Genetic diversity of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout (*Oncorhynchus mykiss*) in Iran

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Abstract

Infectious pancreatic necrosis (IPN) is one of the most important viral diseases in the aquaculture industry. This study aimed to determine the genetic diversity of infectious pancreatic necrosis virus (IPNV) isolated from farmed rainbow trout in Iran during 2011 to 2013 using VP2 gene sequencing. Trout larvae and fingerlings (200 mg-1.5 g) undergoing IPN outbreaks were collected from fish farms and IPNV was detected by reverse-transcription polymerase chain reaction (RT-PCR). In addition, samples of ovarian and seminal fluids were obtained from brood fish and screened for IPNV by RT-PCR. After RT-PCR characterization of the viral isolates, a 205 bp portion of the IPNV VP2 gene were sequenced to determine the genetic diversity of the viral strains detected in Iran. The phylogenetic analysis of the 40 IPNV strains showed that these isolates can be grouped into 6 genogroups. All isolates except genogroup one showed a high genetic similarity (96.57%).

Introduction

Outbreaks of infectious diseases have increased due to the rapid growth of the aquaculture sector. Therefore, an appropriate fish health management strategy is an important element to reduce morbidity and mortality due to these economically important diseases. Outbreaks of viral diseases such as infectious pancreatic necrosis (IPN) also called acute catarrhal enteritis, represent specific issues in fish health management because of the high mortality level reported in the infected fish and lack of effective vaccine as a protective tool (Allnut et al., 2007). IPN is an acute, highly contagious disease that mainly affects first-feeding fry to fingerling lifestages but also adult salmonid and non-salmonid fish (Shivappa et al., 2004) and disease outbreaks are now spread worldwide (Ruane et al., 2009). IPNV is a member of the family *Birnaviridae*, genus *Aquabirnavirus*, having two segments of double-stranded RNA, packaged in a non-enveloped icosahedral shell of 60 nm in diameter (Dobos, 1995). The smaller genome segment B (2784 bp) encodes an internal polypeptide VP1 (94 kDa) while the genomic segment A (approximately 3100 bp) contains 2 partially overlapping open reading

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frames (ORF). The large ORF encodes a 106 kDa polyprotein which is cleaved to produce 3 polypeptides, the precursor of the major outer capsid protein (VP2); a minor capsid protein (VP3); and a non-structural protein (NS). (Havarstein et al., 1990; Bain et al., 2008).

Aquabirnaviruses are classified into two serotypes; A serotype, the major serogroup (A1-A9) and B serotype (B1) (Hill et al., 1995). These serogroups have been detected from various fish species, in particular salmonids from different geographical regions e.g. A1 from USA, A2-A5 from European countries and A6-A9 from Canada (Hill et al., 1995). To study the diversity of IPNV, molecular methods have been demonstrated to be very effective, rapid and cost effective compared to virus isolation in cell culture. Several reports have used genomic and deduced amino acid sequences to study genetic diversity of IPNV (Cutrin et al., 2000; Blake et al., 2001; Shivappa et al., 2004; Bain et al., 2008; Guerrero et al., 2008; Barrera-Mejía et al., 2010; Salgado-Miranda et al., 2014). However, there is no information available concerning the genetic diversity of this wide-host viral agent in the Middle East. Iran is the first country within the Middle East to culture rainbow trout (*Oncorhynchus mykiss*) in freshwater with production of 160 000 tons in 2013 and approximately 200 million trout eggs are imported from different regions such as Europe and America (Aquaculture department, Iranian Fisheries Research Organization, 2013 http://www.ifro.ir/portal.aspx?tabid=105). Outbreaks as a result of IPNV infection is a major worldwide problem in the trout aquaculture industry causing high losses each year (Ortega et al., 2002; Varvarigos et al., 2002; Guerrero et al., 2008; Soltani et al., 2014). To date, there is no data regarding the genotype of the IPNV strains present in Iranian aquaculture. Therefore, this study aimed to assess the genetic diversity of the viral isolates causing disease outbreaks in Iranian farmed rainbow trout during 2011-2013.

