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Continent-Wide Distribution of CMTV-Like Ranavirus, from the Urals to the Atlantic Ocean

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Abstract: Ranavirosis is an emerging infectious disease of amphibians, fish, and reptiles caused by large dsDNA viruses of the genus *Ranavirus* associated with morbidity and mass mortalities worldwide. They are considered to be one of the major drivers of the ongoing amphibian biodiversity crisis. In this study, we investigated the prevalence and genetic diversity of ranaviruses in native and invasive populations of water frogs (*Pelophylax* spp.) across Russia using the DNA sample collection established in 2006–2016. The collection included samples collected in the wild and samples from wild-caught water frogs that had been kept in laboratories for a period of time. Overall, 52 out of 590 (8.8%) of wild frogs from 18 out of 94 (19.1%) sampling sites tested positive, including samples from invasive populations. Among the captive frogs, 71 out of 263 (27.0%) were positive and they had a significantly higher relative viral load. We found six major capsid

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protein gene haplotypes from 22 positive samples, all belonging to the common midwife toad virus (CMTV-like) ranaviruses, at multiple sites within the basins of three of Europe's largest rivers (Volga, Dnieper, and Don). Combined with previously published data, this study provides evidence for a continent-wide distribution of CMTV-like ranaviruses in Europe and strengthens the hypothesis of their endemism on the continent. Our study also highlights that the water frogs are important hosts for ranaviruses and could potentially act as vectors for infection transmission.

Keywords: *Iridoviridae*, alien species, major capsid protein, ranidae, East Europe, emerging disease

INTRODUCTION

The genus *Ranavirus* (*Iridoviridae*) includes large icosahedral double stranded DNA viruses which infect ectothermic vertebrates (bony fish, amphibians and reptiles) (Chinchar, 2002). They have been found on all continents except Antarctica (Brunner et al., 2021). Ranaviruses are considered as emerging pathogens and are an important driver of the current amphibian biodiversity crisis together with the *Batrachochytrium dendrobatidis* and *B. salamandrivorans* fungi (Lips, 2016; Campbell et al., 2018). Numerous cases of morbidity and mass mortality associated with ranaviral infection have been reported both in captivity and in the wild (e.g., Greer et al., 2005; Pasmans et al., 2008; Price et al., 2014; Stark et al., 2014; Butkus et al., 2017; Park et al., 2017). The symptoms include lethargy, anorexia, skin hemorrhages and ulcers, loss of buoyancy and erratic swimming (Miller et al., 2015).

However, the ranaviral infection does not necessarily lead to visible symptoms or death. A number of recent studies found notable ranavirus prevalence in wild populations of various hosts species without signs of clinical disease or mass mortality, as reported for Eastern Europe (Vörös et al., 2020; Palomar et al., 2021), Brazil (Ruggeri et al., 2019, 2023), China (Xu et al., 2010; Herath et al., 2023), South Korea (Roh et al., 2022) and Australia (Wynne 2019; Maclaine et al., 2020). This raises the question of how long the virus has been circulating in ectothermic vertebrate populations and which species or lineages of ranaviruses are endemic or invasive in certain areas.

There are three main phylogenetic lineages of amphibian-associated ranaviruses (following Price et al., 2017): frog virus 3 (FV3)-like ranaviruses, the common midwife toad virus (CMTV)-like variants, and the *Ambystoma tigrinum* virus (ATV)-like group. The last group is paraphyletic (as defined by Price et al., 2017) and includes mostly fish-associated variants. All of these groups have been found in Europe (reviewed in Price et al., 2017; Campbell et al., 2020), although most of the observations came from western and central Europe. Recombination between distinct lineages is a documented phenomenon and is regarded as a significant contributor to the genetic diversity and pathogenicity of ranaviruses (Abrams et al. 2013; Price, 2015; Epstein and Storfer, 2016; Claytor et al., 2017; Vilaca et al., 2019). Furthermore, the acquisition of CMTV-like genes by FV3-like viruses has been linked to an increased virulence (Claytor et al., 2017).

In Russia, despite its large territory, surveys for this pathogen have been scarce. Only two studies dealing with ranaviruses in the country exist to date. A strain of FV3 presumably caused mortality of common toad (*Bufo bufo*) found in the vicinity of Moscow (Reshetnikov et al., 2014), and a CMTV-like variant was reported from a clinically healthy *B. bufo* near the city Tyumen in West Siberia (Lisachov et al., 2022). The rising numbers of ranaviral infection outbreaks world wide makes it important to monitor and screen wild populations of cold-blooded vertebrates for the presence of this pathogen. The understanding of the basic biology, epidemiology, and evolution of ranaviruses also requires tracking their geographic distribution and host range. This information is also crucial for efforts to mitigate their impacts (Brunner et al., 2021).

