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Carp edema virus in Ukraine – The evidence for the furthest east presence of CEV genogroup I in Europe

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ABSTRACT

Carp edema virus (CEV) is a poxvirus infecting gills of common carp (*Cyprinus carpio*) and causes a deadly disease called koi sleepy disease (KSD). Currently thanks to higher vigilance and screening for its presence the virus was confirmed to be widespread on the European continent and affects both ornamental koi, farmed and feral common carp. Phylogenetic research of CEV is however in its infancy. Until recently due to the lack of available sequence information, the phylogenetic analysis was limited to a *p4a* gene. Based on this gene two main clades were established. The genogroup I clade is of European origin and can be found in farmed or feral common carp in Europe. However, the eastern border of the geographic range of CEV from genogroup I is not yet established, therefore we decided to obtain samples from clinically affected carp from Ukraine. Furthermore, we used sequences of the DNA binding viral core protein 8 (VP8) and of the uracil DNA glycosylase (UDGS) to confirm the existence of the genogroups I and II. The results indicate that CEV is highly prevalent in Ukraine. Phylogenetic analysis using the *p4a* gene showed that common carps in Ukraine are infected with CEV genogroup I. The two additional genes used for the phylogenetic analysis supported the phylogenetic findings. This confirms that common carps in continental Europe are hosts for CEV from the genogroup I and the geographic range for this virus is reaching to the most eastern parts of Ukraine.

1. Introduction

Carp edema virus (CEV) is a poxvirus infecting the gills of common carp and it causes a deadly disease called koi sleepy disease (KSD). This disease is associated with mass mortalities of farmed common carp (*Cyprinus carpio*) and the ornamental variety of carp, the koi. Affected specimens develop clinical signs including extreme lethargy, coma like behaviour and a rapid onset of mortality, which are related to the gill damage induced by the infection and which lead to hyponatremia and hyperammonemia (Adamek et al., 2021). Historically, the virus was detected in Japan in the 1970-ies (Murakami et al., 1976) and the initial spread of the virus seemed to be very slow and was contained within this country. Some 20 years post its initial detection in Japan, the virus spread to the USA where it was detected in koi populations (Hedrick et al., 1997). Fuelled by the international trade the disease (re)-emerged into prominence worldwide in the 2010-ies when new detection methods for the virus were developed (Way et al., 2017). At the same time a new variant of the virus was detected affecting feral and farmed common carp populations in Europe (Way et al., 2017). Currently thanks to higher vigilance and screening for its presence, both variants of the virus were confirmed to be widespread on the European continent and affects both ornamental koi, farmed and feral common carp (Adamek et al., 2021; Way et al., 2017).

Phylogenetic research of CEV is in its infancy. Until recently due to the lack of available sequence information, the phylogenetic work was limited to a small fragment of the *p4a* gene (Matras et al., 2017). Still, this fragment was providing a quite high resolution and allowed the detection of two to three distinct clades of CEV depending on the analysis (Baud et al., 2021; Matras et al., 2017). The main clades are robustly established and most likely reflect an initial geographic separation of the virus variants (Zrnčić et al., 2020). The genogroup I clade is of European origin and can be found in farmed or feral common carp all over Europe. The genogroup II clade has up to 9% differences in the *p4a* gene and is of Asian origin (Way et al., 2017). The virus from the

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genogroup II can be mostly found in koi. However, due to the fact that koi are traded globally and the main trading roads are emerging from Japan, the viruses from genogroup II can be detected all over the world (Way et al., 2017). Viruses from the genogroup I have a more limited geographic range and can be located in Europe as far east as Poland (Matras et al., 2017) and in UK and France as the furthest west (Baud et al., 2021; Way et al., 2017). In Western Asia, the virus from this genogroup can be found as far as in Iran (Ziafati Kafi et al., 2022). Interestingly the genogroup I has also a stronghold in invasive common carp in USA where it was most likely introduced together with the introduction of this fish (Lovy et al., 2018; Tolo et al., 2021).