Material and methods

Sample collection

Rainbow trout larvae and fingerlings (200 mg - 1.5 g) that were thought to be undergoing an IPN outbreak were collected from aquaculture facilities during 2011-2013 as part of a monitoring program by the Iran Veterinary Organization. Samples were obtained from 15 states. All fish were dissected in the field and tissues of kidney, liver, spleen were aseptically removed and stored in 75% ethanol (Merck) for RNA extraction. In addition samples of ovarian and seminal fluids were obtained from brood fish. Organ material collected from 10 fish were pooled together, in total, 100 pools (10 pools from ovarian fluids, 13 pools from seminal fluids, 54 from whole fry, 23 from fingerling) were analysed.

RNA extraction

Simply P total RNA extraction kit (BioFlux Cat#BSC52 M1, Hangzhou, China) was used according to the manufacturer's instructions to extract the viral RNA from 50 mg of each mixed sample. In brief, 600 µl of R2 solution was added, mixed thoroughly and the samples were incubated at room temperature for 5 min. The lysates were transferred to spin columns and centrifuged for 30 s. The spin columns were washed twice with wash buffer and transferred to sterile 1.5 ml microcentrifuge tubes. The total RNA was then eluted from the spin columns with 40 µl of elution buffer.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

The cDNA was synthesized by cDNA synthesis kit (Bioer CatBSB09M1). An aliquot of 20 ng of total RNA was reverse transcribed in a volume of 10 µl containing Random Hexamer primer 0.5 µl, AMV enzyme 0.5 µl, 5 x buffer 2 µl, RNase inhibitor 0.5 µl, dNTP mix 1 µl, RNase free water up to 10 µl. The RT reaction was performed at room temperature for 10 min, at 60°C for 45 min, at 95°C for 5 min and on an ice bath for 5 min.

The oligonucleotide primers used in this study were described by Williams et al. (1999). The sequences of primers were WB1: CCGCAACTTACTTGAGATCCATTATGC and WB2: TCTGGTTCAGATTCCACCTGTAGTG. A 205 bp fragment of the VP2 genome was expected to be obtained using these primers. The reaction setup used for PCR contained 10 x buffer 2.5 µl, dNTP mix 0.5 µl, each primer 0.5 µl, Taq mix DNA polymerase 0.5 µl, cDNA 2.5 µl, and ddH2O 18 µl. Amplification reactions were carried out with a thermal program at 94°C for 3 min, at 94°C for 30 s and at 60°C for 30 s for 35 cycles, at 72°C for 45 s and at 72°C for 5 min. The PCR products were then subjected to electrophoresis on a 1.5% agarose gel and visualized by UV doc (Biorad, USA). The PCR product from confirmed IPNV samples were used for the phylogenic analysis.

Phylogenic analysis

All PCR products that matched the expected size for IPNV were purified and commercially sequenced. Multiple sequence alignment was performed by ClustalW analysis using CLC Sequence Viewer (version 6, USA). Genetic distances were calculated by unweighted pair group method with arithmetic mean (UPGMA) and neighbor-joining (NJ) methods (Saitou & Nei, 1987) using MEGA 5. To assess the likelihood of the tree constructions, 1000 bootstrap replicates were performed.

Results

PCR results

An amplified 205 bp portion of the IPNV VP2 gene was detected from 40 samples (40%) originating from 30 aquaculture facilities. The origin/region and accession numbers of these isolates are summarized in Table 1. These isolates were predominately obtained from rainbow trout farms located in the North, North West and West parts of the country (Figure 1).

