using the maximum composite likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included 82 nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The evolutionary divergence of Iranian subgenotype VIIj ranged between 3.9% and 10%

Table 5 Pairwise evolutionary divergence estimation among subgenotype XIIId isolates

	EMM/1/2008	EMM/2/2008	EMM/3/2009	EMM/4/2010	EMM/5/2011	EMM/6/2011	EMM/7/2011
EMM/1/2008		0.003	0.003	0.003	0.002	0.002	0.003
EMM/2/2008	0.014		0.002	0.003	0.003	0.003	0.003
EMM/3/2009	0.009	0.008		0.002	0.001	0.002	0.002
EMM/4/2010	0.011	0.012	0.004		0.001	0.001	0.002
EMM/5/2011	0.009	0.010	0.003	0.004		0.001	0.002
EMM/6/2011	0.009	0.012	0.005	0.003	0.003		0.001
EMM/7/2011	0.012	0.014	0.005	0.004	0.006	0.003	

The number of base substitutions per site between sequences is shown. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). The analysis was conducted using the maximum composite likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included seven nucleotide sequences. The codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. The evolutionary differences among XIIId isolates were estimated

Table 6 Estimate of Evolutionary Divergence between subgenotypes of genotype XIII

	XIIIa	XIIIb	XIIIc	XIIId
XIIIa		0.008	0.009	0.004
XIIIb	0.099		0.010	0.006
XIIIc	0.110	0.117		0.009
XIIId	0.038	0.065	0.103	

The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). The analysis was conducted using the maximum composite likelihood method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included 49 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1662 positions in the final dataset. Evolutionary analysis was conducted in MEGA6. Evolutionary distances between XIIId and other subgenotypes of genotype XIII are compared

in different parts of the world since the 1960s, and ten subgenotypes of this group are distributed throughout Asia, Europe, the Middle East, the United States, Argentina, South Africa, Egypt, Nigeria, Sudan, Ethiopia, Kenya, Russia, Poland and Ukraine [32, 38, 39]. In Iran, subgenotype VIg isolates (isolated from the southwest between 2012 and 2014) share the highest similarity with viruses isolated in Russia (2005-2009) and Poland (2010). Based on these data, these viruses may have a common origin. Furthermore, a recently reported ND virus from India (Bhopal, 2015) [33] showed the lowest divergence from VIj subgenotype isolates (2012) from Iran, which we suggest may share a common ancestor with the Indian isolate. Since the 1990s, in the Far East, and then in Asia, Eastern and Western Europe, South Africa, South America and the Middle East, virulent genotype VII viruses have spread worldwide with variable genetic characteristics [32]. The ancestors of genotype VII isolates of NDV seem to be Indonesian viruses from the 1980s [40]. A wide range of hosts have been reported for