The European water frogs (*Pelophylax* Fitzinger, 1843) are large semiaquatic frogs of the Ranidae family that are common and widespread in the Palearctic region (Frost, 2023). Together with other amphibians, they play an important role in aquatic ecosystems, including transfer of infectious agents (viruses, bacterial pathogens, protozoans, parasitic worms, fungi, etc.) between land and water (Mohneke and Rödel, 2009; Hocking and Babbitt, 2014; Yermokhin et al., 2018). The water frogs are species of conservation priority in several countries (Dufresnes et al., 2020), while some species, such as members of the *P. ridibundus* (Pallas, 1771) complex, are invasive species of high concern (Petrosyan et al., 2023). Worse still, alien species may act as a vector for spreading infectious diseases, including ranaviruses (Sharifian-Fard et al., 2011; Rivera et al., 2019). Therefore, surveillance of *Ranavirus* in the water frogs has important epidemiological and conservation implications.

Here, our aim was to (1) screen native and invasive *Pelophylax* populations across Russia for the presence of ranaviruses using qPCR, and to (2) sequence the viral major capsid protein (MCP) gene to determine phylogenetic position and describe the diversity of *Ranavirus* in the studied area. The findings from this study contribute toward better understanding of *Ranavirus* distribution and host range in Europe for more refined conservation and management practice.

MATERIALS AND METHODS

The collection of extracted DNA samples of water frogs stored in the Laboratory of Molecular Ecology and Animal Systematics of Penza State University (Penza, Russia) were used. The samples were collected in 2006—2016 as part of other studies (see Table S1) and consisted of toe clips of live adult specimens (St-Amour and Lesbarrères, 2007) with DNA extracted using the salt-extraction method (Aljanabi and Martinez, 1997). The collection included the following members of the genus *Pelophylax*: *P. lessonae* (Camerano, 1882), *P.* kl. *esculentus* (Linnaeus, 1758), and two members of *P. ridibundus* complex: *P. ridibundus* sensu stricto and *P.* cf. *bedriagae*, which we refer to as "*P. ridibundus* complex". All three taxa are known to coexist in the same habitats within the East European Plain. However, the majority of the area under study is inhabited by the marsh frog (*P. ridibundus* complex), including a number of invasive populations of the species in the Kamchatka Peninsula, to the east of the Urals, and in West Siberia. The minimal distance between sampling sites in the final dataset was 4.8 km.

According to the information from the collectors, the samples were collected either immediately following capture in the field (hereafter referred to as 'wild') or from frogs that were kept in laboratories for a period of time after capture and before toe clipping (hereafter referred to as 'captive'). The ranavirus prevalence in 'captive' samples were assessed separately, as it was uncertain whether they had been infected prior to captivity.

The qPCR assays were performed with primers and TaqMan probes developed in Leung et al. (2017). One set of primers is specific to a fragment of the *Ranavirus* MCP gene, whereas the second set of primers is specific to the vertebrate non-coding single-copy ultraconservative regulatory element EBF3_Napoleon (EBF3N). We run it as a duplex qPCR with each reaction containing 7.5 µl qPCR master mix (BioMaster HS-qPCR, Biolabmix), each of four primers to concentration of 0.5 pM, each of two probes to concentration of 0.25 pM, 1 µl template DNA and MilliQ water up to the volume of 15 µl. qPCR was performed for 50 cycles with annealing temperature at 60 °C. Amplification was run using the BioRad CFX96 real-time PCR system, and the amplification curves were analyzed using the respective official software.

Each sample was initially run in duplicates. The samples that did not show the amplification of the frog DNA were excluded from the analysis. The samples were considered positive if a robust sigmoid amplification curve of ranavirus DNA was present in both duplicates considering a Ct cut-off value of 40 cycles. If it was present in one replicate, the sample was run again for two more replicates with twice the amount of DNA template. If the sample showed the amplification of the ranavirus DNA in both additional replicates, it was considered positive. Otherwise, it was considered negative. During each run, we prepared two replicates of positive control and negative control (no DNA template). As positive control, we used the DNA obtained from the liver of an infected *P.* kl. *esculentus* individual, kindly provided by Dr. Vojtech Baláž (University of Veterinary Sciences Brno, the Czech Republic). We estimated relative viral load as the ratio between the Ct values of a target gene (viral MCP) and a reference host gene in the same sample averaged over duplicates (i.e., reduction in the ratio corresponds with an increase in the number of viral gene copies relative to the number of host gene copies). Heatmap of ranavirus prevalence based on 'wild' samples only, was created in QGIS Desktop 3.30.0 (QGIS Development Team, 2023).