Phylogenetic analysis

The phylogenetic properties of the identified isolates at nucleic acid level are shown in the phylogenetic tree (Figure 2). Phylogenetic analysis showed that isolates were clustered into 6 genogroups. Genogroup 1 consisted of KO5 (KF414733) showing 41.55% genetic difference with the other genogroups. Genogroup 2 comprised the majority of the IPNV isolates (15 isolates L4 (KF414726), L3 (KF414722), KH3 (KF414728), K2 (KF414727), KO6 (KF414725), AG3 (KF377467), AG2 (KF377466), KE1 (KF897819), KE3 (KF414731), KO2 (KF897820), KO3 (KF897823), KO4 (KF414723), M3 (KF414724), CH2 (KF377465) and CH3 (KF377468)) showing 3.43% genetic difference with genogroups 3, 4, 5 and 6. In addition genogroup 3 (M4 isolate (KF313569)) showed 8.34% differences with genogroups 4, 5 and 6. The isolates of G2 (KF414730), A1 (KF897818), E1 (KF984550), A2 (KF660581), AG1 (KF414729), L2 (KF984551), F1 (KF984549), F2 (KF984552), KG (KF660585) and AS2 (KF660582)

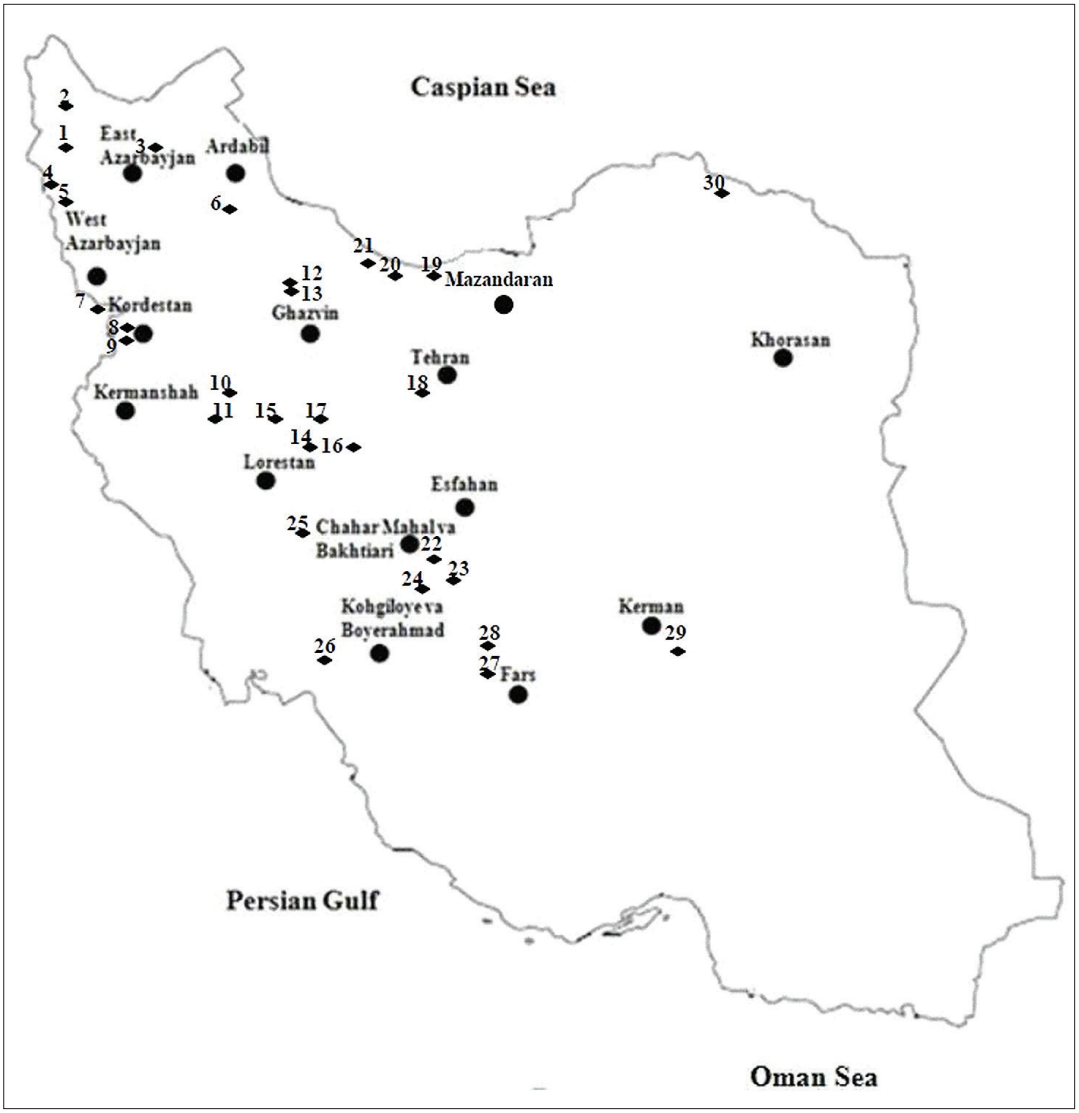

Figure 1. Geographical distribution of IPNV in farmed rainbow trout in different origins in Iran**.** East Azarbayjan, 1=Shabestar, 2= Marand, 3=Haris; West Azarbayjan, 4= Oshnaviyeh, 5= Rashkand, Ardabil, 6= Sarab; Kurdestan, 7=Baneh, 8= Palangan, 9=Sirvan; Kermanshah, 10= Kangavar, 11= Harsin; Ghazvin, 12= Andej, 13= Alamot; Lorestan: 14= Azna, 15= Alashtar, 16= Aligoudarz, 17= Broujerd; Tehran, 18= Tehran; Mazandaran, 19= Amol, 20= Chalus, 21= Tonekabon; Esfahan, 22= Semirom, 23= Mobarakeh; Chahar Mahal va Bakhtiari, 24= Dehno, 25= Koohrang; Kohgiloye va Boyerahmad, 26= Gachsaran; Fars, 27= Delkhan, 28= Sepidan; Kerman, 29= Bardsir; Khorasan, 30= Bojnord.

