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Molecular characterization and phylogenetic analysis of *Cyprinid herpesvirus 3* genotypes in Iran from 2020 to 2023

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The aquaculture industry in Iran contributed to about 1% of the world's aquaculture production in 2020 with a volume of 0.7 million tons. A targeted approach was used to identify positive samples by collecting samples from 342 suspected carp farms showing signs of disease. The carp species was considered as the host for the viruses under investigation and Khuzestan, Mazandaran and Gilan provinces were selected for sampling. A total of 251 farms in Gilan, 68 farms in Mazandaran, and 23 farms in Khuzestan provinces were sampled. *Cyprinid herpesvirus 3* (CyHV-3) was characterized by a combination of sequence analysis and duplex PCR. Genetic analyzes and phylogenetic tree construction were performed using MEGA7 software. Of the 342 farms sampled, 85 were infected with koi herpes virus (KHV). Asian 1 and Asian 2 genotypes were identified by sequence analysis of the *Sph*I-5 and TK gene regions. One of the positive samples showed a match in all motif positions within the TK gene, specifically genotype A1, except for positions 814 – 813 where they had the sequence AT, which was a rare exception. Duplex PCR analysis of two variable marker regions between ORF29 and ORF30 (marker I) and ORF133 and its upstream region (marker II) revealed viruses of genotype J (I^{++} II^{+}), an intermediate genotype (I^{++} II^{-}), and a new genotype, I^{++} II^{-*}, identified in viruses from different farms. This new genotype retains the I^{++} allele of marker I and has a 5-bp deletion in the marker II. The global distribution of CyHV-3 genotypes is not yet fully elucidated. Results indicate the high degree of diversification of CyHV-3 in the West Asian regions, where at least three different genotypes (I^{++} II^{+}, I^{++} II^{-}, and I^{++} $II^{-\Delta}$) currently appear to circulate.

Keywords Cyprinid herpesvirus-3, Genotyping, Cyprinus carpio, Koi herpes virus

Fisheries and aquaculture play a prominent role in ensuring global food security and nutrition in the current century. The common carp, a fish species widely distributed in Asia, Europe and the Middle East, has a long tradition as a valuable food source. Currently, it accounts for about 8% of the world's aquaculture production and is the fourth-produced fish species in the world, producing about 2.4 million tons annually[1]. In Iran, carp fish production represents about 25–30% of the total production of warm-water fish. The koi carp is a color variant of the common carp (*Cyprinus carpio L.*). Additionally, koi have been imported to Iran for approximately 20 years from countries in the Southeast Asian region. Despite the significant growth in aquaculture production, the lack of adequate sanitation infrastructure and disease control measures remains a challenge. Viruses are responsible for about 22.6% of infectious diseases in aquaculture.

Several significant viral diseases pose a threat to carp fish. One of these diseases is koi herpesvirus disease (KHVD), which is caused by *cyprinid herpesvirus 3* (CyHV-3). CyHV-3 belongs to the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*. This newly emerged virus is highly transmissible and causes severe and widespread morbidity and mortality, primarily affecting carp and koi populations[2]. CyHV-3 first appeared in Germany in 1997, although it had already been detected in carp fish in the United Kingdom in 1996[3,4]. Since

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then, CyHV-3 has been reported on a global scale, including in Japan, the United States, European countries, South Africa, East Asia, and Southeast Asian countries[3,5–9]. This disease has been reported in at least 40 countries on all continents except South America, Australia, and North Africa. The virus first broke out in carp farms in Israel in 1998 and then spread rapidly to koi fish stores in the United States, resulting in high mortality rates in carp and wild carp in the U.S. and Canada[3]. In 2000, the causative agent of the disease was identified as herpesvirus in the United States[10]. In Asia, a significant mortality risk was reported in farmed koi fish in Indonesia in 2002 and in farmed carp in Japan in 2003[3]. The disease continued to spread rapidly. In late 2018, the first outbreak of CyHV-3 occurred in the Middle East, resulting in massive mortality among farmed carp in cages along the Euphrates River in Iraq[11,12]. Subsequent investigations revealed that the virus was the main cause of these mortalities. Most of these detections were associated with summer mortality syndrome in carp fish, particularly in Gilan and Khuzestan provinces[13–15]. Due to its economic impact and rapid global spread, cyprinid herpesvirus 3 has been classified as a notifiable disease by the World Organization for Animal Health (WOAH) since 2007.

The CyHV-3 genome comprises 156 protein-coding genes (ORFs) and, with a size of 295 kilobase pairs (kbp), is the largest genome within its taxonomic order. A specific genetic marker, the thymidine kinase gene (TK) or ORF55, has been used to identify target herpesviruses. In conventional PCR for large DNA viruses, the DNA polymerase gene has been used as an ideal target in numerous partial gene sequences submitted in genebank of herpesviruses[16,17]. Currently, 9–11 fully sequenced genomes of the virus are listed in the NCBI database, mainly from viruses isolated from cell cultures. Sequencing of the virus strains isolated from Israel (CyHV-3 I), Japan (CyHV-3 J), and the United States (CyHV-3 U) shows that their sequences are more than 99% similar despite considerable geographic distance. It appears that minor genomic variations, which are common, are generally tolerated and are not necessary for virus replication and integrity[18]. Furthermore, in vitro studies show a relatively slower rate of genetic evolution of this virus[19]. Nevertheless, nine genotypes were identified, including two Asian genotypes (A1 and A2) and seven European genotypes (E1 to E7), which showed clear genetic differentiation. In another analytical approach, Bigarre et al. (2009) developed a duplex PCR assay to distinguish between cyprinid herpesvirus 3 (CyHV-3) genetic lineages U/I and J based on variations in three variable domains within two genetic markers. The first marker (marker I) is detected with primer pairs (oPVP53 and oPVP54) targeting an intergenic region between ORF 29 and ORF 30, resulting in a 168-bp fragment for CyHV-3 strain J. However, for strains I and U, this fragment is shorter (130 bp) because two domains (15 and 23 bp, respectively) were deleted, resulting in a combined deletion of 38 bp. The second marker (marker II) is detected with primers oPVP55 and oPVP56, which amplify a 352-bp fragment in CyHV-3 strain J, whereas a shorter 278-bp fragment is generated in strains I and U because of a 74-bp domain deletion. Based on the presence (+) or absence (–) of each of the three variable domains, Bigarre et al. (2009) proposed a classification system. Strains U/I were designated $I^{--}II^{-}$, representing the absence of all three domains in markers I and II, while strain J was designated I^{++} II $^{+}$ because it contained all three domains. In the Netherlands, a third genotype ($I^{-+}II^{-}$) was discovered that possessed one of the two marker I domains but lacked the marker domain II[20]. Recently, an intermediate genotype ($I^{++}II^{-}$) has been reported in Indonesia[21]. In addition, the study conducted by Chen et al. (2014) reported a novel strain that was classified as a variant of Koi herpesvirus (KHV) based on molecular marker analysis and fell into the J clade. However, this variant had a deletion of 13 nucleotides within the region marker II[22].