As the eastern border of the geographic range of CEV from genogroup I is not yet established, we decided to obtain samples from clinically affected carp from Ukraine to answer the question of how far east in Europe the genogroup I has spread. Is it present in further eastern parts of Ukraine – the Ukrainian Donbas region? Secondly, while analysing the phylogeny of CEV, we propose the use of additional two genes to supplement the *p4a* analyses used routinely. Here we use sequences of the DNA binding viral core protein 8 (VP8) and of the uracil DNA glycosylase (UDGS) to confirm the existence of the genogroups I and II based on an analysis of different parts of the CEV genome.

2. Materials and methods

2.1. Samples

In 2013–2021, gills, kidney and spleen were collected from common carp (*Cyprinus carpio*), bighead carp (*Hypophthalmichthys nobilis*), and silver carp (*Hypophthalmichthys molitrix*) cultured in Ukraine experiencing diverse health issues (Table 1). The samples were pooled from up to 10 fish per case and homogenised thoroughly. The samples were examined for the presence of CEV and cyprinid herpesvirus 3 (CyHV-3) specific sequences by molecular analysis as described earlier (Adamek et al., 2019; Zrnčić et al., 2020). The detection of spring viremia of carp virus (SVCV) was performed by cell culturing using epithelioma papulosum cyprini (EPC) cells and subsequent confirmation with anti-SVCV monoclonal antibody (BIO 331, Biox Diagnostics, Belgium).

2.2. DNA extraction and PCRs

Genomic DNA was extracted with QIAgen DNA Mini Kit (QIAGEN) according to the manufacture's instruction for extraction of DNA from tissues. For detection of CEV and CyHV-3 presence in the samples, a quantitative PCR was performed with the Maxima Probe qPCR Master Mix. The reaction mix contained 1 × Maxima Probe qPCR Mastermix (Thermo Fisher Scientific), 500 nM of each primer, 200 nM of the probe (sequences indicated in Table 2), 5 µl of template DNA and nuclease-free water to a final volume of 20 µl. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s. A standard curve from 10^1 to 10^7 copies of the CEV p4a gene and CyHV-3 ORF89 were prepared as described earlier (Adamek et al., 2019; Zrnčić et al., 2020) and used for quantification of the copy number of CEV or CyHV-3 specific DNA in each sample. The results for virus load are presented as the number of virus specific DNA copies per 250 ng of total DNA. To obtain PCR products for sequencing and phylogeny, an end-point PCR was performed with the KAPA2G Robust HotStart PCR kit (Merck) according to the manufacturer's description using Buffer A. Primers (sequences indicated in Table 2) were designed based on CEV genogroup II genome sequence available under GenBank ID LC613089 (Mekata et al., 2021) and additional sequences from German isolates of CEV from genogroup I obtained by primer waking. The PCR reaction mix contained following: 1 × KAPA2G Buffer A, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 U of KAPA2G Robust HotStart Polymerase, 5.0 µl of DNA (50 ng/µl) and nuclease-free water to a final volume of 20 µl. PCR program included an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. PCR products were directly sequenced by LGC Genomic, Berlin, Germany. Primers used are indicated in the Table 2.

2.3. Phylogenetic analysis

DNA sequences were analysed and aligned using BioEdit 7.2 (Hall, 1999). The phylogenetic analysis of sequences was performed with the package available at http://phylogeny.lirmm.fr (Dereeper et al., 2008). For the *p4a* gene the analysis was performed on the 357 bp long fragment used in earlier studies (Zrnčić et al., 2020). For the gene encoding for uracil DNA glycosylase (UDGS) a 414 bp fragment was used spanning between nucleotide 87 and 500 of the gene encoded by 687 bp. For the gene encoding for DNA binding viral core protein 8 (VP8) a 512 bp fragment was used spanning between nucleotides 179 and 691 the gene encoded by 798 bp.

