**UNIVERSITY OF PÉCS**

Biological and Sportbiological Doctoral School

# Advancing canine distemper virus research through Nanopore Technology for surveillance and genome sequencing

***PhD Thesis***

**Zsófia Lanszki**

**Pécs, 2024**

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## Zsófia Lanszki


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**List of abbreviations**

| | |
|---|---|
| **CAdV-2** | canine adenovirus type 2 |
| **CDV** | canine distemper virus |
| **CIV** | canine influenza virus |
| **CPIV** | canine parainfluenza virus |
| **Ct** | Cycle values |
| **CUB** | Codon Usage Bias |
| **DMV** | dolphin morbillivirus |
| **EEA** | European Environment Agency |
| **F** | Fusion protein |
| **FeMV** | feline morbillivirus |
| **H** | Hemagglutinin protein |
| **HeV** | Hendra virus |
| **ICTV** | International Committee on Taxonomy of Viruses |
| **IUCN** | International Union for Conservation of Nature |
| **L** | Large polymerase protein |
| **M** | Matrix protein |
| **MeV** | measles virus |
| **MPV** | monkeypox virus |
| **MuV** | mumps virus |
| **N** | Nucleocapsid protein |
| **NiV** | Nipah virus |
| **P** | Phosphoprotein |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PDV** | phocine distemper virus |
| **PPRV** | peste des petits ruminants virus |
| **qRT-PCR** | Quantitative Reverse Transcription Polymerase Chain Reaction |
| **RNPs** | Ribonucleocapsids |
| **RPV** | rinderpest virus |
| **RT-PCR** | Reverse Transcription Polymerase Chain Reaction |
| **SLAM** | Signaling Lymphocyte Activation Molecule |

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## 1. Introduction

### *1.1. General introduction about wildlife diseases and surveillance*

Infectious diseases are critical limitation factors regarding the population size and dispersal of wildlife species. Therefore, there has been a growing interest, during the last decades towards the understanding of emerging infectious diseases in wildlife (Cunningham, 2005; Daszak et al., 2000). In several situations, it was clearly shown that most dispersed and small populations of endangered wild animals are more prone to extinction due to stochastic events, such as disease outbreaks (Feng et al., 2016; Roelke-Parker et al., 1996; Williams et al., 1988). Clearly, disease monitoring is deemed important in the conservation of rare species. However, the examination of wild animals, especially carnivores, is often more difficult than that of domestic or zoo animals due to ethical reasons and sampling or detection difficulties. Population-size determination, morbidity, mortality estimation, and the early detection of disease outbreaks are highly challenging, specifically, among wildlife species (Artois et al., 2001).

Detection and investigation of viral diseases are important factors for conserving protected and rare species, however, the elusive nature of several carnivores hampers our understanding of their viruses. Post-mortem examination (autopsy) of animals, furthermore excrements collected on field from passive monitoring programs provides a powerful method to obtain diverse samples, analyse and assess the factors in relation to ecosystem health. The results of retrospective surveillance may provide novel and relevant data for rare and hidden species, including the prevalence and genetic characteristics of certain infectious diseases (Gortázar et al., 2007; Thomas et al., 2020)

### *1.2. General introduction about Paramyxoviridae family, Morbillivirus genus*

The *Paramyxoviridae* family is composed of enveloped RNA viruses that infect a wide range of hosts, including mammals and in some cases, birds, reptiles, and fish. Within this family, many paramyxoviruses exhibit strong host-specificity to its natural host, while several others, such as Hendra virus (HeV), and Nipah virus (NiV), are known zoonotic pathogens. The transmission of these viruses occurs horizontally, primarily through direct contact and airborne routes, without the involvement of vectors (Rima et al., 2019).

Virions range in diameter from 150 to 500 nm and exhibit pleomorphic shapes, although they are predominantly spherical when observed in vitreous ice. These virions are composed of a lipid envelope that surrounds a nucleocapsid. Intracellularly, or in virions, genome-length

RNA is found exclusively encapsidated in ribonucleocapsids (RNPs). Virion is not infectious alone but is infectious if the RNP complex is introduced into the cytoplasm. The genome RNA does not contain a 5′-cap, nor a covalently linked protein. The genome 3′-end is not polyadenylated. The paramyxovirus genome is approximately 14.6–20.1 kb long linear negative-sense, non-segmented RNA. Within this virus family, there are currently 4 subfamilies, 17 genera, and more than 70 species identified and classified (Rima et al., 2019). One of the 4 subfamilies is *Orthoparamyxovirinae*, which includes the *Morbillivirus* genus. Derivation of the family name, *Paramyxoviridae*, is derived from the Greek words "*para*" meaning "by the side of" and "*myxa*" meaning "mucus". The genus name, *Morbillivirus*, is derived from the Latin word "*morbillus*" which is a diminutive form of "*morbus*" meaning "disease".

There are multiple members in *Morbillivirus* genus with significant impact on animal or human health, such as measles virus, canine distemper virus (CDV), peste des petits ruminants virus (PPRV), phocine distemper virus (PDV), rinderpest virus (RPV), feline morbillivirus (FeMV) and dolphin morbillivirus (DMV) (de Vries et al., 2015; Rima et al., 2019). Morbilliviruses possess a P/C/V transcription unit with RNA editing, wherein the templated exact copy mRNA encodes a phosphoprotein (P), and the predominant edited mRNA form with an added G encodes a Zn2+-binding cysteine-rich protein (V). Additionally, all members of the morbillivirus family encode a non-structural protein (C) (Figure 1). In terms of cellular morphology, all morbilliviruses produce both intracytoplasmic and intranuclear inclusion bodies that contain nucleocapsid-like structures (Figure 2). Moreover, these viruses exhibit cross-reactivity in serological tests (Roy et al., 2023).

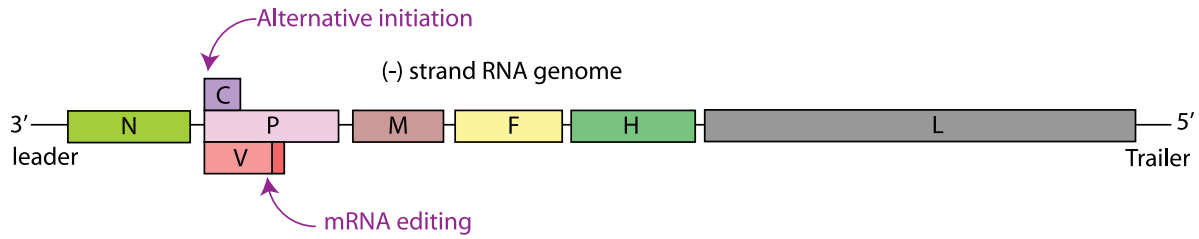



**Figure 1.** Genome organization of Morbillivirus (ViralZone; https://viralzone.expasy.org/86) (Date of Access: 30.12.2022).

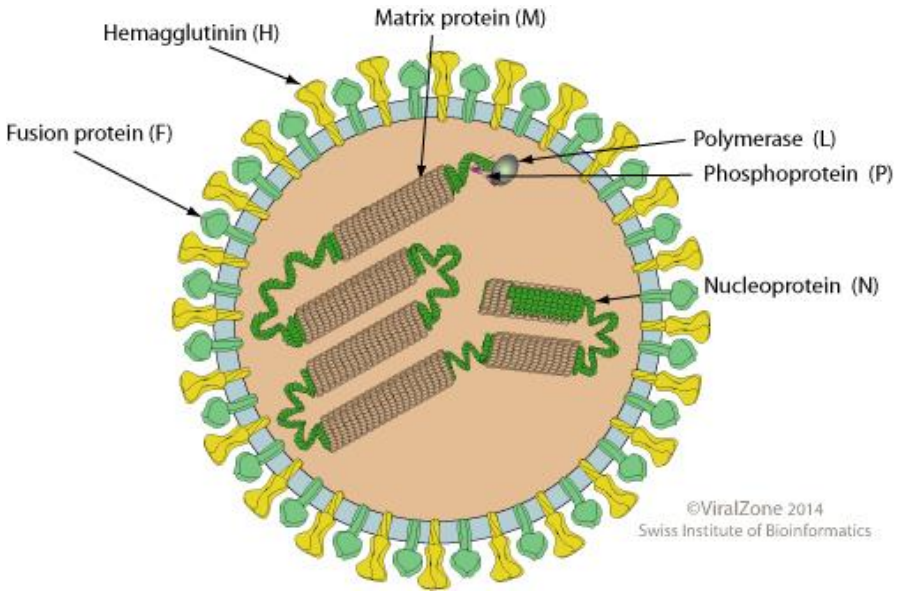



**Figure 2.** Virion structure of *Morbillivirus* (ViralZone; https://viralzone.expasy.org/86) (Date of Access: 30.12.2022)

### *1.3. General introduction about canine distemper virus*

The virus species was named canine distemper virus until 2016 when species name was renamed *Canine morbillivirus.* The nomenclature was changed again in 2022, and now its official name is *Morbillivirus canis*, based on the International Committee on Taxonomy of Viruses (ICTV) [species name: *Morbillivirus canis*, virus name: canine distemper virus]. CDV is a single-stranded negative-sense RNA virus which belongs to the *Paramyxoviridae* family in the *Morbillivirus* genus. Along with the measles virus in humans, CDV is considered the most contagious virus in this family (de Vries et al., 2015; Duque-Valencia et al., 2019) . The CDV genome is approximately 15,690 nucleotides and encodes six structural proteins: two glycoproteins, Hemagglutinin (H) and fusion (F) proteins, one envelope-associated matrix (M) protein, two transcriptase-associated proteins phosphoprotein (P) and large polymerase (L) protein and one nucleocapsid (N) protein (Elia et al., 2006; Martella et al., 2008). The Hemagglutinin protein is a major fusogenicity determinant and plays a key role in the host-specific immunity against CDV (Bolt et al., 1997; Duque-Valencia et al., 2019; Iwatsuki et al., 2000; Martinez-Gutierrez & Ruiz-Saenz, 2016; McCarthy et al., 2007; Romanutti et al., 2016). The Hemagglutinin gene is an attachment protein, it has a key role as a receptor-binding protein. Amino acid variations in the Hemagglutinin protein that bind cellular SLAM (signaling lymphocyte activation molecule) are thought to be important in species specificity (Duque-Valencia et al., 2019).