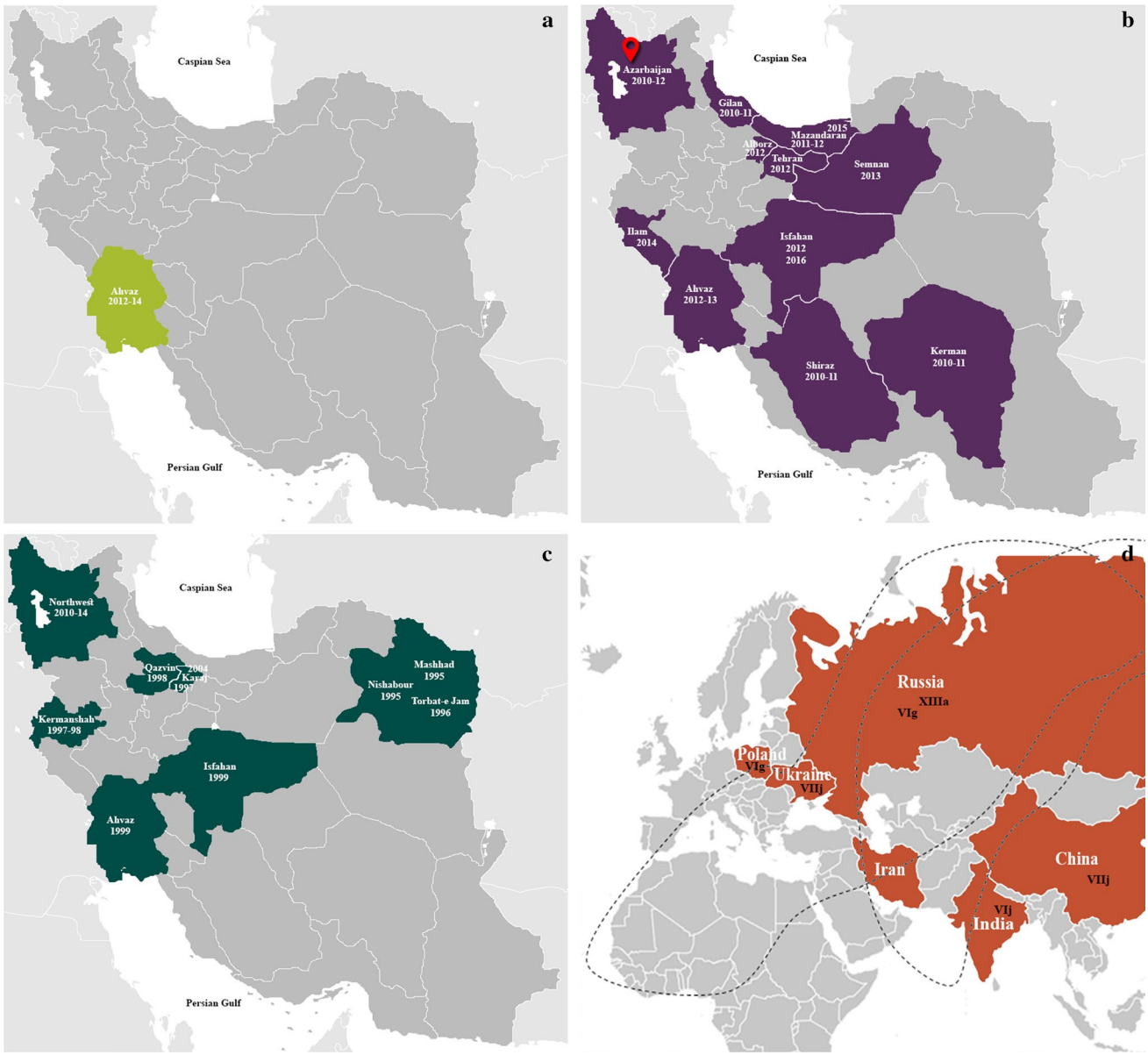

Fig. 6 Epidemiological distribution of Newcastle disease viruses in Iran between 1995 and 2016. **a.** VIg subgenotype distribution in Ahvaz (southwest). **b.** the distribution of VIIj subgenotype isolates from 1999 to 2016, which involves most areas of the country (northwest [Azarbaijan], north [Gilan, Mazandaran], center [Alborz, Tehran, Semnan, Isfahan], west [Ilam], southwest [Ahvaz] and south [Shiraz, Kerman]). In addition, the location pin in the northwest of Iran in panel b. also points out the location of an isolate of subgenotype VIId in 2010. **c.** Geographic distribution (northwest, center [Qazvin, Karaj, Isfahan], west [Kermanshah], southwest [Ahvaz] and east [Mashhad, Nishabour, Torbat-e Jam]) of XIIIa subgenotype isolates circulating in the region from 1995 to 2014. **d**. Flyways by which NDV could be imported from neighboring countries to Iran

genotype VII ND viruses, including poultry and wild species of birds, which, along with their high genetic diversity, makes this group economically worthy of attention [16]. Avian species have been the main hosts for genotype VII isolates circulating in Iran from 1999 to 2016. Thirty-seven virulent viruses [20–24] with the genetic characteristics of the genotype VII group have been isolated in Iran since 1999 from the northwestern-to-northern, central, western, southwestern and southern parts of the country involving a huge geographic area. In fact, sequence differences between VIIj Iranian isolates and other genotype VII isolates indicate the highest similarity to viruses of subgenotypes VIId and VIIe (96.2%), which could suggest the ancestor for evolution of this group of isolates (VIIj). Datamining the genotype VII isolates have also indicated presence of "middle" viruses with equal divergence from subgenotypes VIIj