3. Results & discussion

CEV-specific DNA sequences were detected in 31 out of 43 samples examined (Table 1). The geographical distribution of the sample locations is presented in the Fig. 1. The fish from following regions were tested CEV positive: Cherkasy region, Donetsk region, Kharkiv region, Kherson region, Khmelnitsky region, Kyiv region, Lviv region, Sumy region and Zakarpatya region. In all CEV positive samples, the virus loads ranged from 1 to 9019 copies per 250 ng of extracted DNA, which is considered rather low (Table 1). In only one sample the virus load approached 1 × 10^4 genome copies, which is considered as crude "cut-off point" for clinical signs presentation and disease development based on experimental infections and field observations (Adamek et al., 2019; Adamek et al., 2021). Although it has to be noted that organ pools were used for DNA extraction, while the virus mainly infects gills (Adamek et al., 2017; Adamek et al., 2021). This could explain the low abundance of CEV specific DNA in the examined samples. Nonetheless, this provides only a weak association with the mortality recorded during the sampling, especially when the virus was detected also in cases where no clinical signs were recorded (Table 1). Some mortality could be related with detected spring viremia of carp virus (SVCV), saprolegnia or bacterial infections as well as interaction of multiple pathogens (Table 1). Unfortunately, due to scarcity of archived material the test for cyprinid herpesvirus 3 (CyHV-3) could not be performed in all samples. The selected few samples were tested negative for CyHV-3. Admissibly, CyHV-3 could be responsible for high mortalities in European aquaculture (Zrnčić et al., 2020) and a lack of comprehensive testing for this virus does not allow us to fully pinpoint the pathobiome behind mortality events in common carp in Ukraine (Bass et al., 2019).

Initially the sequencing of a 357 bp *p4a* gene fragment showed that phylogenetically most of the Ukrainian CEV samples from common carp clearly belong to the genogroup I and they resemble similarity to the German isolates used for comparison (Fig. 2). There were however a few samples which belong to the genogroup IIb, which is commonly recognised as a mixed group which could have emerged by a recombination of the two main genogroups (Matras et al., 2017). For the phylogenetic analysis of the virus isolates, in addition to the gene encoding the P4a protein, two new target genes (*vp8* and *udgs)* were used. Using these genes gave a similar separation of the samples from koi and common carp as the tree constructed using the *p4a* sequence information. However, due to low viral load we were not able to obtain PCR products from the additional genes for all samples from Ukraine (Fig. 2). Both *vp8* and *udgs* based phylogeny indicated the presence of two clear clades, which confirms that the genetic diversity between the genogroups I and II is not only limited to the *p4a* gene. Also combining all sequences together in one analysis led to similar results with it seems increased resolution of analysis (Fig. S1). The similar results can be related with the fact that for the phylogenetic analysis of CEV we used gene fragments encoding

Table 1
Detailed description of the fish samplings collected in 2013–2021 from common carp (*Cyprinus carpio*), bighead carp (*Hypophthalmichthys nobilis*), and silver carp (*Hypophthalmichthys molitrix*) with diverse health issues cultured in Ukraine. The virus detection was performed using pooled organ samples of gills, kidney and spleen. Stars indicate the same farm sampled at different time points.