For the amplification and further sequencing of a 703 bp long MCP fragment for phylogenetic analysis we used the primer pair RV2_F/RV1_R from Lisachov et al. (2022). When amplification of the long product failed, two primer pairs RV2_F/RV289_R and RV1_F/RV1_R were used to amplify shorter products (Lisachov et al., 2022). PCR was conducted using the BioMaster HS-Taq PCR-Color master mix (Biolabmix) with the protocol as follows: initial denaturation of 5 min at 96 °C, 40 cycles of amplification (15 s at 96 °C, 30 s at 60 °C and 30 s at 72 °C), and final extension of 5 min at 72 °C. The amplification products were analyzed using the 1.5% agarose gel, cleaned with the PCR cleanup kit (Biolabmix) and sequenced bidirectionally on an ABI 3500 automated capillary sequencer (Applied Biosystems) with the BigDye^{TM} Terminator v.3.1 Cycle Sequencing Kit. We also sequenced the positive control sample to detect potential contamination.

The newly obtained sequences, combined with the ranavirus MCP sequences available in GenBank (NCBI), were aligned using MUSCLE 3.8.31 (Edgar, 2004) and manually checked for unexpected stop codons and errors in SeaView 4 (Gouy et al., 2010). Gene tree reconstruction by maximum likelihood (ML) approach was performed with IQ-TREE 2.2.0 (Minh et al., 2020) using K3Pu + F + I substitution model selected by ModelFinder (Kalyaanamoorthy et al., 2017) and 1 000 non-parametric bootstrap replicates. Identical haplotypes were excluded from the analysis but added to the tree. The tree was visualized and edited with iTOL 6.8.1 (Letunic and Bork, 2021).

RESULTS

A total of 853 samples that showed the amplification of EBF3N (host's reference) were considered in the study. Of these, 590 were from 'wild' *Pelophylax* specimens, and 263 were from 'captive' frogs. Out of the 'wild' frogs, 52 samples (8.8%, 95% Wilson score interval 6.8–11.4%) tested positive for *Ranavirus*, while 71 samples (27%, 22.3–33.1%) tested positive among the 'captive' frogs. Proportion of positive individuals of each species given in Table 1. The 'wild' frogs were sampled from 94 sites, mainly in the Volga basin (Fig. 1). *Ranavirus* was detected in 18 (19.1%) of the sites tested, with prevalence ranging from 11.1% to 100%. Thus, the average prevalence for all tested sites was 8.2%, and within positive sites it was 42.9%. The presence of ranavirus was identified in 'wild' samples collected since 2006 (vicinity of the city Uryupinsk, 50.79° N 41.97° E). The detailed list of locations, numbers of samples and year of collection in each location per species is provided in Table S1.

The presence of the ranavirus was detected in invasive populations of the marsh frog (*P. ridibundus* complex) in the Urals and West Siberia regions. Of the nine invasive sites examined, five (45.5%) were positive. The average prevalence for positive invasive sites (50.9%, $n = 5$) was not significantly different from that observed in positive native populations (39.9%, $n = 13$; Wilcoxon rank-sum test: $Z = 0.298$, $p = 0.766$).

The median ratio of ranavirus MCP Ct to host reference Ct in positive 'wild' frogs was 1.34 (min–max 0.95–1.54), while in positive 'captive' frogs the median ratio was 1.12 (0.48–1.59) (Fig. 2) implying higher relative viral load in the 'captive' frogs (Wilcoxon rank-sum test: $W = 857$, $p < 0.001$).

We successfully sequenced 22 samples: 11 samples from nine sites in the 'wild' group, and 11 'captive' samples from another nine sites (Fig. 1). The analysis of obtained MCP sequences revealed six different haplotypes of *Ranavirus*. All haplotypes were closely related (one to two substitutions difference between each other per the 703 bp

Table 1. Results of qPCR screening for *Ranavirus* among different frog species and condition groups.