Based on historical records and evolutionary analyses, it is likely that CDV have originated from the measles virus (MeV) in the New World (Figure 3). This assumption was supported by multiple molecular analyses, for example, the CDV and MeV genes to human codon usage bias (CUB), suggesting that CDV codon usage is closer to human CUB than canine CUB because the virus or its progenitor, most likely MeV, was initially adapted to humans (Quintero-Gil et al., 2019; Uhl et al., 2019)

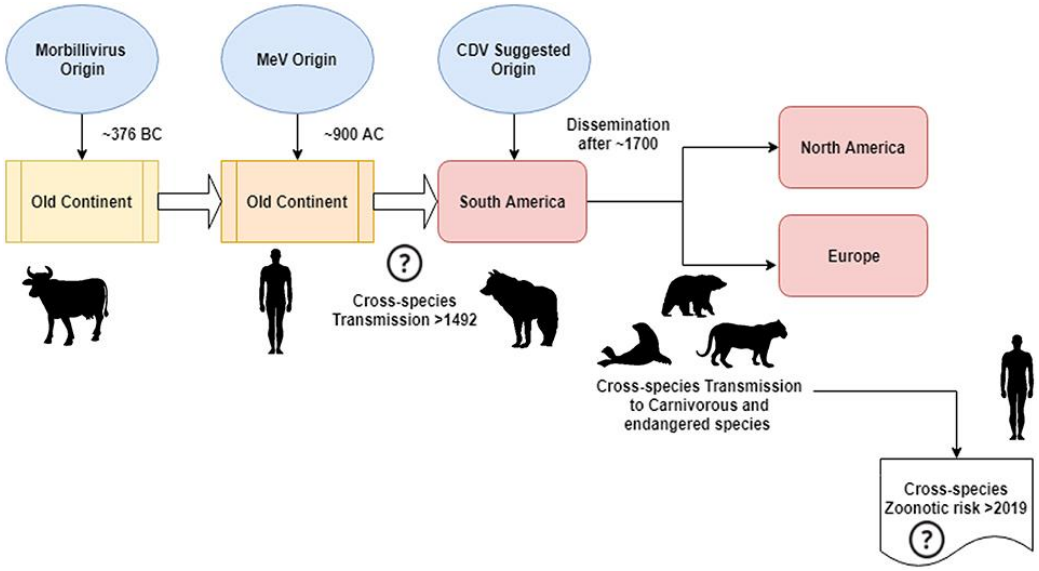



**Figure 3.** Hypothetic emergence scenario for canine distemper virus in the past (Quintero-Gil et al., 2019)

While canine distemper virus is primarily known as an animal pathogen, however recent studies have raised concerns about its potential to infect humans, particularly in communities with low measles vaccination rates and frequent exposure to CDV-positive animals (Quintero-Gil et al., 2019; Uhl et al., 2019). *In vitro* studies confirmed that CDV could potentially become capable of using human cell receptors, and therefore some of the relevant amino acid exchanges in the H gene are already known (Bieringer et al., 2013). Most studies raise the possibility that if measles is eradicated and measles vaccination is stopped, CDV could eventually cross the species barrier into humans and emerge as a new human pathogen, filling this niche. A similar trend is taking place throughout the world as monkeypox virus (MPV) is likely filling the niche vacated through smallpox eradication (Adetifa et al., 2023). Close to humans, CDV can infect nonhuman primates Japanese macaques (*Macaca fuscata*), rhesus macaques (*Macaca mulatta*), and cynomolgus macaques (*Macaca fascicularis*) naturally. *In vivo* experimentally infected cynomolgus macaques, measles virus vaccination induces partial protection against CDV challenge infection (de Vries et al., 2014). However, we are not aware of any studies that specifically showed CDV sequences in human samples or PCR positive human cases so far.

Canine distemper virus is a significant viral pathogen affecting domestic and wild animal species worldwide (Martinez-Gutierrez & Ruiz-Saenz, 2016). CDV is highly prone to cross-species transmission between domestic and wildlife reservoir hosts, representing a significant OneHealth challenge on the wildlife-domestic animals interface (Ludlow et al., 2014; McCarthy et al., 2007). It poses a significant conservation threat to a wide range of endangered animal populations around the world. This virus is a significant veterinary health concern in areas in which the ratio of unvaccinated dogs (*Canis lupus familiaris*) is high and where the virus is also prevalent among wildlife. Young dogs are most commonly infected, but all ages are prone to infection and may quickly fall victim to the disease. The virus is primarily transmitted among animals via a range of body fluids, such as respiratory droplets, ocular discharge, nasal discharge, saliva, urine and feces, including transmission with direct contact (Greene, 2012).

In consideration of the highly contagious nature of this virus, strict quarantine is required in cases of positivity until the clearance of CDV to avoid the spread of the virus to other animals (Willi et al., 2015). In certain instances, when the infection spreads to the central nervous system, it can result in fatal outcomes (Riley & Wilkes, 2015). In contrast, if a dog develops a strong immune response, the animal can completely recover from the infection (Martella et al., 2008; Willi et al., 2015). Clinical signs characteristic of CDV in dogs may include gastrointestinal (vomiting, nausea and diarrhea), respiratory (nose, trachea and pneumonia) or neurological symptoms (mental dullness, lethargy, unresponsiveness, disorientation, blindness, imbalance and seizures) and fever. Among the CDV infections, more than 50% are likely subclinical, depending on the virulence of the virus strain, environmental conditions, host age and immune status (Greene, 2012; Wyllie et al., 2016). Among dogs which survive the infection, the CDV is usually excreted for a few weeks, however, in some cases, the virus persists for up to 3–4 months (Greene, 2012; Sykes & Hartmann, 2014; Willi et al., 2015). The lengthy duration can weaken the immune system and in certain cases contribute to co-infections with other viruses, bacteria or cellular parasites such as canine adenovirus type 2 (CAdV-2), canine influenza virus (CIV), canine parainfluenza virus (CPIV), *Mycoplasma Cynos*, Babesia spp. and *Leishmania infantum* (Chvala et al., 2007; Greene, 2012; Hao et al., 2019; Willi et al., 2015). Currently, approved CDV vaccines are based on attenuated virus strains. Most commercially available modified live CDV vaccines still belong to the America-1 lineage; therefore, many dogs immunized with this vaccine are prone to new CDV infections worldwide. Due to the high genetic diversity of circulating CDV strains, there might be significant antigenic differences which may lead to vaccine escape cases, as was hypothesized in multiple studies

(Budaszewski et al., 2014; Duque-Valencia et al., 2019; Espinal et al., 2014; Iwatsuki et al., 2000; Lan et al., 2006; Martella et al., 2006; Riley & Wilkes, 2015; Romanutti et al., 2016).

In addition to the general veterinary health problem, it is also a significant conservation threat to endangered species worldwide (Gordon et al., 2015; Loots et al., 2017; Terio & Craft, 2013; Viana et al., 2015). Fatal CDV outbreaks are known to occur in wild populations of endangered species. In Africa, CDV caused outbreaks in a diverse range of wild mammals such as the lion (*Panthera leo*), African wild dog (*Lycaon pictus*) and Ethiopian wolf (*Canis simensis*) (Gordon et al., 2015; Roelke-Parker et al., 1996; van de Bildt, 2002; Viana et al., 2015). In Asia, the virus poses a serious threat to the vulnerable giant panda (*Ailuropoda melanoleuca*), red panda (*Ailurus fulgens*) and the endangered amur tiger (*Panthera tigris altaica*) (Feng et al., 2016; Seimon et al., 2013; Wang et al., 2021). Additionally, in Europe, CDV has caused a number of local epizootics among wild carnivores mostly among red foxes (*Vulpes vulpes*) and Eurasian badgers (*Meles meles*; hereafter: badger) (Jo et al., 2019; Martella et al., 2010; Monne et al., 2011; Origgi et al., 2012; Sekulin et al., 2011; Trebbien et al., 2014; Trogu et al., 2021), and CDV infection was also reported in one of the most endangered felid species, the Iberian lynx (*Lynx pardinus*) (Meli et al., 2010). Highlighting its conservational relevance across a number of animal taxa, a large number of Baikal seals (*Pusa sibirica*) were infected with CDV in Lake Baikal between 1987 and 1988, most likely as a result from a spillover event from dogs (Grachev et al., 1989; Mamaev et al., 1995). Caspian seals (*Pusa caspica*) were also seriously affected by the Caspian lineage of CDV in epizootics occurring in the Caspian sea between 1997 and 2000 (Jo et al., 2019; Kuiken et al., 2006).

In the case of mustelids, CDV infection was previously associated with a high mortality rate approaching 100% (Kiupel & Perpiñán, 2014). The most remarkable CDV outbreak in black-footed ferret (*Mustela nigripes*) population occurred in Wyoming, Western USA, seriously affecting a captive breeding program and leading to the extirpation of the species from the wild (Thorne & Williams, 1988; Williams et al., 1988). A recent report from Spain investigated the CDV seroprevalence trends in association to the population size of the critically endangered European mink (*Mustela lutreola*). They found that CDV seroprevalence is an indicator for the population trend of these animals, supporting the hypothesis that CDV may be an important wildlife disease (Fournier-Chambrillon et al., 2022). In Europe, CDV has been reported among multiple species to date, including the stone marten (*Martes foina*), pine marten (*Martes martes*), badger, Eurasian otter (*Lutra lutra*), European mink, European polecat (*Mustela putorius*) and the American mink (*Mustela vison*) (Akdesir et al., 2018; Di Sabatino et al., 2016a; Frölich et al., 2000; Lanszki et al., 2022; Origgi et al., 2012; Pavlacik et al., 2007;

Philippa et al., 2008). All these examples highlight the importance of this virus for nature conservation aspects in multiple continents and distant geographic areas.

