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RESEARCH ARTICLE

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Determination of VP2 sequence-based virulence motifs and phylogenetic analysis of domestic Turkish IPNV ısolates

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Abstract

Infectious pancreatic necrosis (IPN) is a highly contagious disease of young salmonid fish and is one of the most severe economic diseases in aquaculture. In Turkey, an increase in infectious pancreatic necrosis virus (IPNV) outbreaks in freshwater rainbow trout have been reported in recent years. This study aimed to analyze the VP2 gene from recent IPNV isolates from Turkey to determine whether there are epidemiological links between IPNV isolates from rainbow trout (*Oncorhynchus mykiss*; 62) and sea bass (*Dicentrarchus labrax*; 1), wild turbot (*Scophthalmus maximus*; 1) and the environment in order to investigate potential wild and farmed fish interactions. In this study, 62 Turkish IPNV isolates collected over 10 years (2005–2014) from rainbow trout, sea bass and turbot were genotypically characterized. The phylogenetic analysis indicated that Turkish IPNV isolates are closely related to strains from Denmark, Iran and Spain and that all Turkish IPNV isolates belong to genogroup V, serotype A2 (Sp strain). Furthermore, low genetic diversity was found among the Turkish isolates (identity, 95.5%–100% nucleotides and 97.8%–100% amino acids). The result of the analysis of the amino acid residues found at positions 217, 221 and 247 (proline, threonine and alanine, respectively) could be associated with virulence.

KEYWORDS

genotype, IPNV, Turkey, virulence

1 | INTRODUCTION

Infectious pancreatic necrosis virus is a double-stranded and two-segmented *Aquabirnavirus* belonged the *Birnaviridae* family. It causes high mortality in juvenile salmonids/trouts and in post-smolt stages of salmonids after transfer to seawater, and the disease has a worldwide distribution (Akhlaghi & Hosseini, 2007; Benkaroun et al., 2021; Cutrin et al., 2004; Smail et al., 2006; Tamer et al., 2021; Zhu et al., 2017). Although IPN was initially thought to be solely a disease of salmonid species, it was later detected from a variety of freshwater and marine salmonid and non-salmonid species of fish, mollusks and crustacea throughout the world (Moreno et al., 2014; Mortensen, 1993). It is also fairly widespread in Turkey and continues to cause significant economic losses, especially in rainbow trout hatcheries in Turkey (Albayrak & Ozan, 2010; Candan, 2002). There is not too much detailed information about genotypes of IPNV wild field strains causing outbreaks in Turkey. Hence, identification of field strains of IPNV is needed.

Serogroup A and serogroup B are the major serogroups of IPNV. Serogroup A consists of nine serotypes named A1 (WB), A2 (Sp), A3 (Ab), A4 (He), A5 (Te), A6 (C1), A7 (C2), A8 (C3) and A9 (Ja). B1 (TV-1) is the only serotype in Serogroup B. The WB, Sp and Ab serotypes are the common serotypes in the world (Blake et al., 2001; Nishizawa et al., 2005). In the molecular genetic studies conducted in recent years, serological classification has been associated with genogroups, and genotypic classification has been used more widely. According

to a classification based on VP2, there are seven genogroups named genogroup 1 (A1 and A9), genogroup 2 (A3), genogroup 3 (A5 and A6), genogroup 4 (A7 and A8), genogroup 5 (A2 and B1), genogroup 6 (A4) and genogroup 7 (Blake et al., 2001; Cutrin et al., 2004; Duan et al., 2021; Romero-Brey et al., 2009).

Two segmented double-stranded RNA express four viral proteins of IPNV named VP1, VP2, VP3 and VP4. The VP2 gene used for phylogenetic analyses is the major structural and immunogenic polypeptide of IPNV and the most variable gene. It is also known to play a significant role in the virulence of IPNV. Specific amino acid positions (motifs) have been associated with IPNV strains that produce high mortality in challenged fish (Sano et al., 1992; Santi et al., 2004). We previously determined the mortality rate of an IPNV Turkish isolate, which has PTA (Proline at position 217 of VP2, Threonine at position 221 of VP2, Alanine at position 247 of VP2) motif, as moderate virulent for rainbow trout (Tamer et al., 2021). In this study, the VP2 gene from IPNV isolates all we isolate from commercial rainbow trout farms and wild fish in the different geographical areas of Turkey were analyzed, sequenced and classified in terms of virulence factors and amino acid substitutions. Thus, we aimed to investigate the interaction of IPNV between freshwater and wild sea fish in this study.