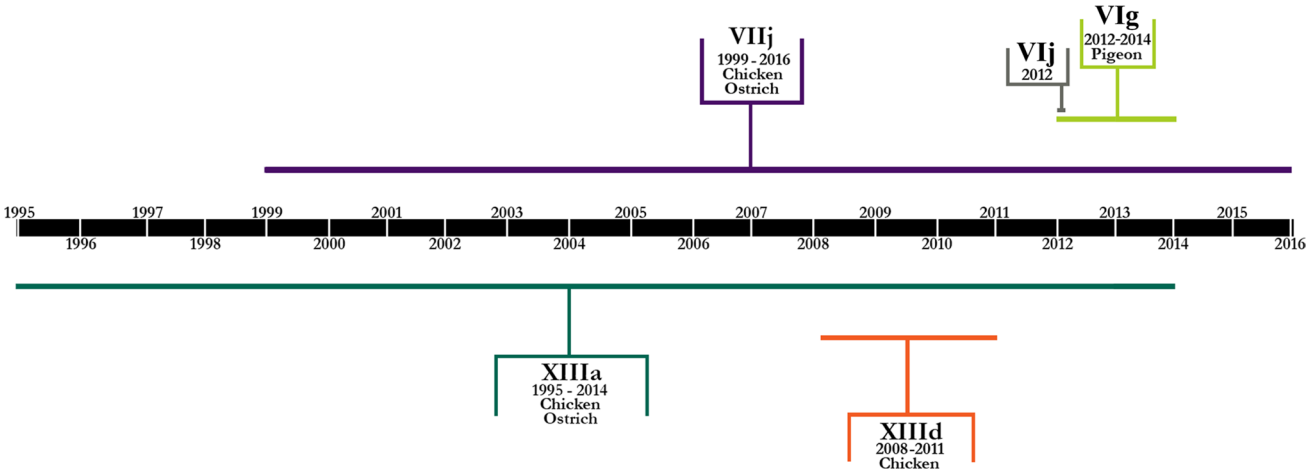

Fig. 7 Timeline related to NDVs of Iran. The timeline demonstrates NDV subgenotypes circulating from 1995 to 2016 in Iran

and VIId. These NDV isolates recovered from Ukraine and China could represent the keys to understanding emergence subgenotype VIIj. Cockatoos were the hosts of the ancestors of virulent genotype XIII viruses isolated in 1982 in India. The low diversity of isolates of this group has led to the classification of genotype XIII isolates into groups a, b and c [32, 34]. The host distribution of this group of NDVs (XIIIa) has been limited to chickens in Europe (1997), Asia (1997-2010), and Africa (1995-2008), but there has been a report of subgenotype XIIIa virus isolation from a wild bird (*Sterna albifrons*) in Russia in 2001 [32]. According to our data, a total of 44 highly virulent (ICPI 1.77-1.97) NDV isolates from Iran (1995-2014) [25–28] belonging to the subgenotype XIIIa cluster were isolated from poultry, while there is a report of ostrich as the host for four isolates recovered from 2001 to 2002 in Iran. Moreover, the geographic distribution of XIIIa isolates indicate that the northwestern, central, western, southwestern and eastern regions of Iran have been involved in XIIIa ND outbreaks since 1995. According to the study of Esmaelizad et al., migratory birds of the species *Sterna albifrons Pallas* the probable source of genotype XIIIa viruses in Iran, based on the high similarity of Iranian isolates to Russian Astr/2755/2001 and VOL95 viruses (XIIIa subgenotype) [41]. Based on various studies, subgenotype XIIIb isolates are limited to Pakistan and India (2003-2013) and have been recovered repeatedly from poultry despite the vaccination programs [32, 42]. Subgenotype XIIIc was also been reported in India in chickens in 2014 [34]. Here, we have characterized a novel XIIId subgenotype whose members are virulent, with an ICPI value of 1.49-1.86. These viruses were isolated between 2008 and 2011 from chickens and reported by the Razi Vaccine and Serum Research Institute (Karaj, Iran) [29]. These documented reports indicate the emergence of an ND outbreak in Iran involving a strain with lowest divergence from subgenotype XIIIa (3.8%) circulating in the country since 1995, and this might be the ancestor of the XIIId viruses. Multiple factors may have affected the host adaptation and emergence of this new subgenotype including the long duration of subgenotype XIIIa circulation in the region.