The herpesvirus affecting koi fish has caused significant economic crises on several occasions due to its highly contagious and transboundary nature, complex epidemiology, negative impact on productivity, trade restrictions, and the need for extensive surveillance and costly control measures. Due to the widespread farming of carp fish in Iran and the lack of effective treatment options, the development of a sensitive, rapid, and specific diagnostic method to identify the virus is essential to prevent widespread transmission. Considering the extensive water connections between Iran and neighboring countries, obtaining virus sequences and studying the phylogeny of each virus can help identify the source and entry routes of these viruses into Iranian waters and farms. The aim of this research is to investigate the presence of prevalent genotypes in the three most important provinces for carp fish farming in Iran.

Materials and methods

Fish sampling and history

Between January 2020 and February 2023, mass mortalities were observed in a number of freshwater fish farms in the main carp culture centers of Iran. Samples of koi carp (Koi *cyprinus carpio*), common carp (*cyprinus carpio*), bighead carp (*Hypophthalmichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), and grass carp (*Ctenopharyngodon idella*) were collected from 342 farms in three provinces of Khuzestan (31.3273°N 48.6940°E), Gilan (37.2774°N 49.5890°E) and Mazandaran provinces (36.5656°N 53.0588°E) from Iran Fig. 1). Of the 342 farms, 251 were in Gilan, 23 in Khuzestan, and 68 in Mazandaran.

Significant mortality rates ranging from 40 to 100% were documented in the fish farms, with surviving fish exhibiting a range of symptoms. These include lethargy, aggregation near the water inlet, lack of coordination (ataxia), pale skin, excessive mucus production at the gills, gills with white spots or severe necrosis, decreased appetite, hemorrhage, skin lesions especially at the base of the fins and on the abdomen), and sunken eyes. Clinical signs of disease are shown in Fig. 2. Most cases were observed during the spring and autumn (Fig. 3), with average temperatures ranging from 22 to 25°C.

The primary tissue samples used in the study were obtained from the kidneys and gills. A total of 1 to 5 kidney or gill tissue samples were pooled from each farm. For confirmation or monitoring of disease and based on previous studies, it was deemed necessary to collect 3 to 5 gill and kidney samples separately[23]. In addition, spleen, intestine, brain, skin lesions, and ovary samples were also collected from some farms. Water quality parameters, including temperature, pH, and O_2, were also thoroughly investigated.

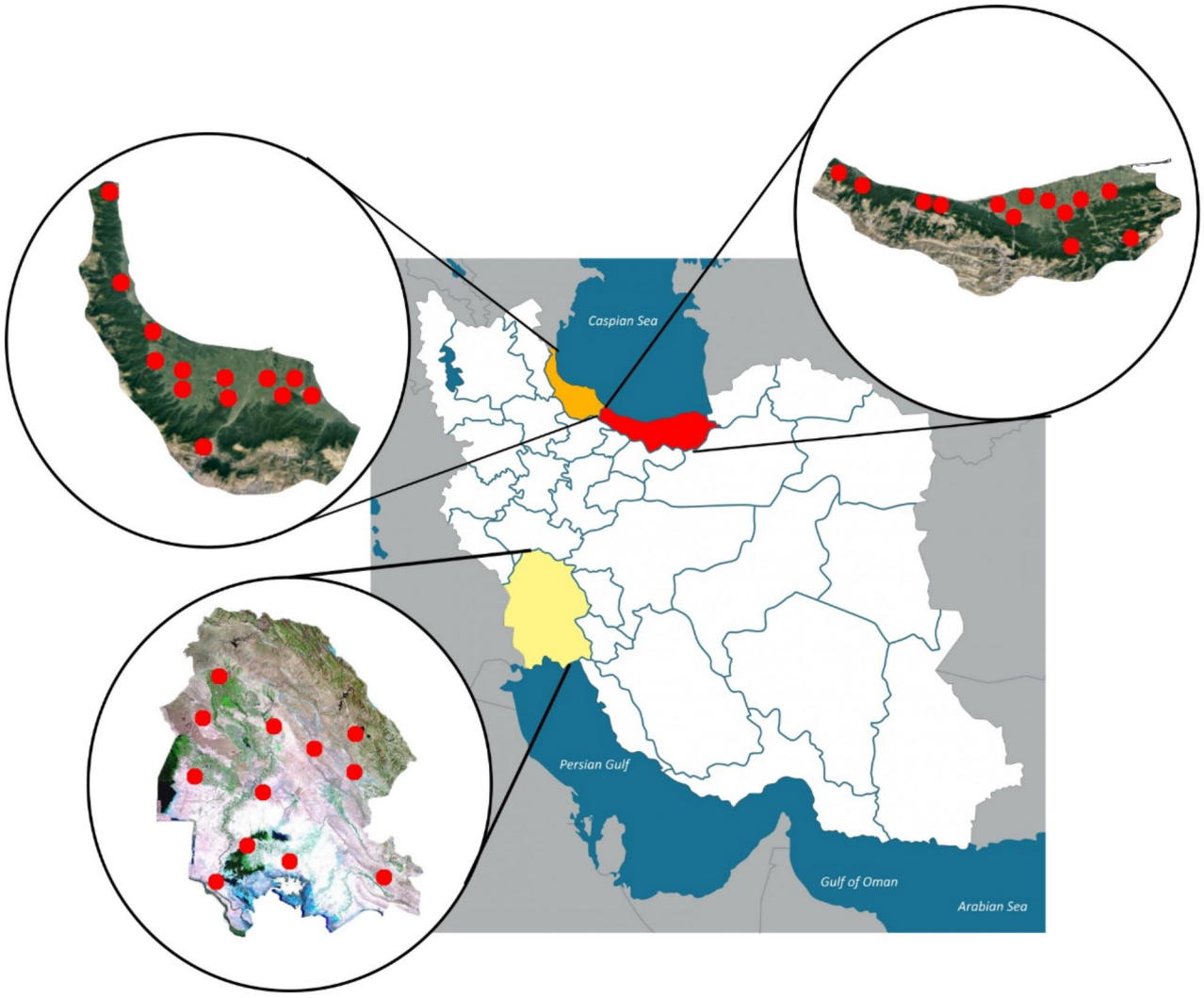

Fig. 1. Geographical locations of Iranian provinces where Koi herpes virus (KHV) was detected. (1, Khuzestan; 2, Gilan; 3, Mazandaran). The areas shown in red are the parts of the province that were sampled. (The map was obtained from https://images.app.goo.gl/oeHXm5td7kJdR6K18, Photoshop version 23.5.5 was used to display magnifications and points).

Detection of CyHV-3 by PCR

The collected tissues were combined and homogenized in PBS, followed by extraction of genomic DNA using a commercial DNA extraction kit (Sinaclon, EX6071, Iran) according to the manufacturer's instructions. A DNA template of 3 µL was used for PCR. For routine screening, KHV-specific PCR was performed with primers targeting the TK gene, resulting in the amplification of a 409-bp amplicon. The final volume of PCR assays was 25 µL and consisted of a 2× ready-to-use master mix containing 2 mM MgCl2, 0.2 mM of each dNTP, 10× PCR buffer, 2 U Taq DNA polymerase (Sinaclon, Iran), and 1 µl of a 10 pmol concentration of each primer (Sinaclon, Iran). To reduce the probability of false-negative results, the subregion of the polymerase gene was amplified as an additional target gene in a nested PCR assay[24].