Several distinct genotypes are known and classified according to different hosts and geographical areas. This classification is based on nucleotide sequence analysis of the Hemagglutinin gene, which characterizes phylogeographic distribution patterns (Duque-Valencia et al., 2019). In Hungary, three different CDV genotypes (Europe-, Arctic-like- and Europe wildlife lineages) were described so far based on the H gene nucleotide sequences in dogs, raccoon (*Procyon lotor*) and ferret (*Mustela putorius furo*) (Demeter et al., 2007, 2009). According to these findings, several different CDV genotypes were present at the same time in the country. The phylogenetic analysis was performed with the full segment of the H gene nucleotide sequence. Thirteen samples from dogs were selected for sequence analysis between 2004–2006. Nine sequences belonged to the group of Arctic-like strains, one group belonged to the Europe isolates cluster and three belonged to the Europe wildlife group; all sequences were from dogs. The nine dogs had unknown vaccination histories. Additionally, CDV infection was also detected in other carnivores, such as red fox, raccoon and ferret; however, these species were not involved in any genetic analysis (Demeter et al., 2007, 2009). Since Arctic-like lineage were detected in Hungary as early as 2004, it may have since become endemic. Large-scale epidemiological surveys are required to obtain a general picture of the prevalence, geographic distribution, and seasonality of the CDV lineages within our region.

The geographical spread of different lineages of CDV can be a serious problem, there can be many reasons for this, such as natural, e.g. diseases that are brought more slowly between countries through wild animals or e.g. during the animal trade. In Italy, three different CDV genotypes (Europe, European wildlife, and Arctic-like lineages) were described so far based on the H gene nucleotide sequences from the last two decades. Only the Europe lineage was present in the Italian dog population until 2000 (Balboni et al., 2014). Then, European wildlife lineages was reported from red foxes with severe neurological signs in 2000 (Martella et al., 2002, 2006). After the year 2000, several cases of Arctic-like CDV infection were detected from domestic dogs and other free-ranging carnivores (Martella et al., 2006).

In Asia, several lineages of CDV have been classified, based on H gene sequences, such as the most common Asia-1, Asia-2, Asia-3, Asia-4, Asia-5 lineages (Feng et al., 2016; Guo et al., 2013; Manandhar et al., 2023; Truong et al., 2022; Zhao et al., 2010). New lineages are constantly being identified, such as from red panda in 2018 from China (Wang et al., 2021). In the South Asia region, in India, CDV were described in several studies (Abirami et al., 2020; Kadam et al., 2022; Pawar et al., 2011; Putty et al., 2020; Swati et al., 2015). The presence of

a novel genetic lineage of CDV based on the H gene was detected, namely: India-1/Asia-5 lineage, which circulates among the dog population in India (Bhatt et al., 2019). CDV caused an epizootic event among Asiatic Lions (*Panthera leo persica*) and leopards (*Panthera pardus*) in 2018 from India, during which based on complete genome sequencing also India-1/Asia-5 strain was detected (Mourya et al., 2019). In Bangladesh, the presence of CDV is known, so far, the relationship has only been identified based on PCR and symptoms (Hossain & Kayesh, 2014; Rahman et al., 2017; Singh et al., 2015; Sultana et al., 2016; Tarafder & Samad, 2010; Yadav et al., 2017; Yousuf et al., 2014), but so far, we have no knowledge of sequence data from the country.

Next generation sequencing (NGS) technologies are increasingly being used to detect and characterize pathogens in wildlife (Arumugam et al., 2019; Conceição-neto et al., 2017; da Costa et al., 2021; Jo et al., 2019). MinION (Oxford Nanopore Technologies, Oxford, UK) has been used in many areas of virology, for instance, metagenomics or sequencing of complete genomes (Batista et al., 2020; Kilianski et al., 2015; Peserico et al., 2019; Young et al., 2021). Amplicon-based NGS sequencing of specific pathogens is a method for rapid detection and genomic characterization of target pathogens which may yield high-coverage genomic sequence information (Freed et al., 2020; Kemenesi et al., 2022; Lanszki et al., 2022; Park et al., 2021; Quick et al., 2016). With the aid of this technology, genomic surveillance is more feasible than before.

### *1.4. Introduction to carnivore species included in the dissertation*

A promising novel concept in biology is focusing on key players of the trophic network, which can indicate parameters of lower and upper levels, making them perfect indicators for ecosystem health. Small and medium size carnivores are positioned in the middle of trophic networks (Figure 4). Small and medium size carnivores are a species-rich, diverse group that is spread all over the world and has a wide range of ecological niches. Most of these species are secondary consumer level, which therefore occupy an intermediate trophic position, respond directly to influences from above and below trophic levels. Furthermore, the short life and higher reproductive rates enable rapid responses to smaller changes than the large carnivore species above them. Due to past and present persecution, large carnivores are often absent in large areas. Most of the species show a hiding behaviour and are difficult to study wild living populations. In summary, small and medium size carnivores are various species with relatively small territories that can be easily non-invasively monitored and investigated with the tools of ecology and molecular biology (Marneweck et al., 2022).

**Dog** has been closely associated with humans for thousands of years and have played various roles in human societies (Hervella et al., 2022). In developed countries, responsible dog ownership and vaccination against the most common infectious diseases of dogs are highly emphasized to ensure the well-being of both dogs and humans (Topál et al., 2009). Rabies, caused by the rabies virus (belongs to the *Lyssavirus* genus) is a major concern, and strict regulations are in place to control its spread. Vaccination not only safeguards individual dogs but also prevents pathogens from being transmitted to other animals or humans. However, the situation is quite different in other parts of the world, for example in southern Asia, where the number of stray or feral dogs is alarmingly high, and there are difficulties in vaccination (Bonwitt et al., 2020).

The **red fox (*Vulpes vulpes***; hereafter: fox**)** is a highly adaptable and one of the most widespread carnivores in the Northern Hemisphere and was introduced to Australia (Macdonald & Sillero-Zubiri, 2004). It is legally hunted year-round in Hungary. An adaptive and opportunistic forager, and due to its population size, the fox is one of the most significant mesopredator globally (Doherty et al., 2016; Soe et al., 2017). Its adaptability to urban habitats has led to frequent sightings of foxes in cities and suburban areas, where they take advantage of available food sources and shelter (Bateman & Fleming, 2012; Doncaster et al., 1990).

The **Eurasian otter (*Lutra lutra***; hereafter: otter**)** is a flagship species of nature conservation efforts throughout Europe, and it is a widely distributed piscivorous mustelid in Eurasia and portions of North Africa (Kruuk, 2006; Mason & Macdonald, 1986). The otter is a characteristic apex predator in aquatic food chains (Kruuk, 2006). It inhabits a wide variety of natural habitats (e.g., rivers, small waterflows, lakes and marshlands) and human-altered areas (fishponds, water reservoirs and recreational lakes). It is characteristically solitary, secretive and nocturnal (Kruuk, 2006; Mason & Macdonald, 1986). Currently, it is a near-threatened species on the IUCN Red List (*Lutra lutra. The IUCN Red List of Threatened Species 2021*, 2021), and it is listed as an animal species of European Community importance (EEA, 2009). The reason for its priority protection is the vulnerability of its population (Kruuk, 2006; Mason & Macdonald, 1986). Recent decades have seen an increase in otter populations in different areas of Europe (Conroy & Chanin, 2002; Yoxon & Yoxon, 2019), which implies that otters are more likely to encounter humans, domestic dogs, and other carnivores. In Hungary, the otter is a widespread but rare, strictly protected species with stable, interconnected populations (Lehoczky et al., 2015; Heltai et al., 2012). In terms of virological examinations, the Eurasian otter is a neglected predator species, therefore it is crucial to understand the dynamics, risks, and evolution of the most common viral diseases regarding this species.

Among terrestrial mustelids, the **steppe polecat (*Mustela eversmanii*)**, **least weasel (*Mustela nivalis*)**, **stoat (*Mustela erminea*)** and **pine marten (*Martes martes*)** are protected species in Hungary, the **European polecat (*Mustela putorius*)** is periodically considered, and the **stone marten (*Martes foina*)** is a legally hunted species throughout the year. Among them, in the Pannonian biogeographic region, the most common species is the stone marten. The stone marten and the European polecat are habitat generalist species (Blandford, 1987; Virgos et al., 2012). They occur in human settlements as well as in fragmented forest-field landscapes and wetlands. Least weasel inhabits various natural habitat types and agricultural areas (King & Powell, 2006). From the more habitat-specialist species, the stoat occurs mainly in wetlands and in mixed mosaic habitats (King & Powell, 2006), the steppe polecat occurs in relatively dry habitats, including steppes, grasslands and adapted to open agricultural areas in the Great Plain (Šálek et al., 2013), the pine marten in various types of forests and shrublands (Zalewski & Jędrzejewski, 2006). These mustelids belong to small mammal consumers and omnivorous trophic guilds (Lanszki et al., 2019). Frequent coexistence of up to 5-6 carnivore species and known killings among smaller related species (Lanszki et al., 2019) indicate interspecific encounters, these direct contacts can cause cross-infection.