Since the 1950s, vaccination programs using live or inactivated viruses have been broadly used worldwide with success [43], but inefficient practices have resulted in insufficient immunity and susceptibility of vaccinated chickens challenged with virulent NDV to disease [43]. Studies have suggested that the genetic evolution of NDV isolates may be a consequence of evasion of the immune response induced by vaccine strains [44], and therefore selective pressure may favor host adaptation and the emergence of different genetic variants that can be transmitted to various species of birds [32]. Most vaccination programs conducted in Iran have been based on live or killed B1 and LaSota vaccines (which are avirulent isolates of NDV) [45]. As Miller et al. have suggested, based on antigenic and genetic variations among NDV strains, as well as their own experimental data, vaccine homology to the challenge virus or the circulating virulent NDV isolates may induce a more efficient immune response [46]. In addition, our previous studies have suggested that there are major differences among virulent and non-virulent isolates of NDV, mainly in the hemagglutinin-neuraminidase protein, in which we have identified a 527-bp fragment in the sialidase region that is useful for pathotyping various groups of NDV isolates [47]. Furthermore, six critical amino acids in the sialidase region have been introduced that could play a crucial role in NDV virulence based on biological parameters [48]. In China, researchers have demonstrated that application of LaSota virus vaccines against ND could be efficient in protection of poultry, but challenging vaccinated chickens with novel emerging viruses sharing new genetic characteristics (substitutions in 347 and 362 residues of HN

protein) did not stop viral shedding [49]. In addition, we have recently identified novel amino acid mutations in circulating Iranian isolates in the same residues of the hemagglutinin-neuraminidase protein (neutralization epitope) as in the Chinese study, raising major concerns for the poultry industry in Iran [50]. The application of biosecurity and control programs using vaccines based on avirulent isolates may be sufficient, but due to the importance of variable residues in major epitopes of different pathotypes, designed vaccines homologous to circulating virulent genotypes may be needed to achieve efficacious levels of antibody production [46]. Recently, studies with DNA vaccines in Iran have suggested that improved immunogenicity can be achieved using a virulent genotype VIII isolate (AF2240) [51]. Therefore, our data analysis and investigation of ND outbreaks, epidemiological links, and phylogenetic relationships from 1995 to 2016 could be a very helpful resource for the design of future recombinant vaccines based on circulating virulent NDVs in Iran and neighboring countries.

In conclusion, we have conducted an investigation of molecular evolution and epidemiological links related to 121 virulent NDV isolates recovered from different provinces of Iran between 1995 and 2016. Based on our study, subgenotype XIIIa and VIIj isolates have been circulating in most areas of Iran for over 15 years now despite vaccination programs. Moreover, we have identified a novel emergence of subgenotype XIIId isolates, and we propose that there is a possibility of host adaptation to the long-term presence of XIIIa subgenotype viruses in the country and possible consequences of evading protective immune responses induced by live vaccines based on avirulent isolates. We also observed that multiple virulent subgenotype VIg and VIj isolates and a single VIId virus are circulating in Iran. The results of this study could be helpful for developing efficient recombinant vaccines that are homologous to viruses that are circulating in the country.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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