Species	Wild				Captive	
	Sample size per site, mean (min–max)	Positive / total sites (%)	Positive / total samples	Prevalence, % (95% CI)	Positive / total samples	Prevalence, % (95% CI)
P. ridibundus complex	5.7 (1–21)	14 / 87 (16.1)	41 / 496	8.3 (6.2–11.0)	34 / 167	20.4 (15.0–27.1)
P. lessonae	5.1 (1–15)	2 / 10 (20.0)	5 / 51	9.8 (4.3–21.0)	16 / 55	29.1 (18.8–42.1)
P. kl. *esculentus*	4.3 (1–20)	3 / 10 (30.0)	6 / 43	14.0 (6.6–27.3)	21 / 41	51.2 (36.5–65.7)
Total	6.3 (1–35)	18 / 94 (19.1)	52 / 590	8.8 (6.8–11.4)	71 / 263	27.0 (22.3–33.1)

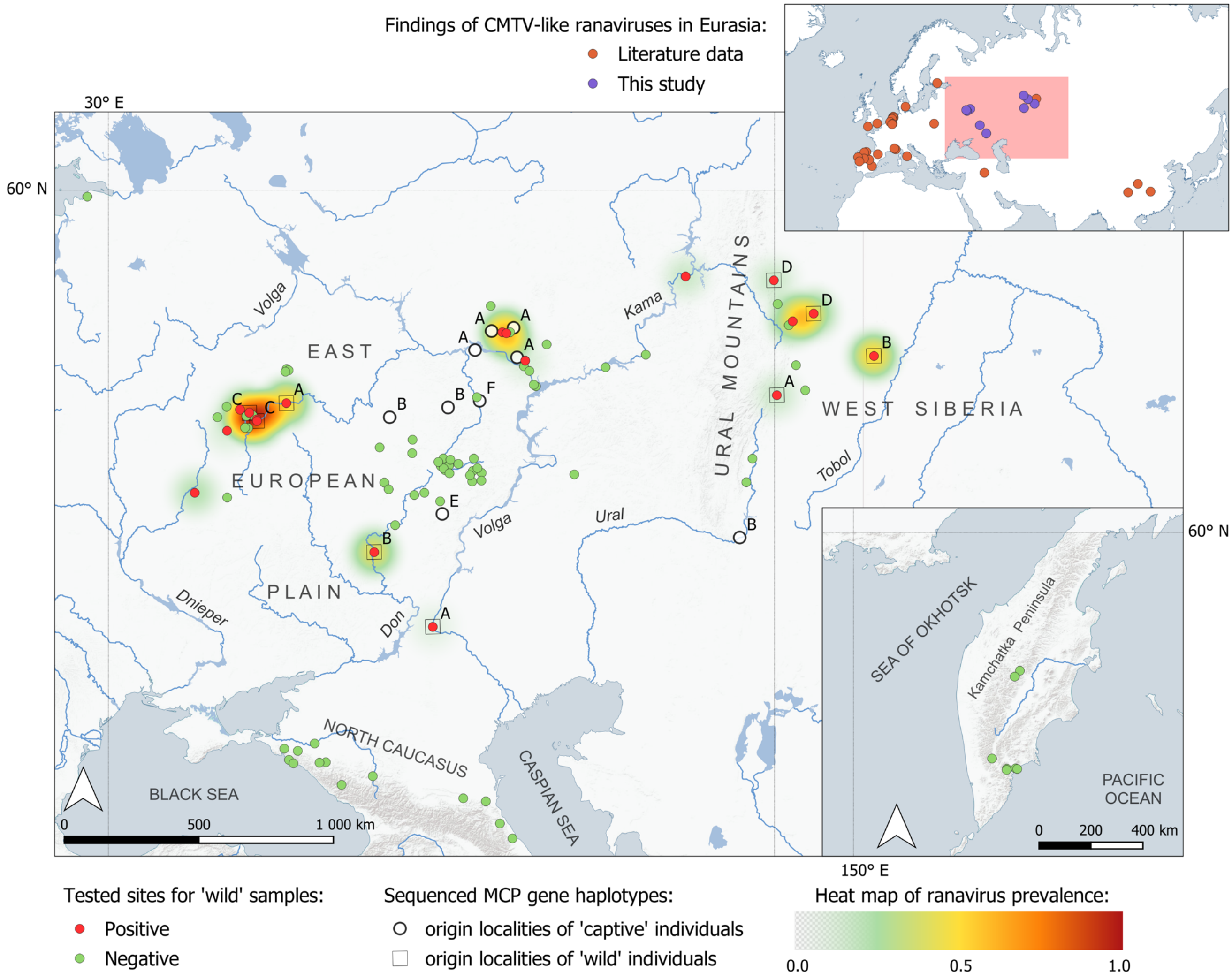

Figure 1. Heat map of the study area showing sampling sites and ranavirus prevalence in *Pelophylax* sp. populations based on 'wild' samples only. The map also shows the sites of origin for the sequenced samples (both 'wild' and 'captive'). Capital letters near sequenced localities indicate detected haplotypes. Localities where identical haplotypes were found are connected by lines of different colors. The map in the top right corner shows locations for literature records of CMTV-like ranavirus along with findings from the present study.