formed genogroup 4 showing 1.98% genetic differences with genogroups 5 and 6. The genogroup 5 comprised the isolates of KE2 (KF984554), T (KF984555), CH1 (KF984553), M2 (KF897822) and L1 (KF897817) revealing 1.61% genetic difference with genogroup 6 (isolates of KO1 (KF984548), KH1 (KF897821), G1 (KF897814), KH2 (KF897815), K1 (KF897816), M1 (KF660584), E2 (KF 414732) and AS1 (KF660583). Moreover, in genogroup 6, isolates AS1 and E2 showed 100% genetic similarity and also the same pattern was seen between KH2 and K1 isolates of this genogroup. In addition, KE2 and T isolates in genogroup 5 showed 100% genetic similarity. Furthermore, among genogroup 4, 100% genetic similarity was seen between A1 and AS2 isolates. With exception of KO5 (genogroup 1) which showed 41.55%

Table 1. Infectious pancreatic necrosis virus isolates identified in farmed rainbow trout and listed by state/origin and accession numbers.

No.	State/origion	Laboratory collection code	Accession number (NCBI)
1	East Azarbayjan	AG1,AG2,AG3	KF4147291,KF3774661,KF3774671
2	West Azarbayjan	AS1,AS2	KF6605834,KF6605821
3	Mazandaran	M1,M2,M3,M4	KF6605843,KF8978222,KF4147244,KF3135691
4	Kohgiloye-va-Boyerahmad	KG	KF6605851
5	Kordestan	KO1,KO2,KO3,KO4,KO5, KO6	KF9845481,KF8978201,KF8978234, KF4147231,KF4147331, KF4147251
6	Ghazvin	G1,G2	KF8978141,KF4147301
7	Kermanshah	K1,K2	KF8978164,KF4147274
8	Lorestan	L1,L2,L3,L4	KF8978174,KF9845511,KF4147221,KF4147261
9	Ardabil	A1,A2	KF8978181,KF6605811
10	Kerman	KE1,KE2,KE3	KF8978191,KF9845543,KF4147312
11	Khorasan	KH1,KH2,KH3	KF8978211,KF8978151,KF4147284
12	Fars	F1,F2	KF9845494,KF9845524
13	Esfahan	E1,E2	KF9845501,KF4147324
14	Chahar Mahal va Bakhtiari	CH1,CH2,CH3	KF9845531,KF3774651,KF3774681
15	Tehran	T	KF9845551

[1]= Pools originating from fry, [2]= Seminal fluids, [3]= Ovarian fluids and [4]= Pools sampled from fingerlings.

genetic difference with the other isolates, a comparison of the nucleotide sequences obtained from all isolates showed a high genetic similarity of 96.57%.