Sampling N№	Fish farm type / Region	Date of sample collection	Fish specie	Fish age, years	Nuumber of fish evaluated (sampled)	Clinical signs	Mortality / morbidity, %	CEV PCR 1st step	CEV PCR 2nd step	CEV qPCR (copies)	Sequencing *p4a/vp8/ udgs*	Presence of other viruses
UKR1	Selection center, Donetsk region* *	05/2013	Carp	0 +	29	SVCV signs	65–70	-	+	2	G_I /na/na	SVCV
UKR2	Selection center, Donetsk region* *	07/2013	Bighead carp	2 +	10	Bacterial infection	15–20	-	+	10	G_IIb/na/ na	No
UKR3	Selection center, Donetsk region* *	07/2013	Silver carp	2 +	10	Bacterial infection	15–20	-	+	< 1	G_IIb/na/ na	No
UKR4	Production center, Kharkiv region	09/2013	Bighead carp	2 +	11	Bacterial infection	20–25	-	+	< 1	G_I/na/na	No
UKR5	Selection center, Kherson region	07/2018	Carp	3 +	10	Lethargy, pale gills, skin lesions	30–40	-	+	< 1	G_IIb/na/ na	No
UKR6	Selection center, Khmelnitsky region	08/2018	Carp	0 +	20	Lethargy	30–35	-	+	9	G_I/na/na	No
UKR7	Fish farm, Odesa region	09/2018	Carp	0 +	15	No	0–5	-	-	Not tested	No	No
UKR8	Fish farm, Lviv region	09/2018	Carp	1 +	10	No	0–5	-	-	Not tested	No	No
UKR9	Fish farm, Lviv region	06/2019	Carp	4 +	5	Saprolegnia	15–20	-	+	3	G_I/na/na	No
UKR10	Fish farm, Lviv region	06/2019	Carp	0 +	10	No	0–5	-	-	Not tested	No	No
UKR11	Selection center, Kyiv region	06/2019	Carp	3 +	6	No	15–20	-	+	17	G_I/na/na	No
UKR12	Selection center, Kyiv region	06/2019	Carp	1 +	10	Lethargy	30–35	-	+	5	G_I/na/na	No
UKR13	Fishing center, Kyiv region	06/2019	Carp	3 +	3	No	15–20	-	+	3	G_I/na/na	No
UKR14	Selection center, Sumy region	06/2019	Carp	2 +	5	No	0–5	-	+	< 1	G_IIb/na/ na	No
UKR15	Fish farm, Sumy region*	06/2019	Carp	2 +	5	No	0–5	-	+	< 1	G_IIb/na/ na	No
UKR16	Fish farm, Kherson region	05/2020	Carp	3 +	7	No	0–5	-	+	1	G_I/na/na	No
UKR17	Fish farm, Kyiv region	05/2020	Carp	1 +	10	Lethargy	10–15	-	+	< 1	G_IIb/na/ na	No
UKR18	Fish farm, Kyiv region	06/2020	Carp	1 +	5	No	0–5	-	+	< 1	G_IIb/na/ na	No
UKR19	Fish farm, Cherkasy region	06/2020	Carp	1 +	10	Lethargy	15–20	-	+	909	G_I/na/na	No
UKR20	Selection center, Volyn region	06/2020	Carp	1 +	10	No	0–5	-	-	Not tested	No	No
UKR21	Fish farm, Odesa region	09/2020	Carp	1 +	10	No	0–5	-	-	Not tested	No	No
UKR22	Selection center, Chernigiv region	09/2020	Carp	2 +	5	No	0–5	-	-	Not tested	No	No
UKR23	Fish farm, Lviv region	10/2020	Carp	0 +	20	No	0–5	-	-	Not tested	No	No
UKR24 (1–10)	Fish farm, Zakarpatya region	10/2020	Carp	0 + , 1 +	10	Lethargy, pale gills, skin lesions	50	+	+	2–9019	G_I/G_I/G_I	No
UKR25	Fish farm, Zakarpatya region	10/2020	Silver carp	0 +	10	No	10–15	-	+	Not tested	No	No
UKR26	Fish farm, Rivne region	10/2020	Carp	1 +	13	No	0–5	-	-	Not tested	No	No
UKR27		04/2021	Carp	1 +	10		20–25	-	+		No	SVCV

(*continued on next page*)

Table 1 (*continued*)

Sampling N№	Fish farm type / Region	Date of sample collection	Fish specie	Fish age, years	Nuumber of fish evaluated (sampled)	Clinical signs	Mortality / morbidity, %	CEV PCR 1st step	CEV PCR 2nd step	CEV qPCR (copies)	Sequencing *p4a/vp8/ udgs*	Presence of other viruses
	Fish farm, Cherkasy region					Lethargy, pale gills, skin lesions				Not tested		
UKR28	Fish farm (fishing centre), Kyiv region	06/2021	Carp	5 +	5	Lethargy	15–20	-	+	Not tested	No	No
UKR29	Fish farm, Sumy region*	06/2021	Carp	2 +	8	No	0–5	-	+	3	G_I/na/na	No
UKR30	Fish farm, Kharkiv region	10/2021	Carp	0 +	15	No	0–5	-	-	Not tested	No	No
UKR31	Selection center, Poltava region	10/2021	Carp	0 +	20	No	0–5	-	-	Not tested	No	No
UKR32	Fish farm, Lviv region	10/2021	Carp	0 +	15	No	0–5	-	-	Not tested	No	No
UKR33	Fish farm, Stara Syniava district, Khmelnitsky region	02/2022	Carp	1 +	10	Gill necrosis, skin lesions	15–20	-	-	Not tested	No	SVCV
UKR34	Fish farm, Medzhibizh district, Khmelnitsky region	02/2022	Carp	1 +	12	Gill necrosis, skin lesions	15–20	-	-	Not tested	No	SVCV

Table 2
Primer sequences used for detection and phylogenetic analysis of CEV and CyHV-3 in Ukraine.