2 | MATERIALS AND METHODS

2.1 | Samples

62 IPNV isolates, which are isolated by Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Virology, Bornova Veterinary Control Institute (National Reference Laboratory), Samsun Veterinary Control Institute and Central Fisheries Research Institute during both epidemics and routine field surveys between 2005 and 2014 years, have been evaluated in terms of VP2 sequences of IPNV in the study. The distribution of the isolates is as follows: a total of 62 isolates; 34 from the Black Sea Region, 12 from the Aegean Region, 9 from the Mediterranean Region, 5 from the Eastern and Southeastern Anatolia Region and 2 from the Central Anatolia Region were included in the study (Table 1).

2.2 | Virus isolations

For cell culture isolation, different sizes of fish were ripped with a scalpel.~ 200 mg of the tissue from the spleen and kidney were extracted. The samples were inoculated the rainbow trout gonad (RTG-2) cells, and the cell cultures were incubated at 16°C in Leibowitz's (L15) medium (Gibco, Cat No: 11415-064) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (Sigma, Cat No: P4333-100ML) and HEPES (Sigma, Cat No: H0887-100ML) and checked daily for cytopathic effects (CPE). When CPE occurred, the supernatant was collected and stored at −70°C until the downstream applications.

2.3 | Conventional RT-PCR, cloning and sequencing

For the RNA extraction, GeneJET RNA Purification Kit (Thermo, Cat No: K0732) was used according to the manufacturer's instructions. To amplify the whole VP2 gene (1326 bp) region, primers with restriction enzymes were designed with the help of Mega X (Version 10.2.6) as follows: SpVP2F BamHI: 5′-CGG GAT CCA TGA ACA CAA ACA AGG CAA CCG A-3′; SpVP2R HindIII: 5′-CCC AAG CTT ATG CCT TTG ACG TGG GCA GGT CAC T-3′.

RT-PCR method was used for in vitro amplification of the VP2 gene encoding the surface glycoprotein of IPNV. To obtain cDNA, Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo, Cat No: K1621) was used. For this purpose,~1 μg double-stranded RNA with 50 ng random hexamer was preheated at 90°C for 7 min, and the samples were immediately cooled on ice. 5× reaction buffer (contain 250 mM Tris-HCl, 250 mM KCl, 50 mM DTT, 20 mM $MgCl_2$), 50 ng random hexamer primer, 200 U Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) and 40 U RNAase inhibitor have been added into the mixture and incubated at 25°C for 5 min, 42°C for 1 h and 70°C for 5 min, respectively. A 50 μl mixture was prepared for PCR reaction using Taq DNA Polymerase kit (Thermo, Cat No: EP0401) according to the manufacturer's instructor: 5 μl 10× buffer, 2.5 mM $MgCl_2$, 200 mM dNTP, 0.5 μM of each primer, 5 U Taq DNA polymerase and 50 ng cDNA mix. The reaction condition was as follow: one cycle for 3 min at 95°C; 35 cycles for 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; and a final extension for 10 min at 72°C. A 10 μl DNA product was run on 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide. The products were evaluated under UV light.

The IPNV VP2 products were cloned into the pGEM-Easy Vector Kit (Promega, Cat No: A1380) according to the blunt-end ligation protocol and were sent to RefGen Technology for commercial Sanger sequencing. All the sequences read bidirectionally using M13 universal primers. Sequence data were edited by using Vector NTI™ Express Software. The consensus sequences of all IPNV isolates were uploaded to the NCBI database with the accession number described in Table 1.

The Bayesian method was used for phylogenetic analysis. 1326 bp of VP2 sequences representing 62 IPNV isolates from Turkey, 1 sp isolate from Denmark, 1 sp isolate form Iran and 1 sp isolate from Spain were evaluated by SeaView Sequence Alignment and Phylogenetic Tree Software 4.7.