Discussion

Despite the significance of IPN disease in many regions, limited data is available concerning the genetic diversity of IPNV isolates particularly in brood fish (Bain et al., 2008; Barrera-Mejía et al., 2011). In the present study the majority of the IPNV positive samples were obtained from either ovarian/seminal fluid or larvae indicating the potential important role of brood fish in the vertical transmission of the virus. As a major structural immunogenic polypeptide of IPNV, the VP2 gene has been used in several phylogenetic studies. Therefore, use of the VP2 genome is a valuable tool to investigate the genetic diversity and molecular epidemiology of IPNV isolates (Heppell et al., 1992; Santi et al., 2004; Glenney et al., 2012).The phylogenetic tree consisting of the Iranian strains of IPNV shows that the 40 analyzed strains can be categorized in 6 genogroups. Based on nucleotide sequences of the VP2 coding region of the virus, the genetic similarities within each genogroup was 91.72% -100%. Fifteen isolates

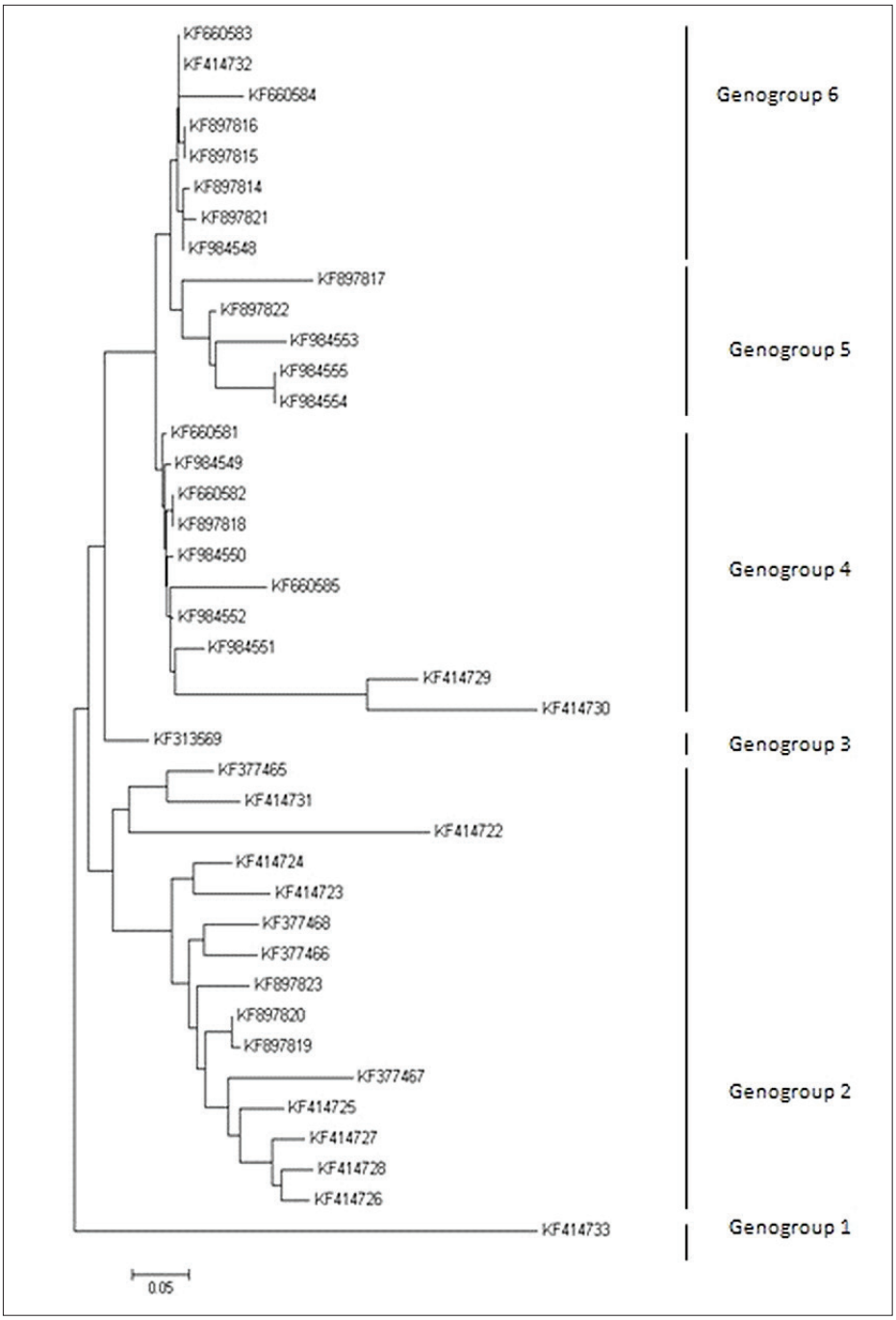

Figure 2. Phylogenetic tree of IPNV isolates from farmed rainbow trout in Iran based on a comparison of the VP2 gene segment sequence utilizing the neighbor-joining method. The length of each pair of branches represents the distance between sequence pairs.

formed one genogroup (genogroup 2) showing 96.57% similarity to the other 5 genogroups. M4 (KF313569) isolate which formed genogroup 3 demonstrated 91.66% similarity to the genogroups 4-6 while genogroup 4 consisting of 10 isolates showed 98.02% similarity to genogroups 5 and 6. Moreover genetic identity between genogroups 5 and 6 was 98.39%. Most of the isolates showed a high similarity (96.57%) to each other except isolate KF414733. Therefore, such a high genetic similarity makes it possible to develop a preventive tool such as

Figure 3. Phylogenetic tree of IPNV isolated from farmed rainbow trout in Iran and IPNV isolates from other countries obtained by neighbor-joining method. The length of each pair of branches represents the distance between sequence pairs.