PCR name	Oligo name	Gene	Sequence (5′−3′)	Product size (bp)
CEFAS end-point	CEFAS_F	*p4a*	ATGGAGTATCCAAAGTACTTAG	528
	CEFAS_R		CTCTTCACTATTGTGACTTTG	
CEFAS nested end-point	CEFAS_nF	*p4a*	GTTATCAATGAAATTTGTGTATTG	478
	CEFAS_nR		TAGCAAAGTACTACCTCATCC	
CEFAS probe qPCR	CEFAS_qF	*p4a*	AGTTTTGTAKATTGTAGCATTTCC	76
	CEFAS_qR		GATTCCTCAAGGAGTTDCAGTAAA	
	CEFAS_q_Probe		[FAM] AGAGTTTGTTTCTTGCCATACAAACT [BHQ1]	
CEV_VP8	CEV_VP8_F1	*vp8*	AATTGTTGGAGGCGAAAGAA	628
	CEV_VP8_R1		TCCAGCTTTACTGGCGAATG	
CEV_UDGS	CEV_UDGS_F2	*udgs*	GATTGGGAACCTATAATGAATGTAGT	495
	CEV_UDGS_R2		RATGAGAACAGTRSTTTTTAAATCT	
CyHV-3 (ORF89)	KHV-86 F	ORF89	GACGCCGGAGACCTTGTG	78
	KHV-163R		CGGGTTCTTATTTTTGTCCTTGTT	
	KHV-probe		[FAM] CTTCCTCTGCTCGGCGAGCACG [BHQ1]	

important proteins for viral replication, P4a, VP8 and UDGS (Gubser et al., 2004). The DNA binding viral core protein VP8 is responsible for the formation of transcriptionally competent viral particles by binding and allowing the packaging of the viral nucleic acids (Bayliss, Smith, 1997). Thus, it seems to be essential for poxvirus replication and therefore mutations within this protein should be tightly controlled. Also, the second new target uracil DNA glycosylase (UDGS) is essential for poxvirus replication (Millns et al., 1994) and mutations in the gene encoding this protein makes the vaccinia virus unable to replicate DNA (Millns et al., 1994). Nevertheless, a multiple alignments analysis showed that in CEV both genes have a high ratio of mutations with 12.3% possible nucleotide bases locations with mutations for *vp8* and 10.8% for *udgs*, which is similar to the 10.1% for *p4a*. This could confirm a relatively high mutation rate in the whole CEV genome. Interestingly, the mutation rate of poxviruses is quite high for DNA viruses and is estimated at 4×10^{-6} substitutions/site/year (Hughes et al., 2010) while for example other large DNA genome possessing alloherpesviruses evolve slower with from 1×10^{-7} to 6×10^{-9} substitutions/site/year depending on the virus and estimation procedure (Donohoe et al., 2021).

Using the different phylogenetic target genes, we could conclude that Ukrainian samples were very similar to samples from other European countries like Germany, Poland or Hungary. It should be noted that the genetic differences in CEV, which separate the isolates into two genogroups, finds also some reflection in differences in virulence towards different strains of common carp. Common carp of an Asian strain, originating from the Amur river, seems to be more resistant to the infection with both genogroups (Adamek et al., 2017). The host species *Cyprinus carpio* is naturally distributed in waters in Eastern Europe and western Asia in rivers draining to the Black, Caspian and Aral Seas (Kottelat and Freyhof, 2008). The carp from Eastern and South-East Asia, which previously were also identified as *C. carpio,* were recently identified as genetically different from the European population (Dong et al., 2015; Kohlmann and Kersten, 2013) and recognised as *C. rubrofuscus* (Kottelat, 2006). Fossil and genetic analyses support an emergence of common carp in East Asia (Mabuchi et al., 2006), from where it spread to Eurasia (Kohlmann and Kersten, 2013). However, the western populations in the Danube and Don Rivers were separated from eastern populations in the Amur drainage basin and China during multiple Pleistocene glaciations (Berg, 1949). The genetic analysis of common carp from several European and Central Asian locations, including the Ukraine, revealed a close relationship between these haplotypes