Genotype analysis

Genotyping analysis was performed on some CyHV-3-positive samples using PCR assays targeting the partial TK gene and the partial polymerase gene. Another PCR reaction was used for amplification of the 610 bp long 'extended *Sph* I-5 region', which includes the known PCR detection target of the 290 bp long '*Sph* I-5' region[25], and the 1,001 bp-length 'enlarged thymidine kinase (TK) gene region,' which includes the 651 bp-length TK gene open reading frame and nested 409 bp-length PCR-detection target, 'TK'[26]. Comparison of DNA sequences obtained with the above primers identified polymorphism patterns that clustered KHV into genotypes and other variants[27]. In addition, a duplex PCR assay developed by Bigarre et al. (2009) was used to distinguish between Iranian KHV gene lineages[20].

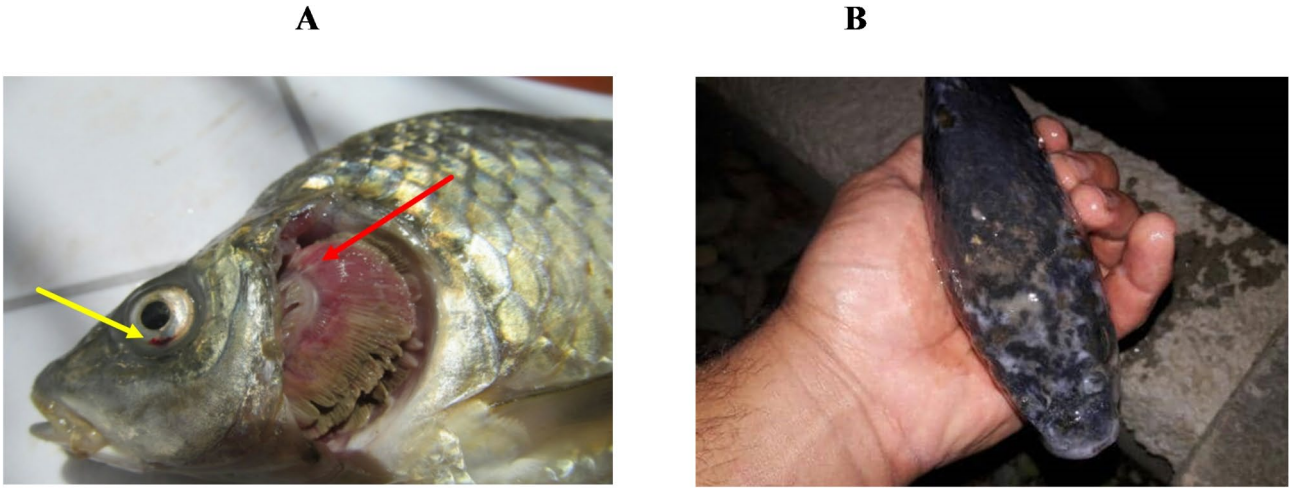

Fig. 2. (**A**) Hemorrhage in the eye (indicated by yellow arrows), watery discharge with congestion, increased mucus on the gill, white spots, and necrosis (indicated by red arrows). (**B**) Irregular patches of pale coloration and sandpaper like skin texture.

Bioinformatics and phylogenetic analysis

Sequence analysis

Positive PCR products were used to collect sequence data with the DNA sequencer (Applied Biosystems). Chromatograms were analyzed individually using Chromas 2.6.5 software. Gene annotation and trimming of sequences were performed using GenSAS v6.0. High-quality sequence data were aimed for, with a maximum size of 1001 bp for the TK gene, 611 bp for the *Sph*I-5 gene, marker I based on ORF29 and ORF 30 (which varied in length from 130 to 168 bp), and marker II based on ORF 133 (which varied in length from 278 to 352). To confirm the positivity of each virus, the confirmed values were evaluated and subsequently examined on the NCBI website in the Nucleotide Blast section. Nucleotide sequences were analyzed using CLC viewer 8.0. The sequences obtained were deposited in GenBank under the accession numbers listed in Table 1.

Phylogenetic analysis

Phylogenetic analyzes were performed using the nucleotide sequence of the TK gene, which spanned 1001 bp and was aligned to closest matches using the BLAST algorithm provided spanned 1001 bp and was aligned with the closest matches using the BLAST algorithm provided.

by the National Center for Biotechnology Information (NCBI) according to rigorous standards. TK gene sequences of different genotypes of KHV were obtained from GenBank, and a FASTA format dataset was assembled. The ClustalW program was used to generate multiple sequence alignments, which were subsequently used to generate distance matrices via the General Time Reversible (GTR) model implemented in MEGA software version 7. Finally, maximum likelihood (ML) trees were generated using MEGA7 and represented with a 1,000 bootstrap approach as described by Kumar et al. (2016) and Nei and Kumar (2000)[28].

Histopathological study

For histological examinations, gill samples were dissected and preserved in 10% buffer formalin (NBF). The 10% NBF fixed samples were dehydrated in a series of ethanol, and finally embedded in paraffin using a paraffin tissue processor (DS 2080/H; Did Sabz Co.) and paraffin dispenser (DS 4LM; Did Sabz Co.). Subsequently, several 4 µm sections (Rotary Microtome RM2145; Leica) were cut and stained with hematoxylin and eosin (H&E). Sections were examined by light microscopy (E600, Nikon), and representative images were taken by a GT 12 microscope camera (Tucsen, Mosaic 2.4 software). Axiovision software (version 4.8) was used to apply the image scale bars.

Result

Water quality

The water quality parameters in the common carp pond were within the normal range, with a pH of 7.5 to 8 and a dissolved O_2 concentration between 6 and 7 mg L^{-1}.

Virus identification

Molecular testing of 342 carp farms showed that 85 of these farms were infected with KHV based on PCR testing. According to Kafi et al.,[29] a certain number of these farms were also infected with carp edema virus. Eleven farms were found to be simultaneously infected with koi herpes virus and carp edema virus. However, in some other farms, the etiology of the clinical symptoms could not be determined. The results of the study showed that Gilan and Mazandaran regions had the highest level of KHV contamination, 84.70% and 15.29%, respectively. In Gilan province, the predominant and definitive host, namely carp, had a KHV infection rate of 93.06%. In addition,