**Family: Canidae**

**Genus: Canis**

Dog

Red fox

**Family: Mustelidae**

**Genus: Lutra**

Eurasian otter

**Genus: Martes**

stone marten

pine marten

**Genus: Mustela**

European polecat

steppe polecat

least weasel

stoat

**Figure 4.** Carnivore species included in the dissertation

## 2. Aims of the thesis

During the thesis work, our primary goal was to identify the knowledge and technology gaps in canine distemper virus research. This was achieved by thoroughly examining clinical cases and epizootic events related to CDV. Our goal was to develop an efficient and versatile genomic surveillance method that could be widely employed. We aimed to showcase the practical application of this method across multiple setups and scenarios to demonstrate its potential effectiveness. Through this research, we sought to contribute valuable insights to the field of CDV research and foster advancements in genomic surveillance techniques for better disease management and control (Figure 5).

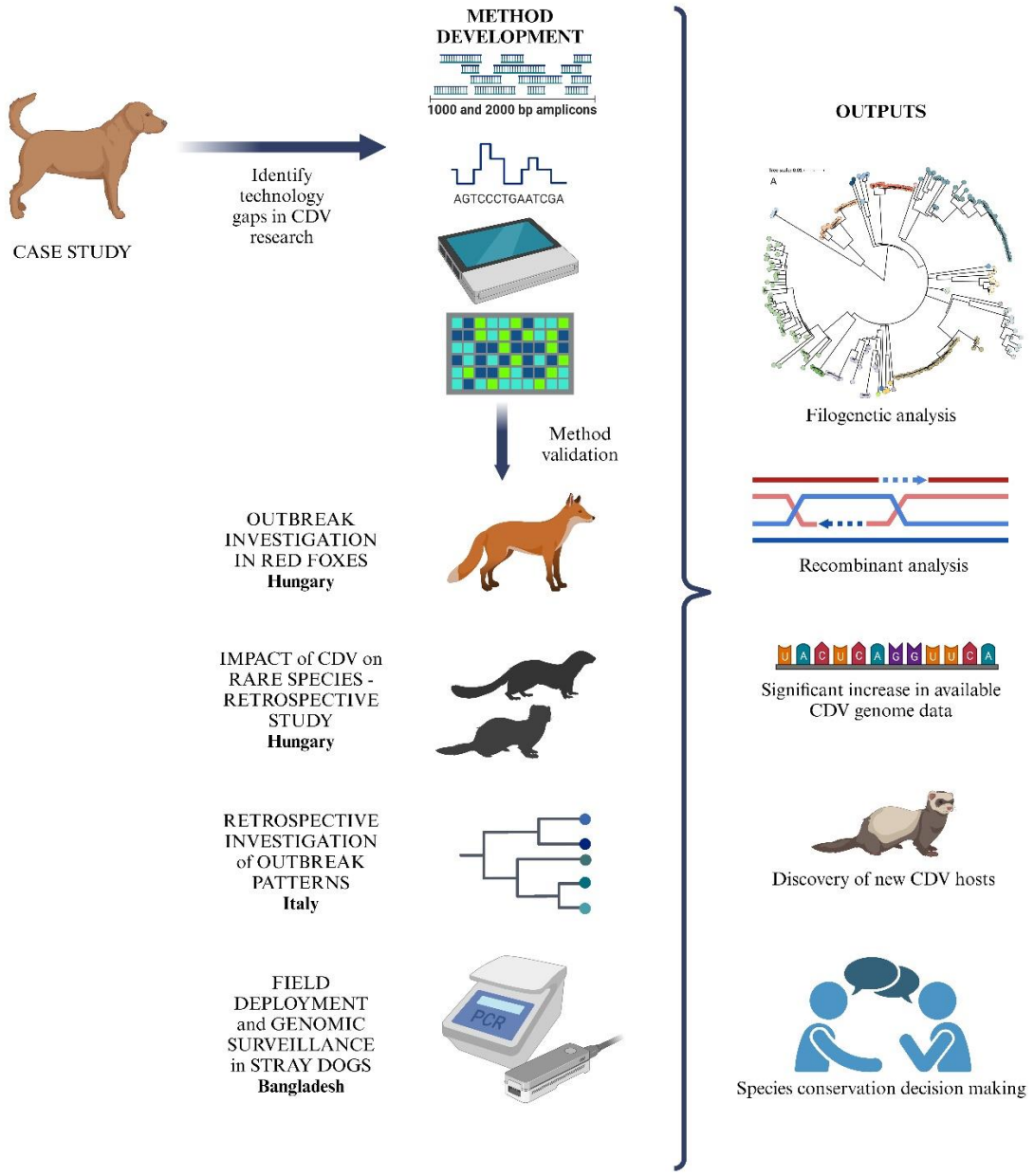



**Figure 5.** Graphical summary of the main elements of the thesis work (made by BioRender)

*Identifying technological gaps in CDV research – Clinical case study with dog*

Our aim was to observe the longevity and quantify the viral RNA shedding in the urine of a mixed-breed dog in Hungary, by monitoring the urine samples of the animal, which was previously described in other studies as the most suitable material for diagnostic purposes of CDV.

*Development of a novel genomic surveillance method and its application in outbreak investigation – CDV in red foxes*

Our aim was to investigate an epizootic event among red foxes in Hungary using our in-house rapid sequencing application and to understand the genomic characteristics of the epizootic CDV strain. We aimed to demonstrate the importance of genomic surveillance tools in outbreak investigation among wildlife, which may be a powerful tool in understanding the nature of recurring outbreaks in Europe.

*Retrospective genomic surveillance of CDV on a sample bank of Eurasian otters*

Our aim was to understand CDV's long-term epidemiology in the Eurasian otter, which has an utmost importance from a conservation perspective, and the presentation of novel sequence data is also highly relevant for better understanding CDV's evolution. We investigated samples from the past 21 years to detect CDV in road-killed Eurasian otter samples and aimed to demonstrate the feasibility of sequencing-based genomic surveillance in understanding the epidemiology of the virus in the past.

*Genomic surveillance among rare and elusive carnivore species - Terrestrial mustelids*

Our goal was to conduct a post-mortem retrospective surveillance study on road-killed mustelids to detect canine distemper virus RNA. Through complete genomic sequencing, phylogenetic analysis, and recombination analysis of the obtained virus sequences, we aimed to gain insight into the impact of CDV on mustelid populations.

*Understanding the genomic patterns of outbreaks in the past - Red foxes and domestic dogs from Italy*

Our objective was to examine the transmission and distribution of the canine distemper virus Arctic-like and Europe lineages in dogs and red foxes throughout Italy over a period of two decades. To achieve this, we conducted a retrospective analysis of CDV samples, focusing on the collection of viral samples for whole genome sequencing. Through our study, we aimed to enhance the existing knowledge on the spread of these two lineages within Italy by providing a comprehensive analysis of their distribution and transmission patterns.

*Mobile genomic surveillance in a snapshot study setup – CDV in stray dogs of Bangladesh*

Our main goal was to incorporate our genome surveillance method into a mobile laboratory capacity, allowing us to gather valuable insights into the prevalence of canine distemper virus and its genomic patterns in low-resource regions. By utilizing this approach, we aimed to expand our understanding of CDV dynamics and genetic diversity in areas with limited resources, contributing to the overall knowledge of CDV epidemiology and assisting in the development of targeted control strategies.

## 3. Materials and Methods

### *3.1. Sample collection*

#### *3.1.1. Clinical Case Study*

In this study a 1-year-old mixed-breed male dog (born in December 2017) afflicted with CDV infection in Hungary was monitored. The dog was kept in poor conditions and therefore was confiscated from its owner and transported to the Noah's Ark Animal Shelter Foundation in Budapest. Based on available data, the animal was infected with the virus prior to being transported to the shelter. Unfortunately, we do not have information regarding the vaccination history of the animal against CDV.

On 27 January 2019, the dog was taken to the veterinary clinic with severe diarrhea and difficulty breathing. During the general health inspection of rescued animals, the *Dirofilaria immitis* infection was discovered with the WITNESS Canine Heartworm Antigen Test (Zoetis). On 1 February 2019, the animal's symptoms improved, and the dog was returned to the shelter quarantine department. In the week following (8 February 2019), the animal was once again taken back to the veterinarian, this time with an intensive nasal discharge, cough and weight loss. The first CDV PCR test was performed with urine. Subsequently, the animal's condition improved steadily and rapidly, requiring no further medical attention. Within a few days, the animal became entirely asymptomatic, and on 14 February 2019 was again placed in the quarantine department of the shelter until the animal was declared healthy, in August 2020, after the PCR confirmed the viral clearance from the animal's urine. Urine samples from the shelter dog were collected multiple times (see details in Results) between February 2019 and August 2020.

#### *3.1.2. Outbreak investigation in red foxes*

Carcasses were collected opportunistically as part of the veterinary investigation of symptomatic cases at an animal rescue center specialized to fox rescue; namely "Állatmentő Szolgálat Alapítvány". After official veterinary diagnostic procedures, the samples for this study were additionally collected by the veterinary practitioner. At the end of the winter of 2021, the first reports of wild living red foxes with CDV symptoms arrived. Between spring and late summer, animal rescuers registered a minimum of 50 cases across the country. Most of the animals were cubs or juveniles at this period. Of these 50 animals, carcasses of 6 cubs and 1 adult fox were obtained during the spring period, and an additional 5 cubs, 10 juveniles and 2 adult foxes during the summer period. Samples were obtained for laboratory examination

after the fatal outcome of the disease. Fox carcasses were stored at -20 degrees at the veterinary clinic. During sampling, oral, nasal, and rectal swabs were collected with sterile sampling sticks into one tube per animal.

Symptomatic live foxes were also sampled with oral and nasal swabs at the rescue center. Sampling was conducted by the veterinary practitioner. Although the foxes were quarantined from the dogs living in the rescue center, saliva samples were taken from the dogs (n=5) as well. The dogs had no symptoms during the season. All samples were received at the request of the animal rescue center, to investigate the origin of the CDV strain.