3 | RESULTS

As a result of the sequencing of the 1326 nucleotides and 442 amino acids-aa encoding the VP2 gene region of the 62 isolates, all the isolates of IPNV clustered in Sp (Figures 1 and 2), which is a common serotype among nine serotypes, and were included in genogroup five in genotype-based classification. It has amino acids similarities varying rate between 97.8%–100%, and nucleotide similarity was detected at a varying rate between 95.5%–100% among themselves. The isolate

TABLE 1 Isolates list includes cities, host, collection date, virulence motifs, GenBank accession numbers, isolates names have been shown

Cities	Host	Year	Virulence motif	GenBank accession number	Isolates name
Trabzon	Rainbow trout	2009	PTA	KY606172	1-Trabzon09
Trabzon	Rainbow trout	2007	PTA	KY606173	2-Trabzon07
Kahramanmaraş	Rainbow trout	2010	PTA	KY606174	3-Maras10
Hatay	Rainbow trout	2006	PTA	KY606175	4-Hatay06
Antalya	Rainbow trout	2006	PTA	KY606176	5-Antalya06
Düzce	Rainbow trout	2009	PTA	KY606177	6-Duzce09
Kahramanmaraş	Rainbow trout	2010	PTA	KY606178	7-Kmaras10
Gaziantep	Rainbow trout	2012	PTA	KY606179	8-Gantep12
Şanlıurfa	Rainbow trout	2012	PTA	KY606180	9-Surfa12
Kahramanmaraş	Rainbow trout	2013	PTE	KY606181	10-Kmaras13
Gaziantep	Rainbow trout	2012	PTE	KY606182	11-Gantep012
Düzce	Rainbow trout	2009	PTA	KY606183	12-Duzce009
Samsun	Rainbow trout	2012	PTA	KY606184	13-Samsun12
Muğla	Rainbow trout	2007	PTA	KY606185	14-Mugla07
Hatay	Rainbow trout	2007	PTA	KY606187	16-Hatay07
Burdur	Rainbow trout	2006	PTA	KY606188	17-Burdur06
Şanlıurfa	Rainbow trout	2012	PTA	KY606189	18-Surfa012
Bolu	Rainbow trout	2008	PTA	KY606190	19-Bolu08
Bolu	Rainbow trout	2008	PTA	KY606191	20-Bolu008
Aydın	Rainbow trout	2007	PTA	KY606192	21-Aydin07
Denizli	Rainbow trout	2005	PTA	KY606193	22-Denizli05
Düzce	Rainbow trout	2010	PTA	KY606194	23-Duzce10
Tokat	Rainbow trout	2007	PTA	KY606195	24-Tokat07
Trabzon	Rainbow trout	2010	PTA	KY606196	25-Trabzon10
Aydın	Rainbow trout	2007	PTE	KY606197	26-Aydın007
Trabzon	Rainbow trout	2010	PTA	KY606198	27-Trabzon010
Kayseri	Rainbow trout	2007	PTA	KY606199	28-Kayseri07
Aydın	Rainbow trout	2007	PTE	KY606200	29-Aydin071
Rize	Rainbow trout	2012	PTA	KY606201	30-Rize12
Trabzon	Rainbow trout	2014	PTA	KY606202	31-Trabzon14
Samsun	Rainbow trout	2014	PTE	KY606203	32-Samsun
Düzce	Rainbow trout	2009	PTA	KY606204	34-Duzce091
Rize	Rainbow trout	2012	PTA	KY606205	35-Rize012
Bolu	Rainbow trout	2010	PTA	KY606206	36-Bolu10
Bolu	Rainbow trout	2009	PTA	KY606207	37-Bolu09
Gaziantep	Rainbow trout	2012	PTA	KY606208	38-Gantep121
Muğla	Rainbow trout	2009	PTA	KY606209	39-Mugla071
Ordu	Rainbow trout	2006	PTA	KY606210	41-Ordu06
Samsun	Rainbow trout	2007	PTA	KY606211	42-Samsun07
Bolu	Rainbow trout	2009	PTA	KY606212	43-Bolu009
Antalya	Rainbow trout	2007	PTA	KY606213	44-Antalya07
Muğla	Rainbow trout	2007	PTA	KY606214	45-Mugla072
Ordu	Sea bass	2014	PTA	KY606215	46-Ordu00

(Continues)

TABLE 1 (Continued)