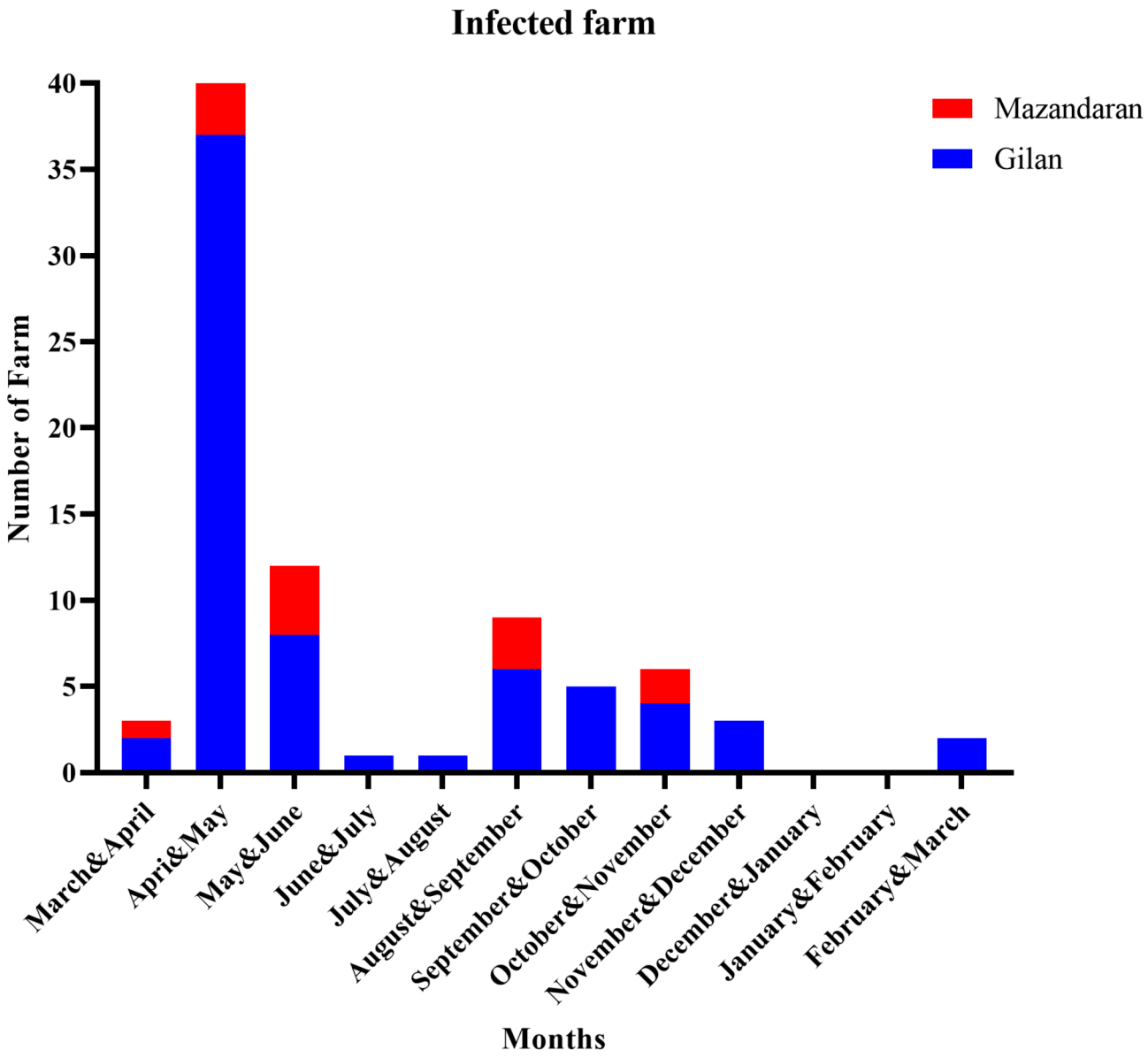

Fig. 3. Number of carp fish farms infected with the KHV, categorized by selected provinces in Iran.

Isolate name	Acc. number	Province	Sampling date
UT-Ziafati 40933	OQ437241	Mazandaran	2021September 4
UT-Ziafati 40295	OQ447505	Gilan	2021 May 16
UT-Ziafati 40489	OQ447506	Gilan	2021 June 14
UT-Ziafati 40357	OQ447507	Gilan	2021 May 24
UT-Ziafati 40851	OQ447512	Gilan	2021 August 12
UT-Ziafati 40271	OQ437242	Gilan	2021 May 12
UT-Ziafati 41097	OQ447508	Gilan	2021 September 14
UT-Ziafati 41098	OQ447509	Mazandaran	2021 September 14
UT-Ziafati 41100	OQ447510	Gilan	2021 September 14
UT-Ziafati 9155	OQ447511	Gilan	2021 October 2

Table 1. Accession numbers for Koi herpesvirus (KHV) sequences (thymidine kinase gene) recorded in GenBank are provided.

silver carp had a KHV infection rate of 5.56%, while grass carp had a rate of 1.39%. Similarly, in Mazandaran province, carp had a KHV infection rate of 92.31% while silver carp had a rate of 7.69%. During April and May, Gilan province had the highest contamination rate of 51.39%, while Mazandaran province had a contamination rate of 30.77% in May and June. It is worth mentioning that the suspect farms were not infected with KHV in August, January and February.

Genotype analysis

Analysis of genetic data based on thymidine kinase gene and Sph I-5

PCR and genotypic analysis were performed on 10 CyHV-3 samples based on the study by Kurita et al. (2009). The aim was to determine the genotype of cyprinid herpes virus 3 by using primers amplifying the *thymidine kinase* gene and the *Sph I-5* gene with a length of 1001 bp and 610 bp, respectively. The sequencing results of these two genes showed that genotype A1 was present in samples UT -Ziafati 40295, UT -Ziafati 40933, UT -Ziafati 40357, and UT -Ziafati 40271. These sequences had repetitive sequence motifs of the genotypes as reported in previous studies. Specifically, the *Sph*I gene had a C nucleotide at position 209 and a deletion of the AAC motif at position 588 – 586. The *thymidine kinase* gene contained typical sequence motifs of genotype A1, including C, A, AA, TTT TTT T, CTT TAA AAA AAA, and AGA TAT T, located at positions 94, 778, 850 – 849, 885 – 877, 956 – 945, and 967 – 961, respectively. Additionally, the motifs AT at positions 814 – 813 and CA at positions 958 – 957 were absent. Notably, the UT -Ziafati 40271 sample had a AT sequence at position 814 – 813, although it corresponded to the A1 genotype at all other positions of the motifs in the *thymidine kinase* gene. Furthermore, sequencing results showed that the A2 genotype was present in samples UT -Ziafati 40489, UT -Ziafati 40851, UT -Ziafati 9155, UT -Ziafati 41097, and UT -Ziafati 41098. These sequences exhibited repetitive sequence motifs of genotypes previously reported in Kurita et al. 2009 studies. The *Sph*I gene had a C nucleotide at position 209 and a deletion of the AAC motif at position 588 – 586. Similarly, the *thymidine kinase* gene contained typical sequence motifs of genotype A2, including C, A, AA, TTT TTT TT, CTT TAA AAA AAA, and AGA TAT T at positions 94, 778, 850 – 849, 885 – 877, 956 – 945, and 967 – 961. In addition, the motifs AT at positions 814 – 813 and CA at positions 958 – 957 were also absent (Table 2).

Analysis of genetic data based on marker I and marker II

Consistent with the study conducted by Bigarré et al. 2009, ten designated sequences were subjected to amplification with molecular markers I and II. Analysis of samples from UT -Ziafati 40933 revealed the presence of band lengths of 352 bp and 168 bp for markers II and I, respectively (Bigarré et al., 2009). This allelic pattern was identified as a J strain, as reported by Bigarré et al. 2009, comprising marker (I^{++} II $^{+}$). In contrast, in the U/I strains reported from America and Israel, the allelic pattern appeared as a deletion in both markers, with marker II showing a band length of 278 bp, while marker I showed a band length of 130 bp. The electrophoresis gel of samples UT -Ziafati 40295, UT -Ziafati 40489, UT-Ziafati 40357, UT -Ziafati 40271, UT -Ziafati 9155, UT -Ziafati 41097 and UT -Ziafati 41098 showed band lengths of 278 and 168 bp for markers II and I, respectively (I^{++} II $^{-}$). Based on the study conducted by Chen et al. in 2014, these strains are categorized as intermediate strains of U/I and J (Chen et al., 2015). Only the sample UT -Ziafati 40851 was identified as an intermediate strain (I^{++} II $^{-}$) and had a 5-nucleotide deletion in marker II, reported as a new strain (I^{++} II $^{-*}$) (Table 3).