sequence) and belonged to the CMTV lineage (Fig. 3). The 'wild' samples harbored four haplotypes. Haplotype A was found in European Russia and the Urals; haplotype B was present in West Siberia and European Russia. Haplotype C was found in European Russia, while haplotype D was observed in localities in the Urals (Fig. 1, Table S1). The 'captive' frogs harbored four *Ranavirus* MCP haplotypes, including A, B, E and F. New haplotypes were deposited into GenBank (NCBI) under accession numbers OR934977–OR934980 (Table S1). None of the detected haplotypes were identical to the positive control sample.

DISCUSSION

Ranaviruses in *Pelophylax* Populations in Russia

The present study reveals a broad spread of ranaviruses in the water frogs of the genus *Pelophylax* in Russia with a prevalence of 9%. Furthermore, the oldest samples available in the examined collection (2006) were tested positive. Our findings are consistent with the results of similar studies recently conducted in other countries, specifically Hungary (Vörös et al., 2020), Poland (Palomar et al., 2021), China (Herath et al., 2023) and Brazil (Ruggeri et al., 2019, 2023). Similarly to these studies, no mortality incidents were observed (per. comm. by the collectors of the samples) despite

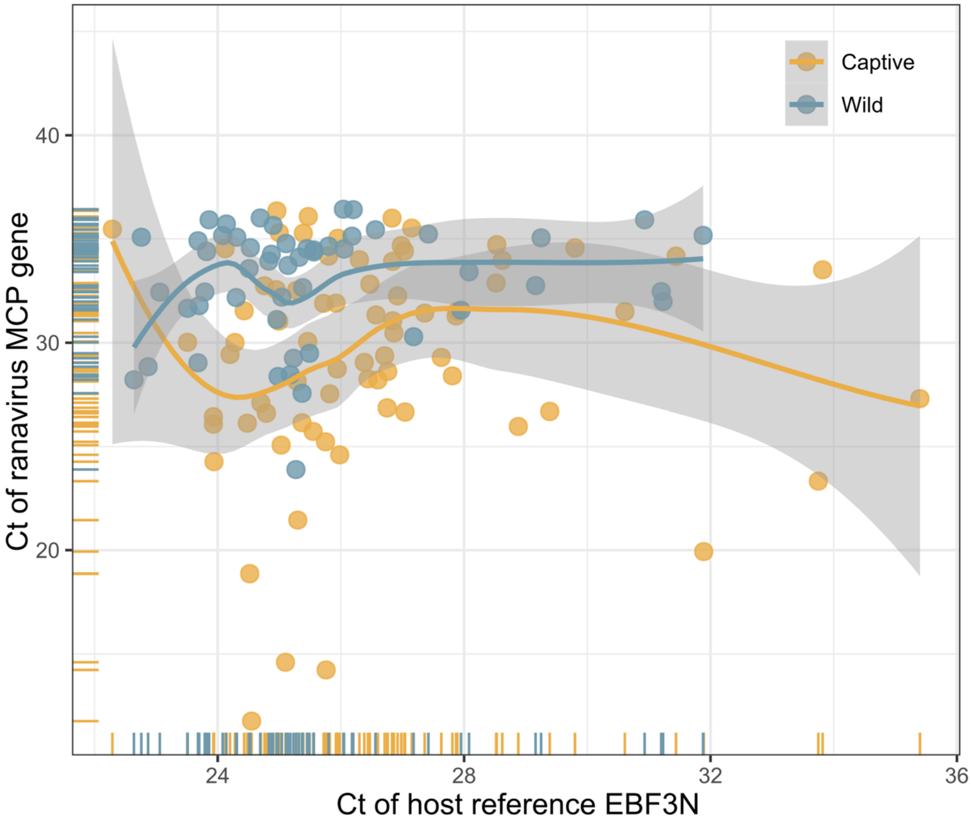

Figure 2. Difference of the relative viral load between 'captive' and 'wild' conditions as the ratio between Ct values of ranavirus MCP gene and host reference marker EBF3N.