Cities	Host	Year	Virulence motif	GenBank accession number	Isolates name
Trabzon	Rainbow trout	2010	PTA	KY606216	47-Trabzon0101
Düzce	Rainbow trout	2009	PTE	KY606217	48-Duzce092
Düzce	Rainbow trout	2009	PTA	KY606218	49-Duzce093
Tokat	Rainbow trout	2007	PTA	KY606219	50-Tokat007
Zonguldak	Rainbow trout	2009	PTA	KY606220	51-Zonguldak09
Ankara	Rainbow trout	2010	PTA	KY606221	52-Ankara10
Trabzon	Rainbow trout	2012	PTA	KY606222	54-Trabzon12
Muğla	Rainbow trout	2014	PTA	KY606223	55-Mugla14
Trabzon	Rainbow trout	2014	PTE	KY606224	56-Trabzon14
Trabzon	Rainbow trout	2014	PTE	KY606225	57-Trabzon141
Denizli	Rainbow trout	2014	PTA	KY606226	58-Denizli14
İzmir	Rainbow trout	2012	PTA	KY606227	59-Izmir12
Denizli	Rainbow trout	2013	PTA	KY606228	60-Denizli13
Uşak	Rainbow trout	2005	PTA	KY606229	61-Usak05
Hatay	Rainbow trout	2006	PTA	KY606230	62-Hatay006
Trabzon	Turbot	2010	PTA	KM972673	63-HAH-2
Trabzon	Rainbow trout	2010	PTA	KM972674	64-HAH-3
Trabzon	Rainbow trout	2010	PTA	KM972675	65-HAH-4
Tokat	Rainbow trout	2013	PTA	KM972672	66-Almus

with GenBank number AF342728, originating from Denmark, has amino acid similarity between 98.1%–100% and nucleotide similarity between 96.3%–100% with our isolates. The isolate with GenBank number AJ489222, originating from Spain, has amino acid similarity between 98.4%–99.6% and nucleotide similarity 96.9%–99.4% with our isolates. The isolate with GenBank number KF279643 originated from Iran has amino acid similarities.

It was determined that the isolates that caused the outbreaks carried both PTA and PTE (Proline at position 217 of VP2, Threonine at position 221 of VP2, Glutamic acid at position 247 of VP2) virulence motifs at amino acids 217, 221 and 247. 8 isolates have PTE virulence motifs, and 54 isolates have PTA virulence motifs (Figure 3, Table 1).

4 | DISCUSSION

Turkey is one of the biggest rainbow trout producers in the world and carries out both export and import of rainbow trout eggs and fry. Almost every region of Turkey was contaminated with IPNV causes significant economic loss in the aquaculture industry in Turkey (Albayrak & Ozan, 2010; Isıdan et al., 2019). These results constitute important evidence that our isolates are of European origin., Dadar et al. (2013; 2014) also claimed that the Iranian IPNV isolates belonged to the A2 (Sp) serotype, were 99% similar to Spanish isolate and that the viruses circulating in Iran originated in Europe. However, IPNV, first isolated in 2007 in Iran, belonged to the A3 (Ab) serotype (Akhlaghi & Hosseini, 2007). Besides, Genogroup 6 (A4) was also detected from rainbow trout farms in Iran (Soltani et al., 2015). Turkey and Iran are exporting rainbow trout eggs from many countries such as the United States of America, the United Kingdom, the Republic of South Africa, Azerbaijan and apart from the European continent, which A2 (Sp) are widely found (Ahmadivand et al., 2020; Cutrin et al., 2004; Romero-Brey et al., 2009). And it is crucial that A3 (Ab) and A4 (He) serotypes, previously reported in Iran, Europe and America, have not been reported in Turkey yet (Cutrin et al., 2004, Soltani et al., 2015). Since the IPNV virus is a segmented virus, a new serotype or genotype may enter Turkey as a result of reassortment. The emergence of new serotypes of IPNV will make it very difficult to implement disease control and control measures.

One of the virulence markers of the IPNV is amino acid position at 217, 221 and 247 on VP2 (Santi et al., 2004). All the isolates evaluated in this study have been shown to have PTA and PTE virulence motifs. Recent outbreaks in rainbow trout, caused by PTA and PTE motifs of IPNV, known previously nonvirulent for Atlantic salmons, are moderate virulent for rainbow trouts (Ahmadivand et al., 2020; Panzarin et al., 2018; Tamer et al., 2021). When the epidemiological data and sequence results are compared, it is thought that both virulence motifs (PTA and PTE) can be pathogenic for rainbow trout.