Genotype/Variant	Enlarged TK gene region	Enlarged SphI-5 region								References
Nucleotide	209	586–588	94	778	813–814	877–885	945–956	957–958	961–967	
A1	C	–	C	A	–	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	[27]
A2	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	[27]
E1	C	AAC	C	G	AT	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
E2	T	AAC	C	G	AT	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
E3	C	AAC	C	G	AT	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
E4	C	AAC	C	G	AT	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
E5	C	AAC	C	G	AT	TTTTTTTTT	–	–	–	[27]
E6	C	AAC	T	G	AT	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
E7	C	AAC	C	G	AT	TTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	[27]
UT-Ziafati 40933	C	–	C	A	–	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 40295	C	–	C	A	–	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 40489	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 40357	C	–	C	A	–	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 40851	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 40271	C	–	C	A	**AT**	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 41097	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 41098	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 41100	C	–	C	A	–	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study
UT-Ziafati 9155	C	–	C	A	–	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	This study

Table 2. Genotype analyses of cyprinid herpesvirus 3 at the enlarged *SphI-5* and TK regions.

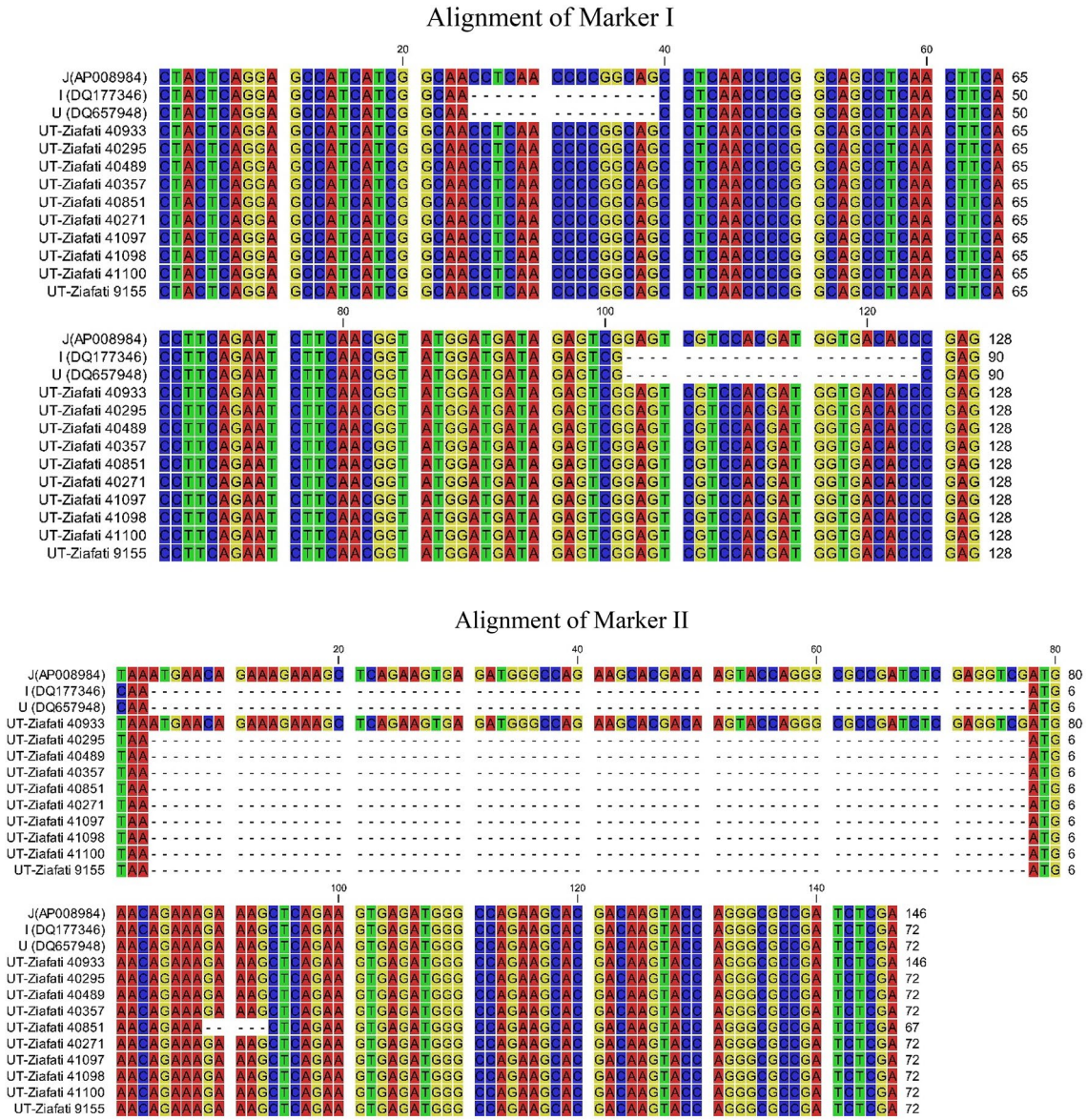

Table 3. Sequence alignment and analysis of cyprinid herpesvirus 3 genotypes focused on marker I and marker II.
Specific motifs for marker I and marker II were identified and highlighted.

Summary of genotyping
The merging of the results of both genotyping methods as explained by Bigrre et al. (2009) and Kurita et al. (2009) is shown in Table 4. Identification of UT -Ziafati 40933 samples was reported to have genotype A1 and J strain (I^{++} II $^{+}$). In addition, UT -Ziafati 40295, UT -Ziafati 40291 and UT -Ziafati 40357 samples were observed to have genotype A1 with intermediate strain (I^{++} II $^{-}$). Similarly, UT -Ziafati 40489, UT -Ziafati 9155, UT -Ziafati 41097 and UT -Ziafati 41098 samples were identified as intermediate strain of genotype A2 (I^{++} II $^{-}$). The UT-Ziafati 40851 sample of the intermediate strain of genotype A2 was identified with a new mutation(I^{++} II $^{-*}$) (Table 4). Furthermore, the isolate UT-Ziafati 40933 was found to have a T nucleotide at position 51 of the second marker, corresponding to the J strain. In contrast, other isolates in this study had the nucleotide C replaced by T at position 51, more similar to the J strain than to the U/I strain.