the wide occurrence of ranavirus. Specifically, in Hungary 29.2% of all tested amphibians were positive for *Ranavirus* and none of them were clinically ill (Vörös et al., 2020). In Poland, 2.4% of all tested amphibians were infected, with a prevalence of 5.2% in *P. lessonae*, 1.3% in *P. esculentus*, and 1.1% in *P. ridibundus*. In North-East Australia, 2.7% of examined individuals from 47% of sampled sites were infected, with no external signs of infection (Wynne, 2019).

In the study of ranavirus surveillance in West Siberia (Russia), only one infected individual, an adult common toad (*Bufo bufo*), out of the 252 amphibian samples was detected (Lisachov et al., 2022). This low prevalence could be attributed to the milder climate in the European part of Russia compared to Siberia, which may affect the distribution and prevalence of ranaviruses. However, it is important to note that the sample did not include any *Pelophylax* species. Furthermore, it is worth noting that the Siberian samples were primarily collected during or shortly after spawning in the spring, whereas ranavirus prevalence typically peaks in the summer (Miaud et al., 2019). The warmer temperatures are also known to decrease systemic infection of amphibians by ranavirus (Brand et al. 2016). These factors may have contributed to the significant difference between the results of the two studies.

Invasive Marsh Frogs as a Potential Spreader of the *Ranavirus* Infection

The frogs of *P. ridibundus* complex are highly concerning invasive species. In West Siberia and the eastern Urals, the populations established over the last 50–60 years (Kuzmin, 2012). The main source of introduction is believed to be the fish farms located in the European regions of Russia and neighboring countries, from where the tadpoles were inadvertently introduced with the fish fry (Ivanova and Berzin, 2019; Ualiyeva et al., 2022). We detected the presence of *Ranavirus* in five invasive populations of the marsh frog in south-western West Siberia and the Urals, suggesting that the marsh frog may also act as an important spreader of ranavirus infection. In contrast, all 57 samples from seven marsh frog invasion sites on the Kamchatka Peninsula tested negative for the virus. Although ranaviruses found in the invasive populations of marsh frog east to Urals may be native to these areas, it is possible that it