*3.1.3. Retrospective collection of Eurasian otter samples*

The Eurasian otter carcasses were collected between 2000 and 2021 in Hungary. These animals were primarily (90%) road-killed individuals, whilst the remaining animals were found dead at their natural habitats (Lanszki et al., 2008; 2009; 2018). Animal collection localities cover two habitat types (stagnant waters or watercourses) and highlight the distribution of these animals within the country (Lehoczky et al., 2015). The animal carcasses were collected by the staff of the ten National Park Directorates and stored at −20° C until processing. The post-mortem examination was carried out and tissue samples of different organs were stored at −20° C by the Carnivore Ecology Research Group, Kaposvár University Campus, with permission from the competent authorities. A total of 339 lung tissue samples were collected from the carcasses using general dissection procedures (Simpson, 2000). The body condition (K-index) based on body mass and total length data was calculated for both sexes after Kruuk (2006) (Kruuk, 2006). The initial sampling strategy for these otter specimens was not linked to virological studies, therefore from these animals only lung samples were taken during dissection.

Identification numbers of research permits for years: 2002: 3498/2002 (KvVM-KJHF), 2003: 215/2003 (KJHF), 2004: 189/3/2004 (KJHF), 2005-2009: KJHF-837/6/2005 and 14/3347/3/2005, 2010-2013: 14/1239-1/2010 (OKTVF), 2014-2017: 14/8553-17/2013 (OKTVF), 2019-2022: PE-KTFO/508-4/2019 (PMKH).

*3.1.4. Sample collection from multiple species of the terrestrial mustelids*

Road-killed mustelids (n=170) were collected in Hungary between 1997 and 2022 by the staff of National Park Directorates and volunteers and stored at −20 °C until processing. Tissue samples from spleen (and lung; in case spleen sample was not available) via general dissection procedures were collected from the steppe polecat (n=64), European polecat (n=36),

stone marten (n=36), pine marten (n=18), least weasel (n=10) and stoat (n=6). The post-mortem examination (Figure 6) was carried out and tissue samples were stored at −20 °C by the Carnivore Ecology Research Group at the Kaposvár University Campus and by the Hungarian Natural History Museum, Budapest. We scored the body condition based on fat deposit over flanks between 1 (poor), 2 (average) and 3 (good) (Simpson, 2000). A few months before nucleic acid extraction, they were deposited in the National Laboratory of Virology at −80 °C.

Research and sample collection permits were issued by the relevant authorities to the Kaposvár University Campus (SO-04Z/TO/392-2/2019) and to the Hungarian Natural History Museum (14/6156/7/2011, OKTF-KP/6903-21/2015, PE-KTF/736-6/2017, PE-KTFO/329-16/2019, PE-KTFO/1568-18/2020, PE-KTFO/1403-3/2022).

**Figure 6.** Dissection of polecats in the Hungarian Natural History Museum, Budapest

### *3.1.5. Sample collection from red foxes and dogs in Italy*

In Italy, the collection of samples from dogs and foxes took place between 2005 and 2019 (Table 1). The storage and processing of the samples were carried out at the University of Bari at the Department of Veterinary Medicine of Bari.

**Table 1.** Sample numbers indicated for years and species

| Year | 2005 | 2006 | 2008 | 2009 | 2010 | 2011 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dog | 2 | 2 | 1 | 1 | 1 | 1 | 3 | 1 | 2 | 2 | 1 | 1 | 2 |
| Red fox | - | - | - | 2 | 1 | - | - | - | - | - | - | - | - |

### *3.1.6. Sample collection from stray dogs in Bangladesh*

We collected oral swab samples (n=257) from stray dogs in two major cities of Bangladesh, Rajshahi and Chattogram (Figure 7) in 2023. Random sampling in Rajshahi was performed between 20-28 February (n=135) and in Chattogram between 4-5 March (n=122) among stray dogs with the aid of local veterinary students and animal rescuers. The samples were collected using a sterile stick and placed in 500 µl phosphate-buffered saline (PBS). In the case of morning collection, the samples were processed immediately on the day of collection in a mobile laboratory setup, whilst in the case of evening collection, they were stored the next day at −20 °C until processing.

**Figure 7.** Stray dogs in Bangladesh

*3.2. Nucleic acid extraction*

In the case of the shelter **dog**, the total RNA was extracted using a Quick-RNA MiniPrep kit (Zymo Research, USA).

In the case of the **red foxes**, all swabs were homogenized in 500 µl of phosphate-buffered saline. 100 µl of the supernatant was used for RNA extraction using the Monarch total RNA miniprep kit (NEB, USA).

In the case of the **Eurasian otters**, lung tissues were homogenized in 500 µl of phosphate buffered saline (PBS), using the Bertin Minilys machine at maximum speed for 3 min, supplemented with two glass beads per sample to facilitate tissue disruption. Following brief centrifugation, 100 µL of the supernatant was used for RNA extraction using the Monarch total RNA miniprep kit (NEB, USA).

In the case of the **terrestrial mustelids**, for most animals, nucleic acids were extracted from the spleen, but lung was substituted when spleen was not available. Tissue samples were homogenized in 500 µl phosphate buffered saline, using a TissueLyser LT device (Qiagen, Hilden, Germany) at maximum speed for three minutes, supplemented with two glass beads per sample to facilitate tissue disruption. The total RNA was extracted using the Monarch Total RNA Miniprep Kit (NEB, USA) in full adherence to the manufacturer's recommended guidance.

In the case of the **dog and fox samples collected and stored in Italy**, nucleic acid extraction was carried out at the University of Bari.

In the case of **stray dogs from Bangladesh**, the total RNA we extracted from oral swab supernatants after throughout vortexing and spinning down, using Direct-Zol RNA MiniPrep (Zymo Research, USA).

*3.3. PCR reactions*

All the samples were screened with a CDV-specific real-time RT-PCR method (Elia et al., 2006). All PCRs were performed using the QIAGEN One-Step RT-PCR Kit (Qiagen, Germany) in full compliance with the manufacturer's recommendations by using 5x Qiagen OneStep RT-PCR Buffer 5 µl, 600 nM of each primer (forward and reverse) 0,5-0,5 µl, 400 nM of probe 0,5 µl, 10 mM dNTPs 1 µl, Qiagene OneStep RT-PCR Enzyme Mix 1 µl, RNA 5 µl and nuclease free water to make a total volume of 25 µl and subjected to a thermal cycler at one cycle of 50 °C for 30 min for the reverse transcription of RNA to cDNA, followed by one cycle at 95 °C for 15 min. The cDNA was amplified by PCR for 50 cycles, each cycle consisting of

denaturation at 94 °C for20 sec, annealing at 46 °C for 30 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 10 min. All PCRs were run on the MyGo Pro PCR system platform (IT-IS Life Science, Ireland). RT-PCRs were performed immediately following RNA extraction without freeze-thawing the nucleic acid to avoid possible RNA degradation for improved output in complete genomic sequencing activities. For negative PCR control, we used nuclease-free water, whilst the positive control was a previously CDV-positive sample.

In the case of one otter sample, in which the virus titer was too low for complete viral genome sequencing, and in the case of the dog, where only H gene sequencing was performed we applied a specific PCRs reaction, targeting only the Hemagglutinin gene (1824 bp) of CDV with previously published primer sets (Sekulin et al., 2011). Due to multiple unsuccessful PCR amplification attempts in the case of the dog sample, the primer pair (472f and 1172r) was replaced by newly designed primers (649f: 5'-CGCCTAGTAAGATCAAAGTG-3' and 1216r: 5'-ACTTGATCCATAGGTGTTGC-3').

For the dog and positive otter samples, RT-PCR standard curve was generated by serial 10-fold dilutions of a corresponding CDV amplicon with a known copy number in a range of $1 \times 10^{10}$ to $1 \times 10^{1}$. These dilutions were measured in triplicate, and the measured results were used to construct the standard curve, which was subsequently used to determine the copy number from threshold cycle values (Ct) of the samples.

### *3.4. Sanger Sequencing*

Final CDV amplicons from dog samples in the clinical case study were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit in full accordance with the manufacturers' protocol regarding ABI Prism 310 DNA Sequencer platform (Applied Biosystems, USA) and CDV amplicons from otter sample, when the virus titer was too low, was performed by an external service provider (Eurofins Genomics, Germany).

### *3.5. MinION library preparation, sequencing, and data analysis*

The complete genome sequencing was performed with MinION nanopore sequencing technology (Oxford Nanopore Technologies, UK). We developed an amplicon-based sequencing method based on previous protocols (Quick, 2019, 2020). The detailed protocol and the primers are available at our laboratory protocols.io page (Protocols.io, 2021). cDNA preparation from the CDV positive RNA sample was conducted with Superscript IV (Invitrogen, USA) using random hexamers. Two sets of primers were used to generate

overlapping genome fragments that differ in the length of amplicons (1000 bp, 2000 bp). These multiplex PCRs were conducted directly from the cDNA with the usage of Q5 Hot Start HF Polymerase (New England Biolabs, USA). Following the amplicon PCR DNA from the same primer set (1000 or 2000) were purified with AMPure XP beads (Beckman Coulter, USA) as per manufacturer's instructions. The end-repair and dA tailing were performed with the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs, USA). End-prepped DNA were transferred to the next reaction directly and the barcode derived from EXP-NBD196 (Oxford Nanopore Technologies, UK) were ligated with NEBNext Ultra II Ligation Module (NEB, USA). After, the pooled barcoded samples were jointly cleaned up with Ampure XP beads, the AMII sequencing adapters were ligated with NEBNext Quick Ligation Module. The final library was quantified with Qubit dsDNA HS Assay Kit (Invitrogen, USA) on Qubit 3 fluorometer. The sequencing runs were performed on a R9.4.1. (FLO-MIN106D) flow cell with the AMX-F motor protein from SQK-LSK110 kit (Nanopore Technologies, UK).