Phylogenetic analysis
The phylogenetic tree of koi herpesvirus (KHV) reveals the molecular relationship between viral strains from two geographical regions of carp fish farming in Iran and strains existing worldwide. The tree was constructed using the maximum likelihood method with the general time reversible model and 1000 bootstrap repeats based on the thymidine kinase gene using MEGA7 software (Fig. 4). The nucleotide similarity levels of the isolates in this study with those in Genbank are shown in Table 5. In the current study, *thymidin kinase* gene sequences were used to analyze strains with similarity level of 98–100% compared to Asian and European sequences. According

Strain	Alignment of Marker I
J(AP008984)	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
I (DQ177346)	CTACTCAGGAGCCATCATCGGCAA----------CCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCG----------CGAG
U (DQ657948)	CTACTCAGGAGCCATCATCGGCAA----------CCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCG----------CGAG
40933	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
40295	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
40489	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
40357	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
40851	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
40271	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
41097	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
41098	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
41100	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG
9155	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAG

Strain	Alignment of Marker II
J(AP008984)	TAAATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGAGGTCGATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGA TCTCGA
I (DQ177346)	CAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
U (DQ657948)	CAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
40933	TAAATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGAGGTCGATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGA TCTCGA
40295	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
40489	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
40357	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
40851	TAA----------ATGAACAGAAA-----CTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
40271	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
41097	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
41098	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
41100	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA
9155	TAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGA

Figure 3. (continued)

Name of isolate	MarkerI/II	Strain	Lineage TK gene and Enlarged SphI-5
J	$I^{++}II^{+}$	J	Variable
U/I	$I^{-}II^{-}$	U/I	Variable
Intermediate	$I^{++}II^{-}$	Intermediate	Variable
Intermediate*	$I^{++}I^{-*}$	Intermediate*	Variable
UT-Ziafati 40933	$I^{++}II^{+}$	J	A1
UT-Ziafati 40295	$I^{++}II^{-}$	Intermediate	A1
UT-Ziafati 40489	$I^{++}II^{-}$	Intermediate	A2
UT-Ziafati 40357	$I^{++}II^{-}$	Intermediate	A1
UT-Ziafati 40851	$I^{++}I^{-*}$	Intermediate*	A2
UT-Ziafati 40271	$I^{++}II^{-}$	Intermediate	A1
UT-Ziafati 41097	$I^{++}II^{-}$	Intermediate	A2
UT-Ziafati 41098	$I^{++}II^{-}$	Intermediate	A2
UT-Ziafati 41100	$I^{++}II^{-}$	Intermediate	A2

Table 4. Summary of genotyping cyprinid herpesvirus 3 based on marker I, marker II, *SphI-5*, and TK gene regions.

to the phylogenetic analysis results, the sequences from this study clustered within the branches of previously reported Asian and European strains, indicating their placement in the phylogenetic trees of Asia and Europe.

Histopathological analysis

Histopathological investigations of gills revealed fusion of secondary lamellae and clubbing of the gill filaments. Numerous necrotic respiratory epithelial cells (Degeneration with eosinophilic cytoplasm and pyknotic nuclei) and mild proliferative and lymphoplasmacytic branchitis were also observed (Fig. 5a–c). Microscopically, intranuclear inclusion bodies and marginated chromatin at the nuclear membrane were present (Fig. 5d).

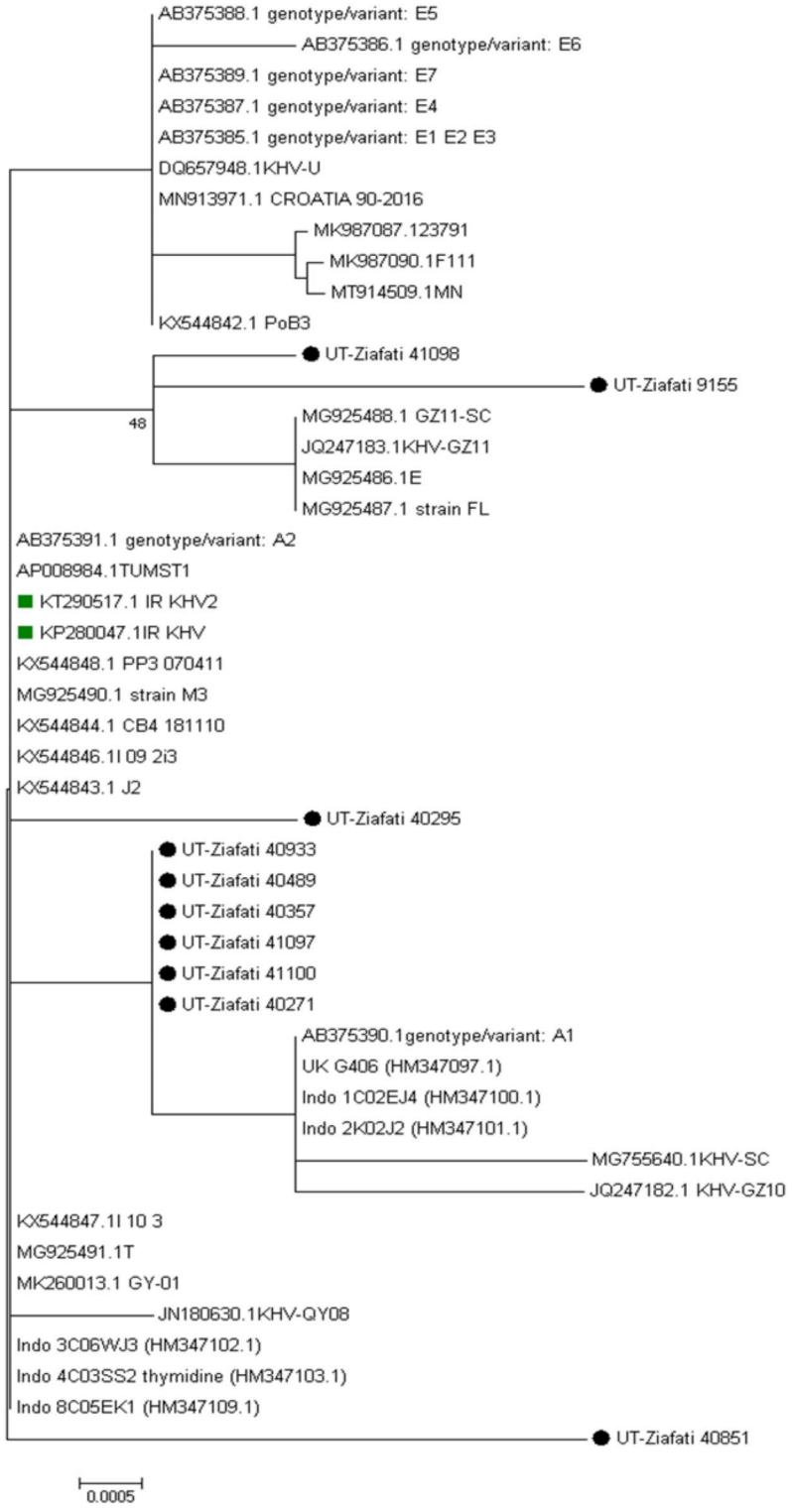

Fig. 4. A molecular phylogenetic analysis of Iranian Koi herpesvirus (KHV) was conducted using the maximum likelihood method, focusing on the partial TK gene. The evolutionary history was inferred using the General Time Reversible model and MEGA software version 7. The analysis generated a bootstrap consensus tree based on 1000 replicates, providing insights into the evolutionary relationships among the analyzed taxa. The branches of the tree are annotated with percentage values representing the clustering of associated taxa in the bootstrap test. The phylogenetic tree reveals the presence of three distinct genogroups. The dataset used in the analysis consisted of a total of 1001 positions. Iranian KHV isolates are denoted by black circles, and they were found in common carp hosts. The previous Iranian isolates are highlighted with a green square. The scale bar represents a rate of 0.050 substitutions per site.