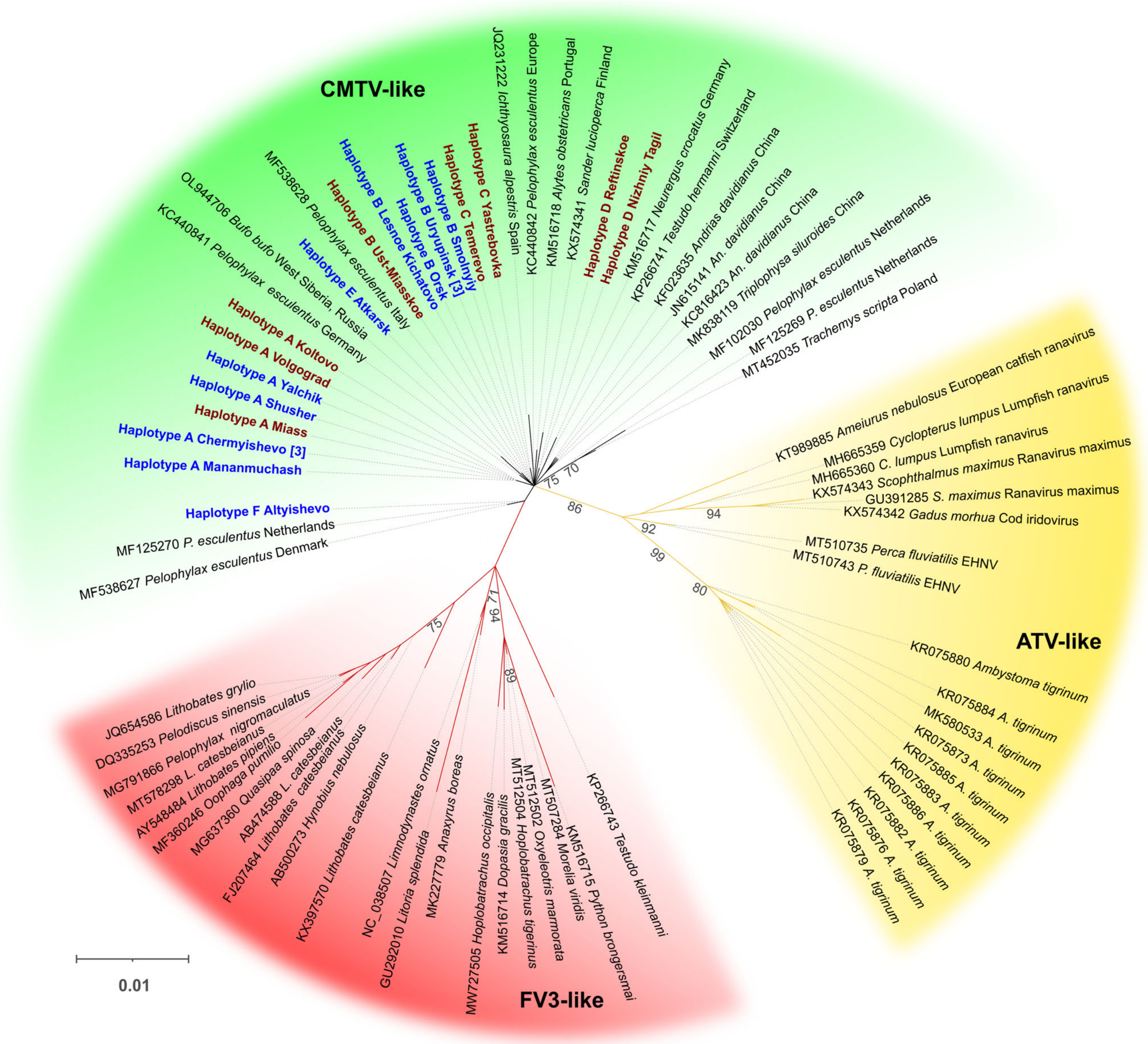

Figure 3. Unrooted ML phylogenetic tree based on partial major capsid protein (MCP) gene DNA sequences of the ranaviruses from *Pelophylax* species in this study (in bold color font) and from GenBank. Non-parametric bootstrap values above 70% are indicated. Haplotype designations and locality names for 'wild' and 'captive' samples are red and blue, respectively (refer to Table S1 for locality names). The square brackets indicate the number of samples sequenced for a site when it exceeds one. The *Ranavirus* groups highlighted sensu Price et al. 2017.

also spreads together with frogs, given its occurrence in the native range. Our study did not include samples from invasive *P. ridibundus* complex populations known in the south and south-east of West Siberia (Omsk oblast, Novosibirsk oblast, Altai Krai, Altai Republic) (Simonov et al., 2022). It is possible that these populations may harbor ranavirus as well and require further efforts for surveillance.

There are examples of other aquatic invasive frog species suggested to serve as ranavirus spreaders and reservoirs. For instance, mass mortality events of farmed North American bullfrog *(Lithobates catesbeianus)* tadpoles due to FV3-like viruses were detected in Japan in 2008 (Une et al., 2009) and in Brazil in 2003–2005 (Mazzoni et al., 2009). Prevalence of ranavirus in *L. catesbeianus* imported for food to the US was estimated as 8.5% in the years 2000–2005 (Schloegel et al., 2009). In Chile, invasive populations of the African clawed frog (*Xenopus laevis*) were suggested to be the source of *Ranavirus* introduction to the country (Peñafiel-Ricaurte et al., 2023). This highlights the additional damage that inadvertent introductions of highly invasive frogs like *Pelophylax* can cause to the local communities of ectotherms.

Continent-Wide Distribution of CMTV-like Ranaviruses in Europe

It has previously been illustrated that CMTV-like ranaviruses are mostly observed in Europe (reviewed in Price et al., 2017) with just a few records from Asia and North America (Majji et al., 2006; Chen et al., 2013). First discovered and described in Spain (Balseiro et al., 2009), they were subsequently found in Portugal, Italy, the UK, Germany, the Netherlands, Belgium, Denmark, Finland, and Poland (Kik et al., 2011; Sharifian-Fard et al., 2011; Holopainen et al., 2016; Miaud et al., 2016; Rosa et al., 2017; Borzym et al., 2020). These ranaviruses are known to affect amphibians, fish, reptiles, thus considered as "important pathogens with extremely broad host ranges" (Price et al., 2017). An invasive origin of CMTV ranaviruses in Europe was initially proposed (Price, 2015; Price et al., 2017; Campbell et al., 2020). However, recent analyses of numerous ranaviral genomes have revealed deep evolutionary diversity associated with specific geographic regions in CMTV-like ranaviruses, arguing against a recent invasion and in favor of a natural distribution restricted to Europe (Owen, 2021).