The ONT guppy software was run under Ubuntu Linux 18.04. Base-calling with super-accuracy basecaller algorithm (dna_r9.4.1_450bps_sup config file), were carried out with Guppy basecaller (version 5.0.7. and 6.0.1.). Demultiplexing and trimming of barcodes were performed with Guppy using default parameters of "guppy_barcoder" runcode. The demultiplexed reads were length filtered when reads under 800 basepair in the case of 1000 primer set and under 1800 basepair by the 2000 primer set were eliminated from the dataset. Additional 50 basepair were trimmed from the both ends of the reads and were mapped to the MN267060 to generate preconsensus with the usage of Genious mapper (version Geneious Prime 2021.2.2 and 2022.1.1.). To obtain polished consensus sequences, the trimmed reads were mapped against the preconsensus using Medaka versions (version 1.4.2 and 1.6.0.). Finally, the generated consensus sequences were manually checked for base-calling errors especially in the homopolymeric regions.

*3.6. Phylogenetic analysis*

Prior to the phylogenetic reconstruction, cognate sequences were retrieved from the GenBank database (NCBI, Bethesda, USA) and aligned with our sequences in the MUSCLE alignment webserver (Edgar, 2004). As the GenBank database has expanded over time, incorporating numerous new sequences, variations can be observed in phylogenetic analyses.

Case of the **dog**, the final dataset comprised 59 either complete or partial H gene sequences; the final sequence length was 1824 nucleotides. Subsequently, the maximum likelihood phylogenetic tree was constructed under the Tamura 3-parameter with gamma-

distributed rate heterogeneity (T92 + G) substitution model and 1000 bootstrap replicates using MEGA X (MEGA, Pennsylvania, PA, USA) (Tamura et al., 2021).

Case of the **red foxes**, sequences for both datasets (complete genomes and H genes) were first aligned in MAFFT webserver using default parameters. Thereafter, IQTREE webserver was used for both best substitution model selection and maximum likelihood phylogenetic tree reconstruction using ultrafast bootstrapping. The complete genomes phylogenetic tree analysis was performed under the GTR+F+I+G4 substitution model chosen according to Bayesian Information Criterion (BIC). Whereas, the H gene phylogeny was implemented under the TVM+F+I+G4 according to BIC.

Case of the **Eurasian otter**, two datasets were used for phylogenetic tree analysis comprising 180 and 843 complete genomic and complete Hemagglutinin gene sequences, respectively. Sequences were aligned in MAFFT webserver using default parameters. Subsequently, in IQ-TREE webserver, both best substitution model selection and maximum-likelihood phylogenetic tree reconstruction were performed with ultrafast bootstrapping.

Case of the **terrestrial mustelids**, two datasets were used for phylogenetic tree analysis comprising 221 complete genomes and 969 complete Hemagglutinin gene sequences, respectively. Subsequently, the Maximum Likelihood phylogenetic tree was constructed under the General Time Reversible Model, Gamma Distributed with Invariant Sites (GTR+G+I) substitution model with best model selection in MEGA X (MEGA, Pennsylvania, USA) (Tamura et al., 2021).

Case of the **dogs and foxes from Italy**, one dataset was used for phylogenetic tree analysis comprising 256 complete genomes. All sequences were aligned with MAFFT v. 7.505 (Katoh & Standley, 2013). Model parameters (GTR+I+G) were determined with ModelTest-NG v0.1.7 (Darriba et al., 2020). The tree was generated using RaxML-NG v. 1.2.0 (Kozlov et al., 2019) with 1000 bootstraps.

Phocine distemper virus (PDV) was used as an outgroup for all phylogenies. The resultant trees were edited in iTOL (iTOL, Heidelberg, Germany) (Letunic & Bork, 2021).

### *3.7. Recombinant analysis*

The potential recombinant CDV genomes were tested through recombination analysis using similarity plot and bootscan analyses in SimPlot software package (version 3.5.1.) (Lole et al., 1999). The recombination analysis was modeled with Kimura 2-parameter distance model using a window size of 600 bp and step size of 20 bp in the case of complete genomes and H gene sequences.

## 4. Results

### *4.1. Clinical Case Study*

#### *4.1.1. PCR detection and sequencing*

The urine samples were PCR-positive in February, March, April, May, July, August, September, October and November 2019 and January, February and June 2020 and finally tested negative in August 2020. The dog excreted the virus RNA for 17 months, which we successfully verified and quantified with qRT-PCR (Table 2).

**Table 2.** Detection of canine distemper virus RNA by real-time RT-PCR with corresponding viral genomic copy numbers (Lanszki et al., 2021).

| | **2019** | | | | | | | | | **2020** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | February | March | April | May | July | August | September | October | November | January | February | June |
| Ct value | 18,48 | 22,66 | 20,37 | 23,9 | 32,66 | 39,75 | 38,3 | 39,3 | 40 | 39,39 | 40 | 41 |
| cRNA (*n* copies) | 2510955 | 207819 | 813841 | 99235 | 536 | <100 | <100 | <100 | <100 | <100 | <100 | <100 |

The full-length Hemagglutinin nucleotide sequence (2044 nt) was sequenced and submitted to GenBank database (accession number: MW248101). Based on the GenBank BLASTn search, the sequence depicted the highest nucleotide similarity (99.34%) with a representative sequence of the CDV Arctic-like lineage (KR002657) identified previously from a domestic dog which was transported from Hungary to Switzerland in 2013 (Willi et al., 2015). It displayed 99.23% nucleotide identity with the KX943323 sequence that originated from a domestic dog in 2015 (Mira et al., 2018). Similarly, 99.01% identity was observed with a sequence characterized from Apennine grey wolves (*Canis lupus italicus*) in 2013 (KC966928) (Di Sabatino et al., 2014). Likewise, it shared 98.52% and 98.83% identity regarding the nucleotide level with Italian sequences obtained from domestic dogs in 2005 (DQ226088) (Martella et al., 2006) and 2008 (HM443706) (Monne et al., 2011), respectively. It further represented a high nucleotide similarity 98.19% to an Arctic-like lineage formerly described in a domestic dog (DQ889184) from Hungary in 2005 (Demeter et al., 2007).

*4.1.2. Phylogenetic analysis*

Based on the phylogenetic analysis involving all known genotypes (Figure 8), the CDV strain explored in the case study belongs to the Arctic-like genetic lineage of the virus. It is positioned in the genetic cluster of previously reported CDV sequences from Italy, Austria, Hungary and Switzerland.

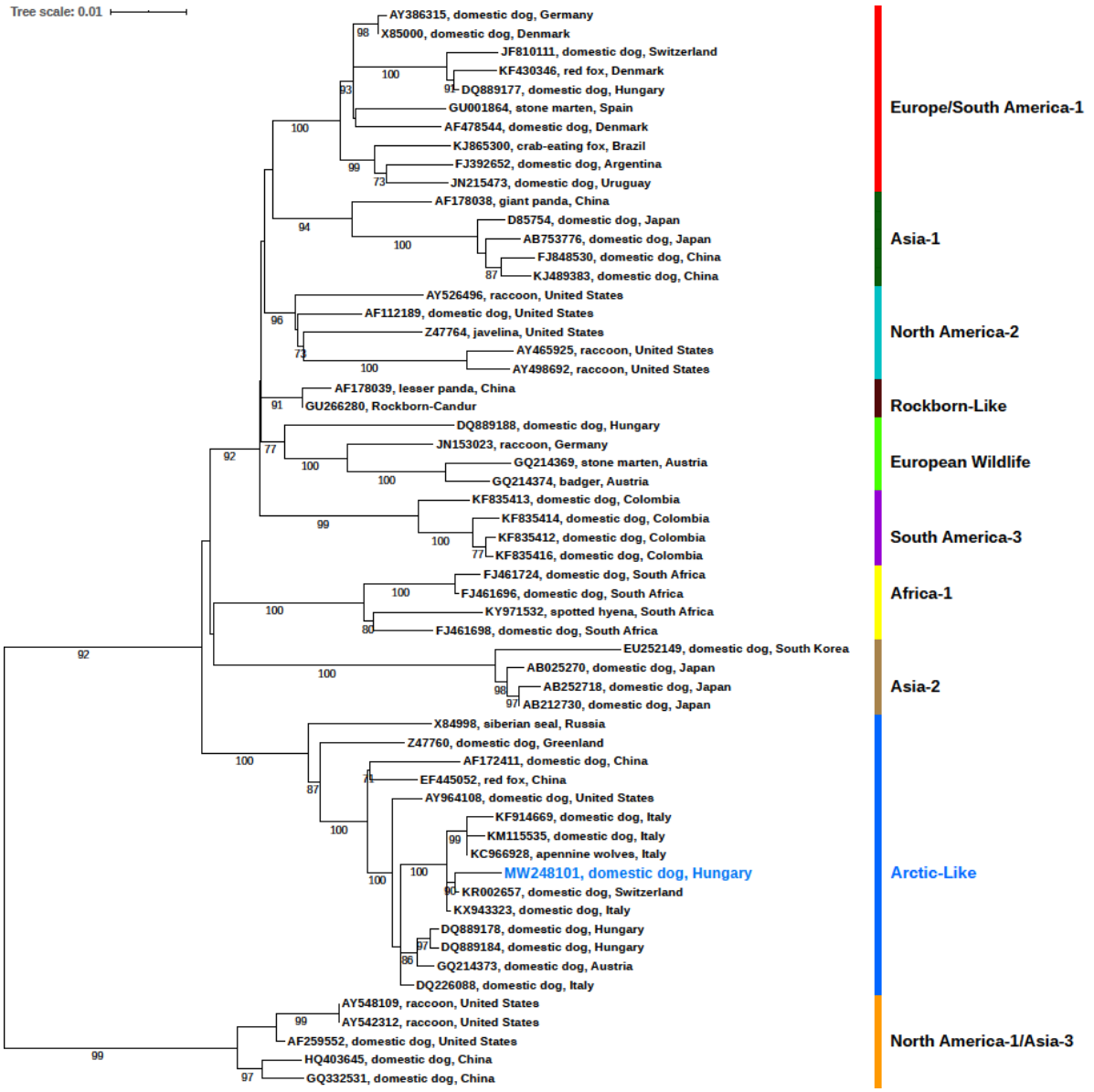



**Figure 8.** Phylogenetic tree based on the full-length Hemagglutinin (H) nucleotide sequences. Phocine distemper virus (PDV) (GenBank accession number: AF479277) was used as an outgroup to root the phylogenetic trees. Bootstrap values lower than 70% are not shown. The sequence of interest is highlighted in bold and blue color (Lanszki et al., 2021).