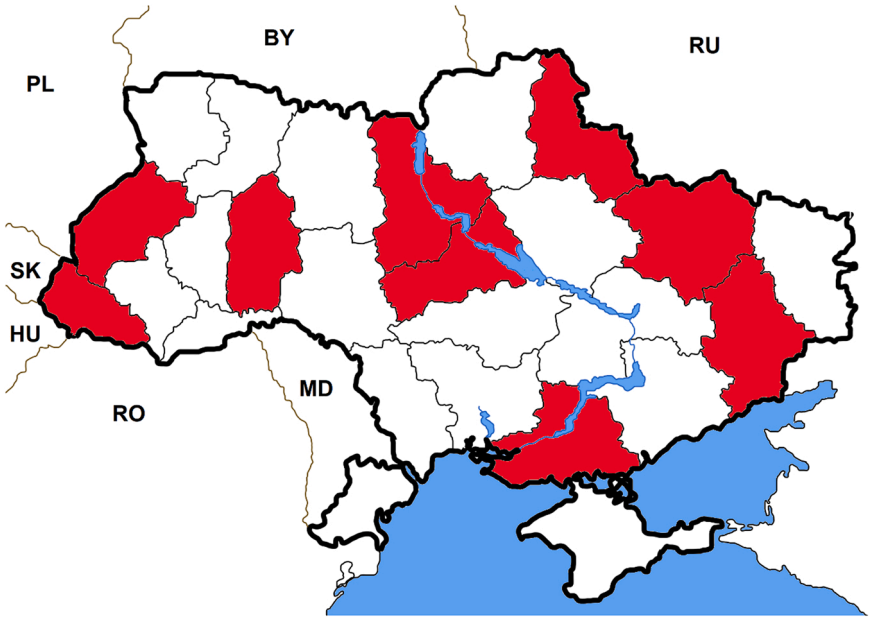

Fig. 1. The map of the Ukrainian regions where the CEV positive samples were collected. Red shedding indicates regions which were tested positive for CEV: Cherkasy region, Donetsk region, Kharkiv region, Kherson region, Khmelnitsky region, Kyiv region, Lviv region, Sumy region and Zakarpatya region. MD – Moldova, RO – Romania, HU – Hungary, SK – Slovakia, PL – Poland, BY – Belarus, RU – Russia.