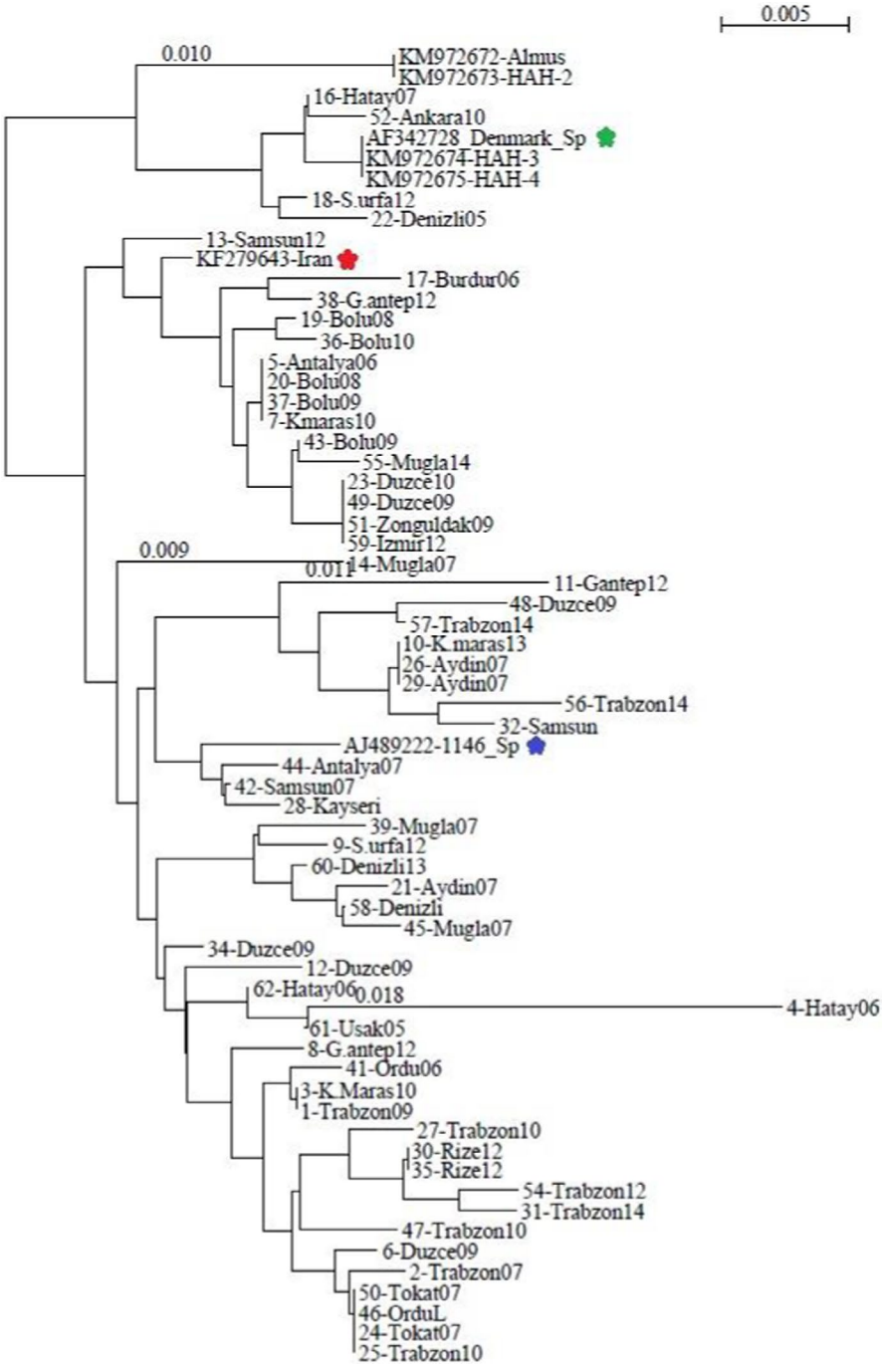

FIGURE 1 The phylogenetic tree of 62 IPNV isolates, constructed by the neighbour-joining method and based on VP2 among Sp serotypes, was performed using SeaView Sequence Alignment and Phylogenetic Tree Software 4.7 with bootstrap values being calculated from 1000 trees. Green star ★: Danish isolate, red star ★: Iranian isolate, blue star ★ : Spanish isolate

Therefore, the determination of virulence and genogroups of IPNV is vital for Turkey.