*4.2. Red foxes from Hungary*

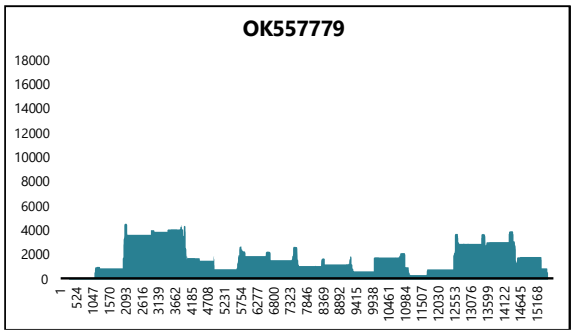
*4.2.1. PCR detection and sequencing*

Carcasses of 6/5 cubs and 1/1 adult fox from the spring period, and 5/3 cubs, 10/10 juvenile and 2/2 adult foxes from the summer period were positive for CDV with RT-PCR. A total of 21 of the 24 foxes tested were positive. After the results, 19 samples were selected for further sequencing, based on their low Ct (correlates with higher viral load) during the real-time PCR reaction (Table 3). Finally, 19 complete CDV genomes were sequenced from foxes with a pan-genotype CDV-specific amplicon-based sequencing method resulting in high sequencing coverage (Figure 9). We retrieved the complete genomic data of all 19 samples and submitted these to the GenBank (NCBI) database. The dog samples collected at the rescue center were tested negative for CDV.

The development of the sequencing protocol is a main result of the current thesis. Following the principles of open science, the complete protocol is available at protocols.io website (Protocols.io, 2021).

**Table 3.** Sequencing and diagnostic parameters of the investigated fox samples. Most relevant next-generation sequencing quality data as the mapped reads and mean coverage per sample is presented. Number of multiplex PCR cycles is relevant for the amplicon-based NGS sequencing workflow (Lanszki et al., 2022).

| Accession Number | Season | Age Category | RT-qPCR Ct value | Number of multiplex PCR cycles | Processed and Mapped reads | Mean coverage on the targeted region (reads) |
|---|---|---|---|---|---|---|
| OK557779 | Summer | Cub | 21,50 | 25 | 19846 | 1868,1 |
| OK557780 | Summer | Cub | 24,15 | 25 | 14192 | 1347,4 |
| OK557781 | Summer | Juvenile | 29,25 | 28 | 14244 | 1398,3 |
| OK557782 | Summer | Cub | 26,02 | 28 | 14491 | 1231,2 |
| OK557783 | Summer | Juvenile | 27,12 | 28 | 12588 | 1090,2 |
| OK557784 | Summer | Juvenile | 25,46 | 28 | 6513 | 579,0 |
| OK557785 | Summer | Adult | 36,90 | 36 | 14186 | 1263,0 |
| OK557786 | Summer | Juvenile | 27,83 | 28 | 11827 | 1011,4 |
| OK557787 | Summer | Juvenile | 38,58 | 36 | 10750 | 1102,3 |
| OK557788 | Summer | Juvenile | 28,15 | 28 | 8871 | 810,3 |
| OK557789 | Summer | Adult | 38,25 | 36 | 4485 | 320,2 |
| OK557790 | Summer | Juvenile | 28,32 | 28 | 10203 | 1114,6 |
| OK557791 | Summer | Juvenile | 26,74 | 28 | 8494 | 801,7 |
| OK557792 | Summer | Juvenile | 22,83 | 25 | 7461 | 674,2 |
| OK557793 | Summer | Juvenile | 21,49 | 25 | 12266 | 1232,6 |
| OK557794 | Spring | Cub | 31,33 | 32 | 93228 | 5662,0 |
| OK557795 | Spring | Cub | 31,91 | 33 | 63959 | 3984,3 |
| OK557796 | Spring | Cub | 30,78 | 32 | 56054 | 3460,9 |
| OK557797 | Spring | Adult | 32,69 | 33 | 80721 | 4696,6 |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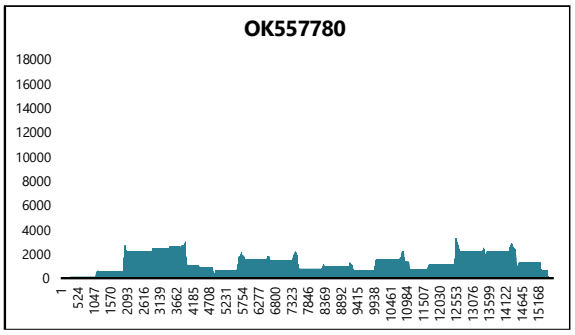
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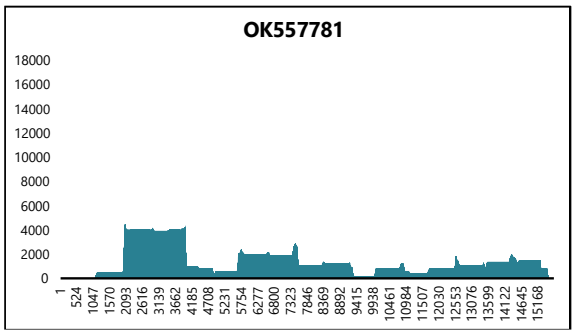
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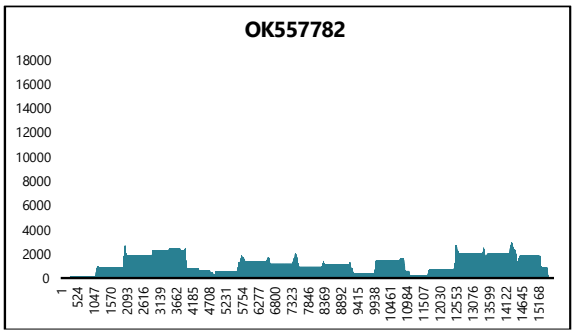
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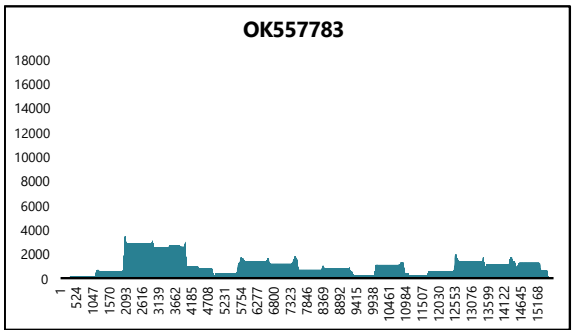
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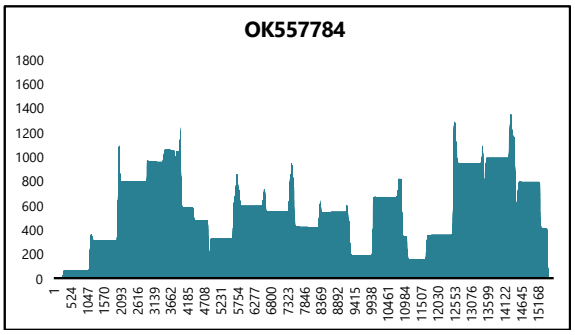
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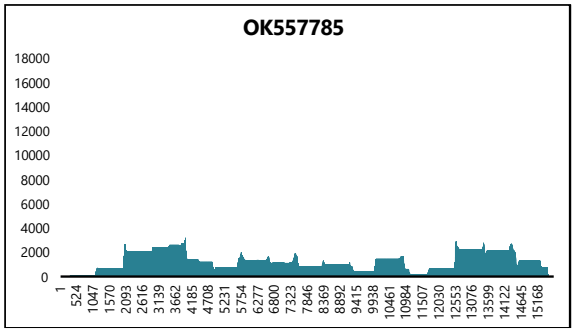
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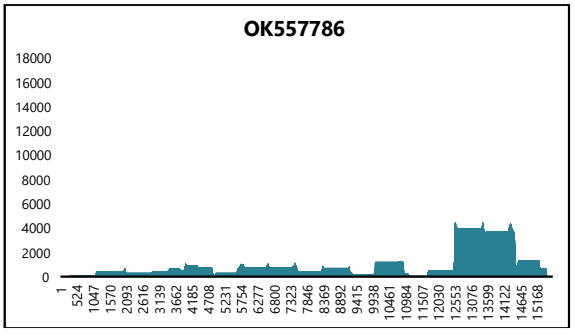
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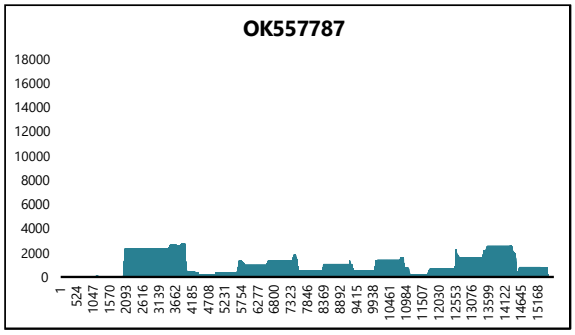
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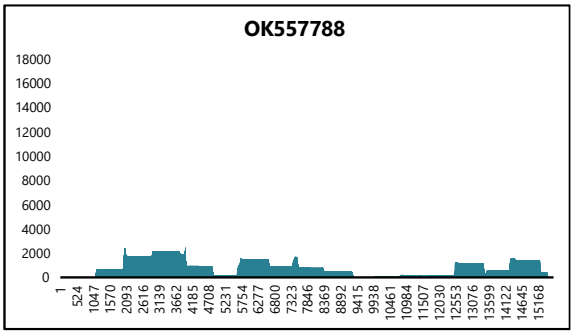
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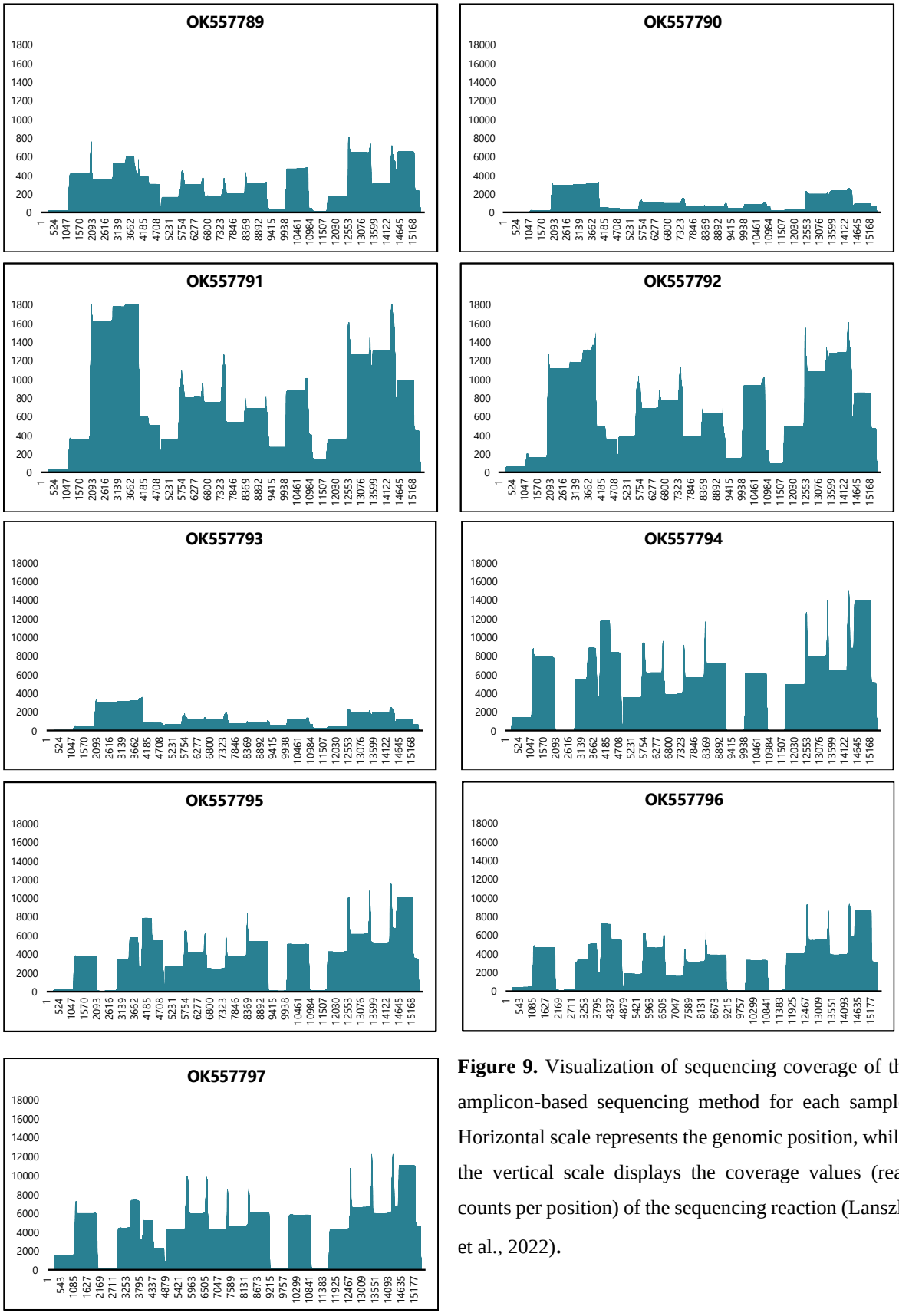