All haplotypes of *Ranavirus* observed in this study belong to the CMTV-like lineage and are close to other previously discovered haplotypes of the same lineage. The haplotype B was detected in both 'wild' and 'captive' samples and was previously reported from Italy in 2002 (Ariel et al., 2017; MF538628), while the haplotype F was only detected in the 'captive' group and was previously reported from the Netherlands in 2016 (Saucedo et al., 2018; MF125270). Other haplotypes were not reported before. Low variability of the sequenced fragment does not allow to resolve their position within the CMTV-like clade. Nevertheless, the presence of six haplotypes of a conservative gene fragment in the surveyed area indicates relatively high diversity of the CMTV-like ranaviruses. We found various CMTV-like haplotypes occurring at multiple sites within the basin of Europe's largest river (Volga) and in the basins of other largest European rivers (Dnieper, Don). This is probably the evidence of a relatively long and presumably natural presence of these ranaviruses in the area. In combination with previously published data, the findings of the present study provide further support toward the endemism and continent-wide distribution of CMTV-like ranaviruses in Europe, and possibly Eurasia, notably extending the area of their detection in the wild.

Higher Prevalence and Relative Virus Load in Frogs from Captivity

In the sample of 'captive' frogs, the prevalence was three times greater than in the 'wild' frogs. The ratio of ranavirus Ct to reference Ct was also lower in 'captive' frogs meaning higher relative viral load in the samples. The samples examined in this study were sourced from three distinct research laboratories. In each of these settings, the frogs were maintained in groups and subjected to a degree of crowding prior to the sampling. The observed picture is consistent with that observed in the University of Zürich during the *Ranavirus* outbreaks in the captive frog colonies established for research purposes: the captured frogs that were initially clinically healthy developed high viral load and clinical ranavirosis in captivity (Stöhr et al., 2013). In China and Thailand, animals at frog farms, food and pet markets, where they are frequently kept crowded and in suboptimal conditions, were also shown to have higher frequency of *Ranavirus* infection than wild animals in surrounding areas (Sriwanayos et al., 2020; Herath et al., 2023). Other emerging pathogens are also known to spread within laboratory colonies of amphibians, for instance the *Elizabethkingia miricola* bacterial infection has been noted in laboratory-maintained dwarf African frog *Hymenochirus* sp. (Yang et al., 2023).

In research and educational laboratories, frogs are commonly kept in more crowded conditions than those found in the wild. Additionally, during capture and transportation, they may be subjected to stress and even higher levels of crowding. It is hence likely that frogs that were released or have escaped from captivity present a higher potential risk of being carriers of pathogens, unlike the wild frogs that were accidentally transported with aquaculture. The biological and medical university departments in the Siberian parts of Russia have also been sources of some invasive marsh frog populations. The frogs were previously kept in these departments for classes and experiments (Kassal, 2022). Thus, we highlight the need of introducing the practice of testing frogs for ranavirus and other pathogens (such as chytrid fungi) when preparing batches of frogs for educational and other purposes to reduce disease and mortality of frogs in the labs and occasional spreading of pathogens into the wild.

CONCLUSIONS

Our study is the first large-scale survey of ranaviruses in the European part of Russia. Although limited to water frogs of the genus *Pelophylax*, it shows that ranaviruses are widespread in both native and invasive populations in the basins of some of Europe's largest rivers, extending the detection range of CMTV-like ranaviruses to the easternmost parts of Europe. Since we detected several different haplotypes in a short fragment of the conservative MCP gene, we suppose that the presence of CMTV-like ranaviruses in East European Plain and the Urals is probably old and natural. We also found that frogs kept in laboratory conditions exhibit higher viral prevalence and relative viral load than wild frogs. Thus, releasing the frogs back to their original capture sites after experimentation could result in the spread of ranaviruses if not properly controlled.

Although many recent studies report ranavirus detection only by means of qPCR, our study underlines importance of their further identification by the means of DNA sequencing to advance our knowledge of distribution and evolutionary history of distinct *Ranavirus* species and lineages. To further trace their spread history and phylogeography, whole genome sequencing and analysis have proven to be an effective tool, as in the case of the extensive studies on FV3-like ranaviruses (Price et al., 2016; Owen, 2021).

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DECLARATIONS

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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