	Isolate name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	UT-Ziafati_40933 (OQ437241)	######																					
2	UT-Ziafati_40295(OQ447505)	99.67																					
3	UT-Ziafati_40489(OQ447506)	100.00	99.67																				
4	UT-Ziafati_40357(OQ447507)	100.00	100.00	100.00																			
5	UT-Ziafati_40851(OQ447512)	99.67	99.33	99.67	99.88																		
6	UT-Ziafati_40271(OQ437242)	100.00	100.00	100.00	100.00	99.89																	
7	UT-Ziafati_41097(OQ447508)	100.00	99.67	100.00	100.00	99.67	100.00																
8	UT-Ziafati_41098(OQ447509)	99.67	99.67	99.67	99.88	99.33	99.89	99.67															
9	UT-Ziafati_41100(OQ447510)	100.00	99.67	100.00	100.00	99.67	100.00	100.00	99.67														
10	UT-Ziafati_9155(OQ447511)	99.44	99.33	99.44	99.54	99.10	99.54	99.44	99.55	99.44													
11	AB375390.1genotype/variant: _A1	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55												
12	AB375391.1_genotype/variant: _A2	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00											
13	MG925488.1_GZ11-SC	99.67	99.55	99.67	99.77	99.33	99.77	99.67	99.78	99.67	99.55	99.78	99.78										
14	KX544843.1_J2	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78									
15	MK260013.1_GY-01	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00								
16	MG925487.1_strain_FL	99.67	99.55	99.67	99.77	99.33	99.77	99.67	99.78	99.67	99.55	99.78	99.78	100.00	99.78	99.78							
17	MG925490.1_strain_M3	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78						
18	UK_G406_(HM347097.1)	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78	100.00					
19	Indo_3C06WJ3_(HM347102.1)	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78	100.00	100.00				
20	KP280047.1 IR_KHV	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78	100.00	100.00	100.00			
21	KT290517.1_IR_KHV2	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78	100.00	100.00	100.00	100.00		
22	AP008984.1 TUMST1	99.89	99.78	99.89	100.00	99.55	100.00	99.89	99.78	99.89	99.55	100.00	100.00	99.78	100.00	100.00	99.78	100.00	100.00	100.00	100.00	100.00	

Table 5. Genetic homology analysis was conducted on Iranian and other selected Koi herpesvirus (KHV) isolates, based on the *thymidine kinase* (TK) gene, using the maximum composite likelihood substitution model.

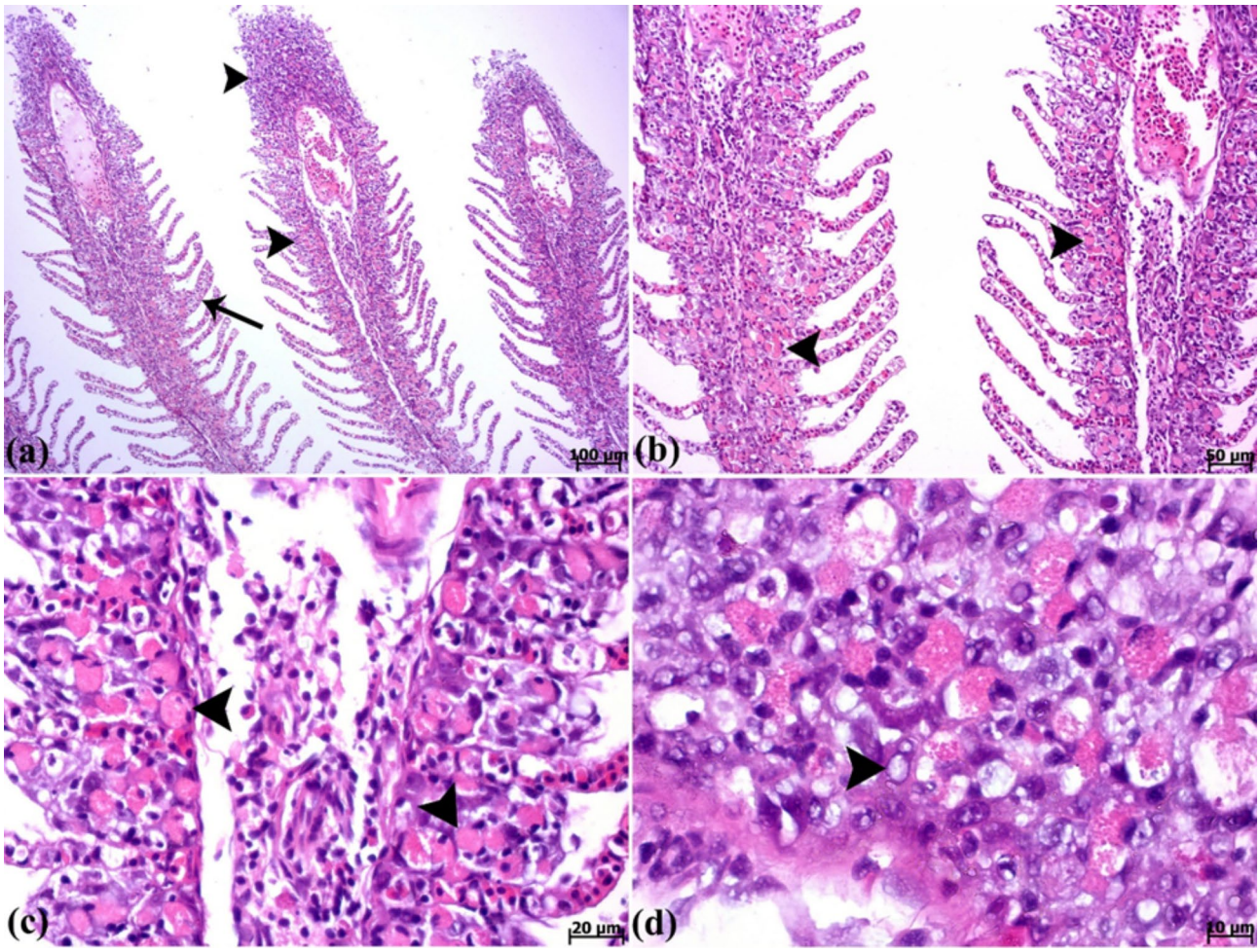

Fig. 5. (**a**–**d**) Microscopic findings in the gills of Koi herpesvirus (KHV)-infected carp (*Cyprinus carpio*). (**a**) Fusion of secondary lamellae (arrow) and lymphoplasmacytic branchitis (arrowheads). (**b**, **c**) Necrotic epithelial cells (arrowheads). (**d**) Intranuclear inclusion body (arrowhead) (H&E stain).