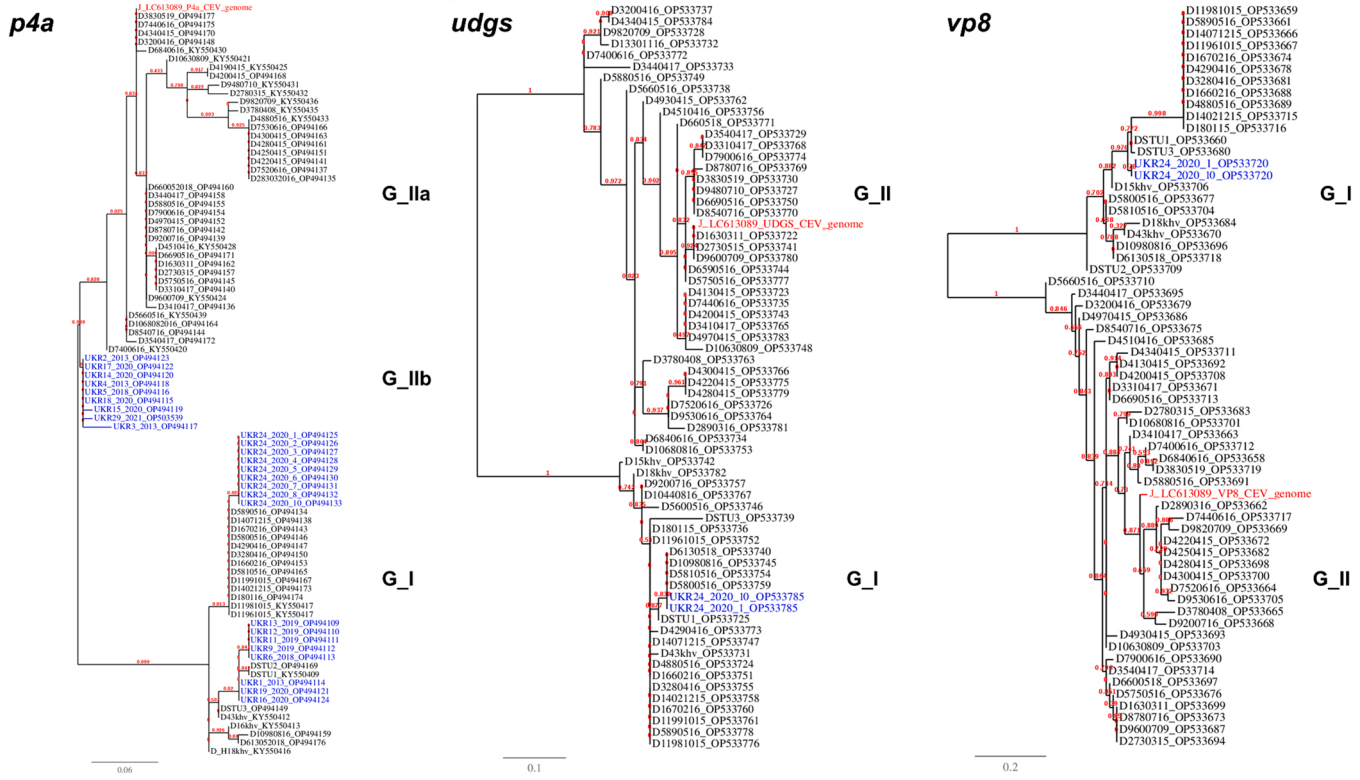

Fig. 2. Phylogenetic (PhyML, http://phylogeny.lirmm.fr) analysis of Ukrainian (indicated in blue) and German (indicated in black) isolates of CEV using *p4a*, *vp8* and *udgs*. The red colour indicates the sequences extracted from the full genome obtained for CEV genogroup II in Japan (Mekata et al., 2021). Leaf (isolates) names indicate the country of origin (UKR - Ukraine, D – Germany, J – Japan), followed by the isolate number and GenBank ID. G_I indicates genogroup I, G_II indicates genogroup II. The branch length is proportional to the number of substitutions per site. The branch supporting values are indicated in red numbers next to the branch.

from the European carp clade, which was also present in rivers in Uzbekistan (Kohlmann and Kersten, 2013). Currently, CEV from the genogroup I was mainly detected in European carp populations, including the Ukraine, but also in carp from Iranian waters connected to the Caspian sea (Ziafati Kafi et al., 2022). The distribution of CEV of genogroup I thus coincides with the distribution of European common carp, suggesting a co-evolution of the virus of this genogroup with this carp variety.

The recognition of the geographic border between the different virus genogroups can help explaining the evolution of the virus and its

emergence despite the effects of the global mobility of the host (Pybus et al., 2015). Remarkably, even at its most eastern border in Iran the isolates from genogroup I had significant genetic differences depending on the location the virus was detected. In the Khuzestan Province at the Persian Gulf isolates were significantly different that in the Gilan and Mazandaran Provinces near the Caspian Sea (Ziafati Kafi et al., 2022). The viruses at these provinces were more similar to European isolates. The results linking these isolates with others from carp producing countries like Germany, Hungary or Poland indicate that the intra-continental trade is facilitating the spread of the virus from the genogroup I.

4. Conclusions

The common carps in continental Europe aquaculture are hosts for the CEV from the genogroup I and the geographic range for this virus is reaching to the most eastern parts of Ukraine. Based on the current biogeographic findings, we hypothesise that CEV genogroup I co-evolved with the western European/ central Asian variety of common carp and can be found in populations of this carp variety. The two additional genes used in the current work for the phylogenetic analysis support the previous phylogenetic findings and could be used for a global phylogeny of CEV.

CRediT authorship contribution statement

Yuriy Rud, Olga Zaloilo, Leonid Buchatsky collected the samples, clinical description of the cases and run initial diagnostics. Marek Matras, Dieter Steinhagen, Mikolaj Adamek re-examine the samples. Mikolaj Adamek performed the phylogenetic analysis. Yuriy Rud, Dieter Steinhagen and Mikolaj Adamek wrote the initial version of the manuscript. All authors edited and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101500.

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