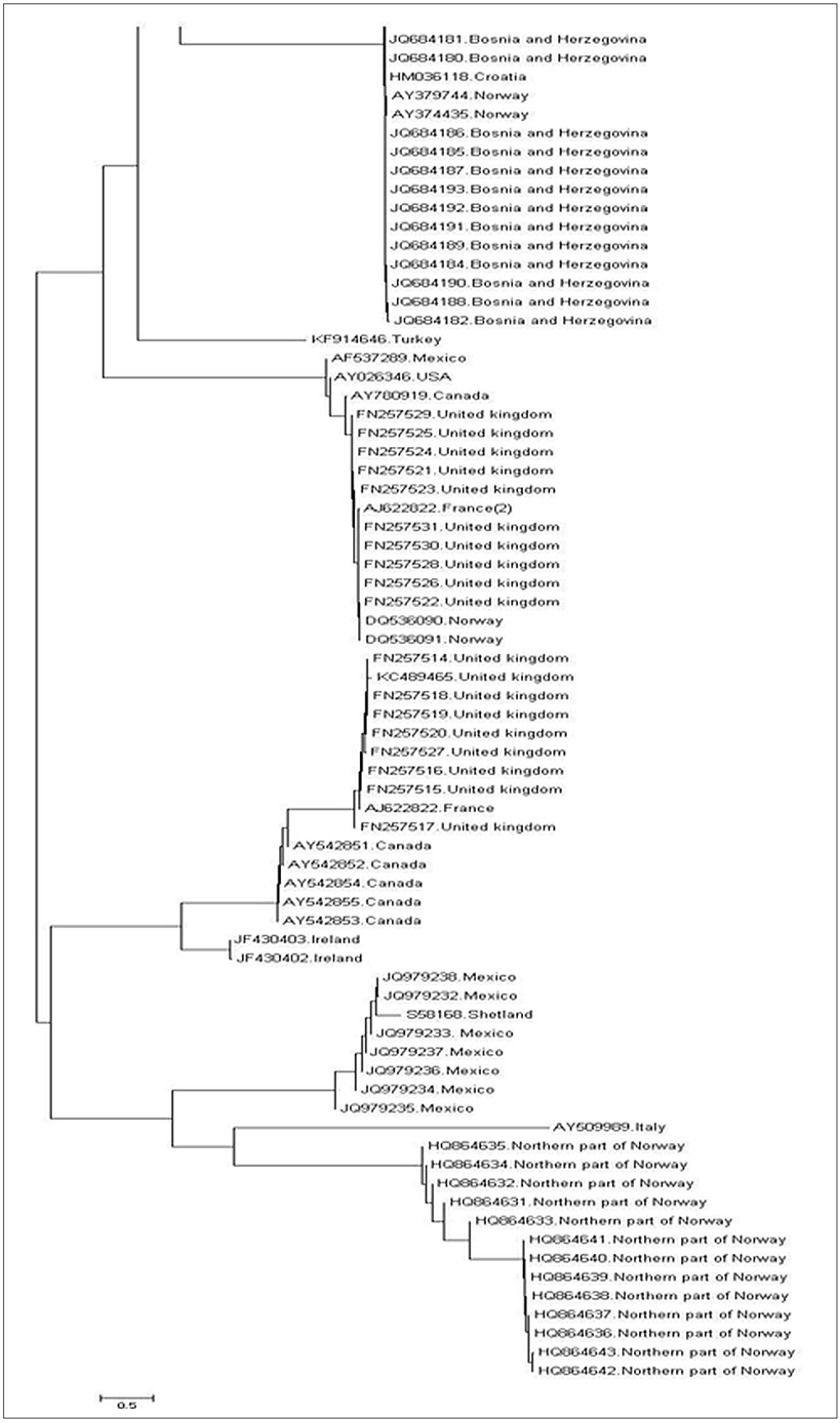

Figure 3. Continued.

production of a vaccine against disease caused by IPNV. The phylogenic tree consisting of published genogroups/isolates in the National Center for Biotechnology Information (NCBI) database plus Iranian isolates obtained in this study is demonstrated in Figure 3. The reason for such genetic diversity among Iranian isolates warrants further study to investigate the introduction and spread of IPNV within Iran. However, different environmental conditions and health management criteria may give rise to an increase in the diversity of a particular pathogenic micro-organism such as IPNV. This study was conducted on farms that underwent a serious disease outbreak, resulting in a high mortality in not only larvae-fingerling size but also in larger fish of > 200 g weight. According to the phylogenetic tree (Figure 3), the high genetic similarity of the Iranian isolates reported in this study with both European and USA strains could potentially indicate the multi-introduction of the virus via importation of infected ova from these regions.

Although the Iranian Veterinary Organization attempts to follow the biosecurity criteria recommended by OIE, (2003) e.g. routine sampling at the quarantine ports, establishment of high quality diagnostic laboratories in different parts of the country, having surveillance plans for detecting the pathogen and conducting epidemiological surveys to limit the spread of a given pathogen, as well as providing workshop/seminar/training courses for the fish farmers, such attempts appear to be inadequate. Also, some Iranian fish farmers hesitate to undertake the appropriate bio-security measures and this has undoubtedly contributed to the spread of IPNV within the country.

In conclusion, Global aquaculture development has raised the necessity for the implementation of robust biosecurity measures to prevent the spread of trans-boundary diseases such as IPN. Gaps in its implementation can cause high economic losses in rainbow trout cultivation. Data on genetic diversity of IPNV can contribute to the detection of the disease pathway and it can be a starting point for development of effective commercial vaccines.

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