Discussion

Fish and fishery products are among the most traded food products in the world, with trade in fishery and aquaculture products showing remarkable growth on a global scale. Iran ranks 26th in the world in the fishing industry. Based on the available records of the Iranian Veterinary Organization, a significant and steady increase in carp mortality has been observed since the beginning of summer 2017. Between 2016 and 2019, a large number of carp mortality reports were reported in the provinces of Gilan, Mazandaran, and Khuzestan. Koi herpesvirus in koi fish from Iran was first reported by Rahmati et al.[13]. Ahmadivand et al. (2020) later reported the occurrence of koi herpesvirus in carp farms in Alborz, Tehran and, Khuzestan provinces, and Taheri Mirghaed et al. 2019 confirmed their findings. Between 2020 and 2023, disease outbreaks with fatalities occurred in carp farms in various parts of the country. The investigation of three environmental factors raised suspicion of viral diseases such as CEV, SVC, or KHV. The first factor was the mortality rate, which ranged between 40% and 100%. The second reason was the season in which the epidemics occurred: Spring and fall, when the water temperature was between 16 and 28 °C, the optimal temperature for CyHV-3 pathogenicity[30,31]. The third factor was the host-specific nature of the epidemic: in some affected farms, other carp species besides common carp were cultured, including *Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix* and, *Ctenopharyngodon idella*, but none of these species showed clinical signs of the disease or mortality[32].

In addition to the environmental factors, the clinical signs raised suspicion of a viral disease. Affected carp showed increased mucus secretion on the skin and, in many cases, slight gill necrosis, which occurs in CEV, SVC, or KHV infections[33,34].

Therefore, we attempted to detect CEV, SVC, or KHV in our samples. CEV and KHV were detected in the tested farms. Since the data on detected CEV were reported in our previous study[29], this paper presents the obtained results on molecular diagnosis, genetic analysis, histopathology, and phylogenetic analysis of the identified KHVs.

In this study, viral DNA was detected in both WOAH most recommended tissues including the gills and kidneys, and also other tissues such as the intestines, spleen, and ovaries in some cases[23]. The pathological

findings of this study, including necrotic respiratory epithelial and lymphoplasmacytic branchitis were in accordance with previous studies[12,35].

The definitive diagnosis of KHV is based on identifying viral DNA or isolating and characterizing the virus using immunological and molecular techniques. Among all the methods recommended by WOAH[23], real-time PCR, conventional PCR, and nested PCR are the most accurate and sensitive assays for both monitoring and confirming the diagnosis. These methods efficiently detect the virus in the early life stages of fish, as well as in juvenile and adult stages[3,36,37]. In this study, the *thymidine kinase* (TK) gene or ORF55 was used as a specific genetic marker for identifying koi herpesvirus. Another important gene is DNA polymerase, which affects the severity of the virus, clinical symptoms and mortality[34]. Moreover, this gene belongs to the early or immediate early genes that are transcribed within 2-4 h after infection[38]. There is a considerable number of the submitted DNA pol partial gene sequences of herpesviruses, which is an ideal target for conventional PCR for large DNA viruses[16,17].We targeted both TK and *DNA polymerase* genes, and the results of comparative genome analysis showed that the thymidine kinase sequence is completely specific and unique to koi herpesvirus (CyHV-3), which aligns with previous reports[25,30].

When analysing the polymorphism patterns in the TK and *SphI-5* genes, the KHV samples could be broadly classified into two groups: Asian and European genotypes. The Asian genotype contained two variants, while the European genotype had seven variants[27]. Accordingly, two Asian genotypes were identified in the present study. In addition, we discovered a novel Asian variant (UT -Ziafati40271) that is generally similar to genotype A1, except for two nucleotides at positions 813–814 (Table 2).

Based on variations in three variable regions within two genetic markers, five genotypes, including lines U/I ($I^{-}II^{-}$), J (I^{++} II^{+}), the third genotype (I^{-+} II^{-}), an intermediate genotype (I^{++} II^{-}) and the fifth genotype in which marker I occurred as I^{++} and marker II had a 13-bp deletion[20–22]. The discovery of the fifth strain shows that this genotype represents another new variant of CyHV-3. Our research results revealed the existence of a sixth strain, designated I^{++} II^{-*}, which differs from the known intermediate genotype. Our strain (UT-Ziafati 40851) has a 5-bp deletion (GAAAG) in the allele of marker II. Remarkably, this study is the first to describe a deletion in the second tandem repeat motif of the II marker. The exact evolutionary event that led to this duplication or deletion of the II marker motif in the virus remains unknown[20,22].

Moreover, in all samples in which marker II was sequenced, the first nucleotide is a T (TAA), whereas in the established is a C (CAA) (Table 3).

We concluded that, out of four genotype A1 viruses detected in our study, three were intermediate strains, and one was a novel intermediate strain (I + + II-*). On the other hand, all five genotype A2 viruses were classified as intermediate strains (Tables 3 and 4). Although the genotypes were Asian, the sequence of markers indicated the predominance of intermediate strains. Differences caused by various classification methods have already been reported. In 2017, Wang et al. reported the occurrence of the GY-01 strain in carp during an epidemic. Research comparing the TK gene of KHV strains revealed that strain GY01 was initially considered close to the Asian lineage. However, Duan et al. 2020 found that GY01 was more closely related to the European lineages KHV E and KHV-I based on full-genome analysis. This study suggested that GY01 may be descended from the same ancestor as KHV E and KHV-I[39].

In our phylogenetic tree based on TK genes, sequences from Asia and Europe were used for comparison. Eleven whole-genome sequences from seven geographic regions from China (GZ11, GZ11-SC), Israel (I, Cavoy), Belgium (FL, M3), the United Kingdom (E), the United States (U) and Japan (J) were included. T, J, and M3 were assigned to the Asian lineage, while the remaining seven strains, including KHV-U, E, I, KHV-I, FL, Cavoy, GZ11, and GZ11-SC, were classified as European lineages[40]. It has been established that our strains UT -Ziafati 9155 and UT -Ziafati 41098 have a common ancestry with the European strains FL and GZ11. Seven other strains were positioned as intermediaries between the common Asian and European lineages. The last strain, UT-Ziafati 40851, was located at a considerable distance from the other strains in this work.

Genetic analyses using markers I and II confirmed the full TK-based phylogenetic results, which were not entirely consistent with the analysis of the polymorphism patterns of the TK and *SphI-5* genes. The results emphasize the importance of exercising caution when making evolutionary predictions based on specific short regions or limited genomic data, as they may lead to conclusions different from those of analyses using large numbers of whole genomes. Therefore, it is crucial to consider multiple comparisons and perform thorough phylogenetic analyses to make accurate evolutionary inferences about a particular strain.

Conclusions

The global distribution of CyHV-3 genotypes is not yet fully understood. The results of our study show a high degree of diversification of CyHV-3 in Iran as part of the Asian regions, where at least three different strains (I^{++} II^{+}, I^{++} II^{-} and $I^{++}I^{-*}$) are currently circulating. As the trade in ornamental fish leads to increased animal movements in Asia, further variants of CyHV-3 will likely emerge. Continuous surveillance, strict biosecurity, and quarantine measures are crucial to control the spread of this economically important pathogen. Finally, our study data will contribute to understanding the global geographic distribution of CyHV-3 genotypes, focusing on the West-East Asian regions.

Data availability

The datasets generated and/or analyzed during the current study are available in the NCBI database with the accession numbers provided in Table 1.

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Author contributions

Z.Z: Methodology, Data analysis and Wrote the MS text, A.GH.: Study design, Supervision, M.A: Advisor, H.R: Methodology, S. Sh: Methodology, H.N: Wrote the MS text, supervision. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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