In this study, a trace of IPNV between freshwater and wild sea fish was also aimed to investigate by a large-scale phylogenetic analysis based on the VP2 gene region of 62 Turkish isolates. The high percentage of identity among the VP2 genes from geographically distant IPNV isolates and the evidence of wide distribution in Turkey might have been facilitated by carrier trout without being checked in terms of significant diseases. This hypothesis is supported by the identification of the amino acid threonine at position 221 in all Turkish isolates, a factor related to the carrier state for IPNV, as reported by other studies (Buyukekiz et al. 2018; Durmaz & Albayrak, 2019; Isıdan et al., 2019). Interaction between wild and freshwater transmission of IPNV clearly indicated with this study, which isolates from Trabzon with the accession number: KM972673, isolated from wild turbot is %100 identical with rainbow trout isolate from Tokat. The IPNV with the accession number KY606215 isolated from sea bass in Ordu has also been found closely related to freshwater rainbow trout isolates from Tokat and Trabzon. Thus, controlling borders and importation are not enough to eliminate IPNV. Anadromous fish should also be checked in terms of IPNV to prevent wild sea fish-borne IPNV contamination to freshwater rainbow trout farms.

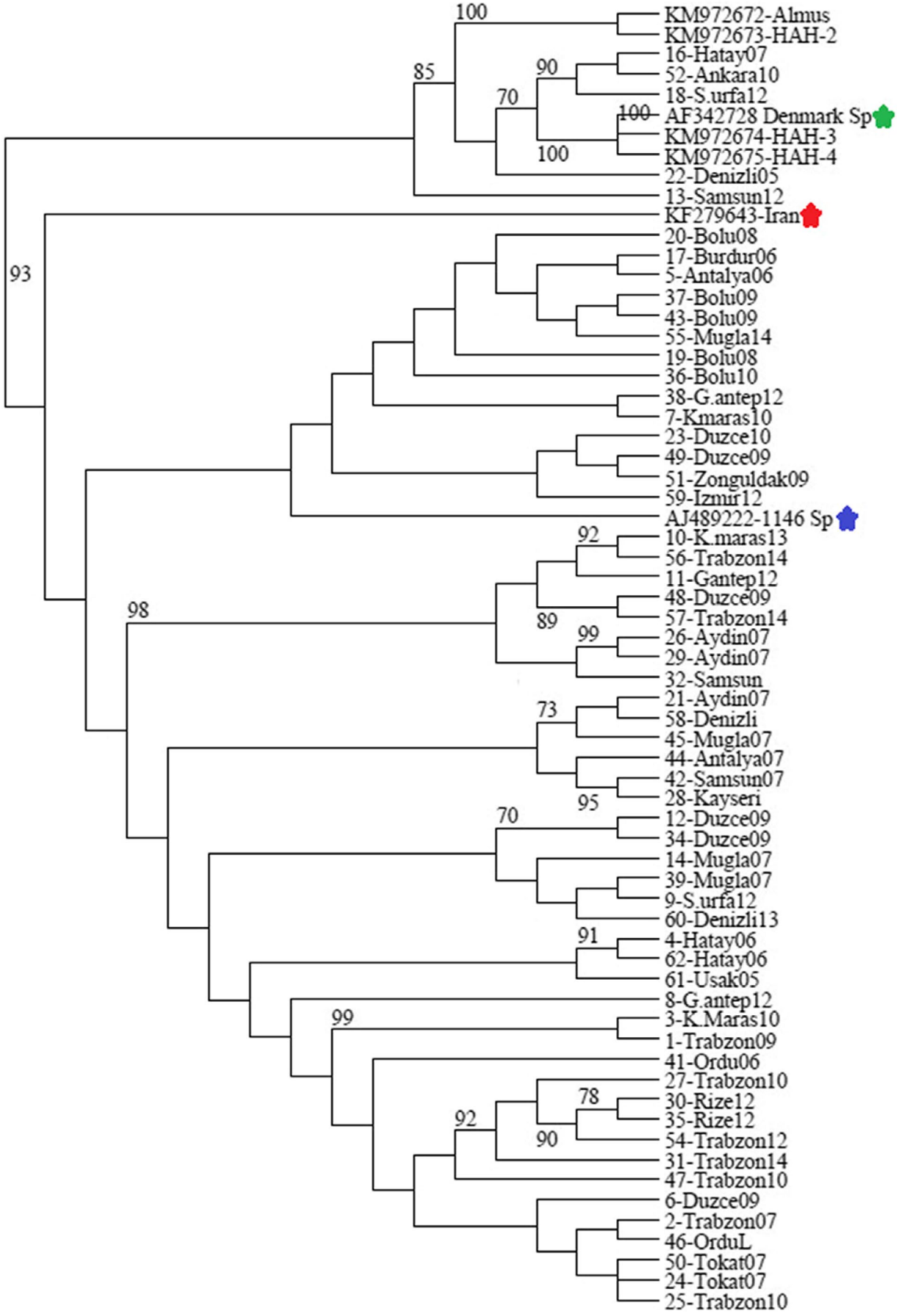

FIGURE 2 The phylogenetic analyses of 62 IPNV isolate according to the VP2 nucleotide sequences. The phylogenetic tree was constructed in the same way as Figure 1. Green star ★: Danish isolate, red star ★: Iranian isolate, blue star ★: Spanish isolate

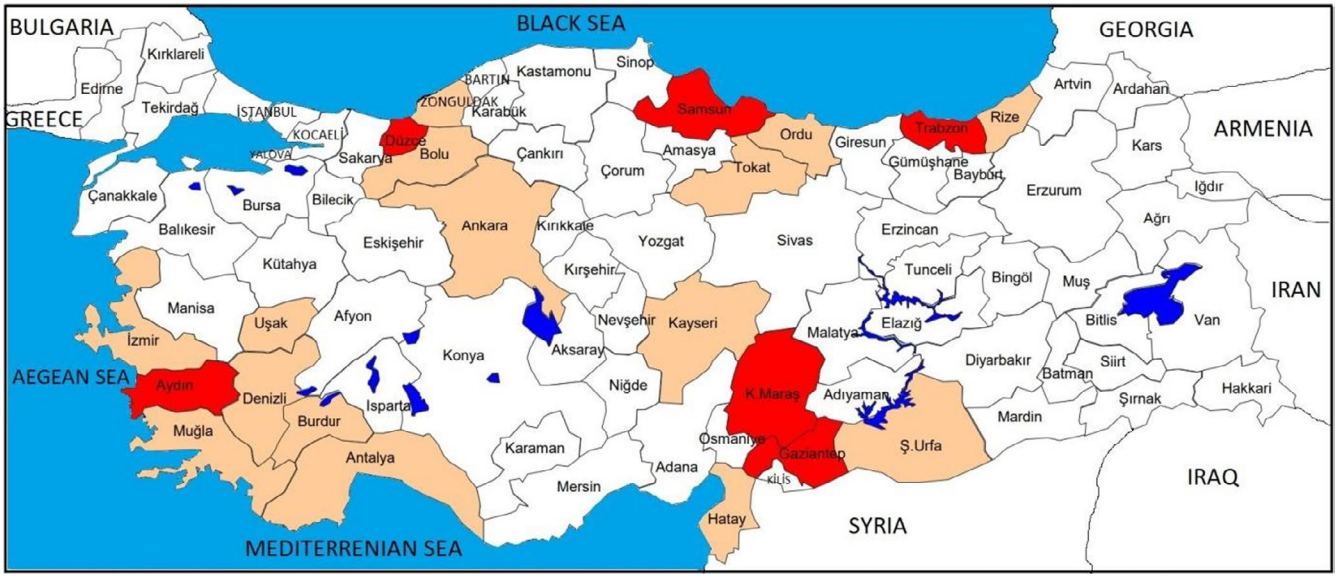

FIGURE 3 Geographical distribution of IPNV isolates according to the virulence motifs. The cities in which both PTE and PTA virulence motifs have been detected are marked with red colour ■. The cities in which only PTA virulence motifs have been detected are marked with yellow colour ■

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All the data supporting the findings of this study are available on request from the corresponding author.

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