**Figure 9.** Visualization of sequencing coverage of the amplicon-based sequencing method for each sample. Horizontal scale represents the genomic position, whilst the vertical scale displays the coverage values (read counts per position) of the sequencing reaction (Lanszki et al., 2022).

*4.2.2. Phylogenetic analysis and amino acid differences in the H gene proteins*

Based on the phylogenetic analysis of all currently recognized genotypes (Figure 10 and 11), the CDV strains of this study belong to the Europe lineage. They are positioned in the genetic cluster of previously reported CDV sequences in Europe. We present the closest relation of the epizootic sequence cluster to a Hungarian dog sample from 2004, that is a unique branch within the Europe lineage of CDV and sequences of foxes from Germany in 2008, however the node connecting to this sequence cluster clearly indicates the lack of sequence data from previous years (Figure 11). Therefore the source of the current epizootic strain remains unknown, nevertheless we present the closest genetic relation to regional sequences, supporting the epizootic potential of locally circulating CDV strains. Phylogenetic analysis of both the complete genome and the H protein sequence clearly showed that the current epizootic sequences are genetically related to the enzootic Europe genetic lineage of CDV.

All of the 19 H gene sequences from foxes contained G at position 530 and Y at position 549 which correlates with the constellation in the CDV sequence from a dog in Hungary, 2004, the closest known relative to the current epizootic strains.

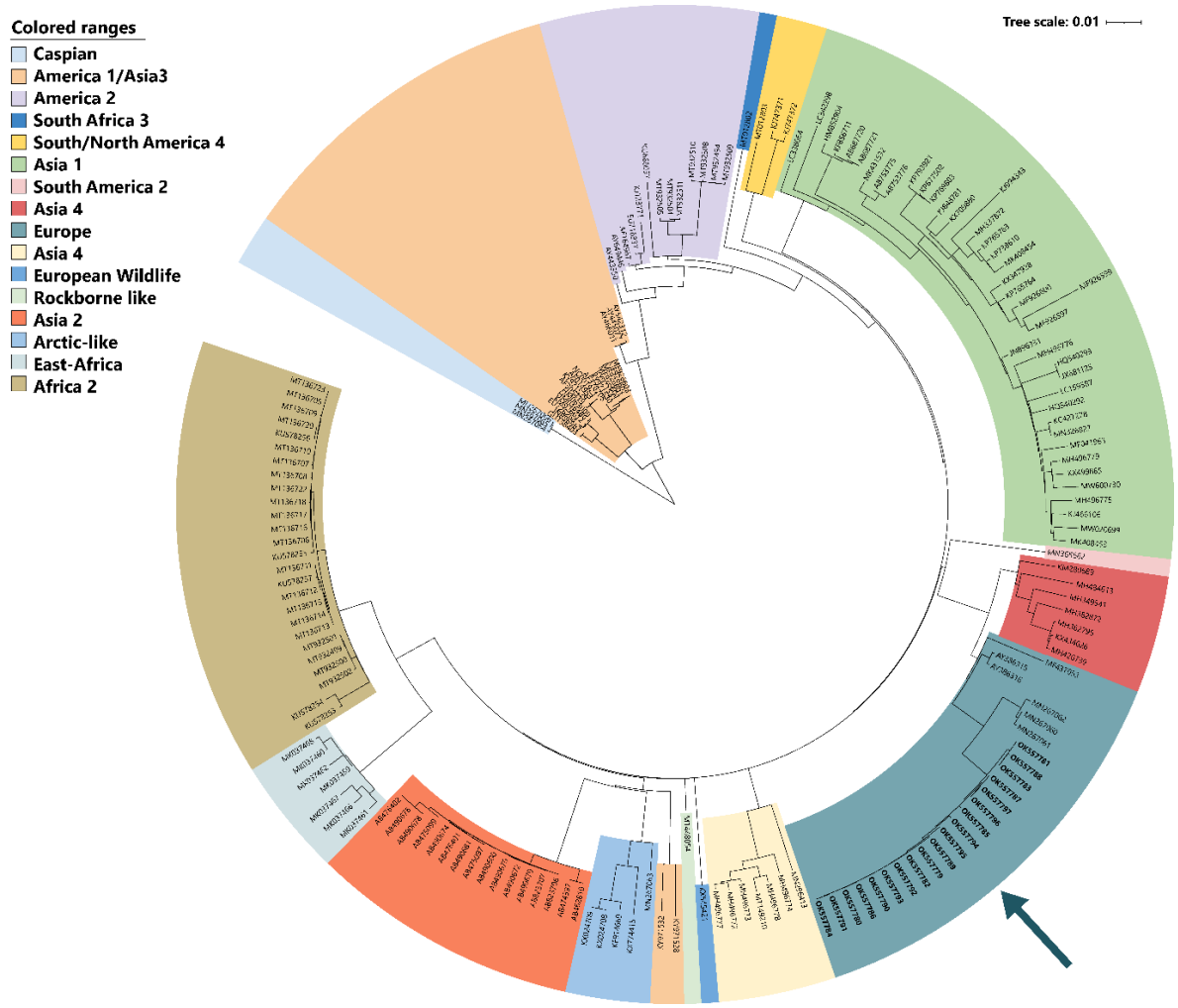


**Figure 10.** Maximum Likelihood phylogenetic tree based on 179 CDV complete genomes nucleotide sequences. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root the phylogenetic tree. The Europe lineage of interest is highlighted in light blue (Lanszki et al., 2022).

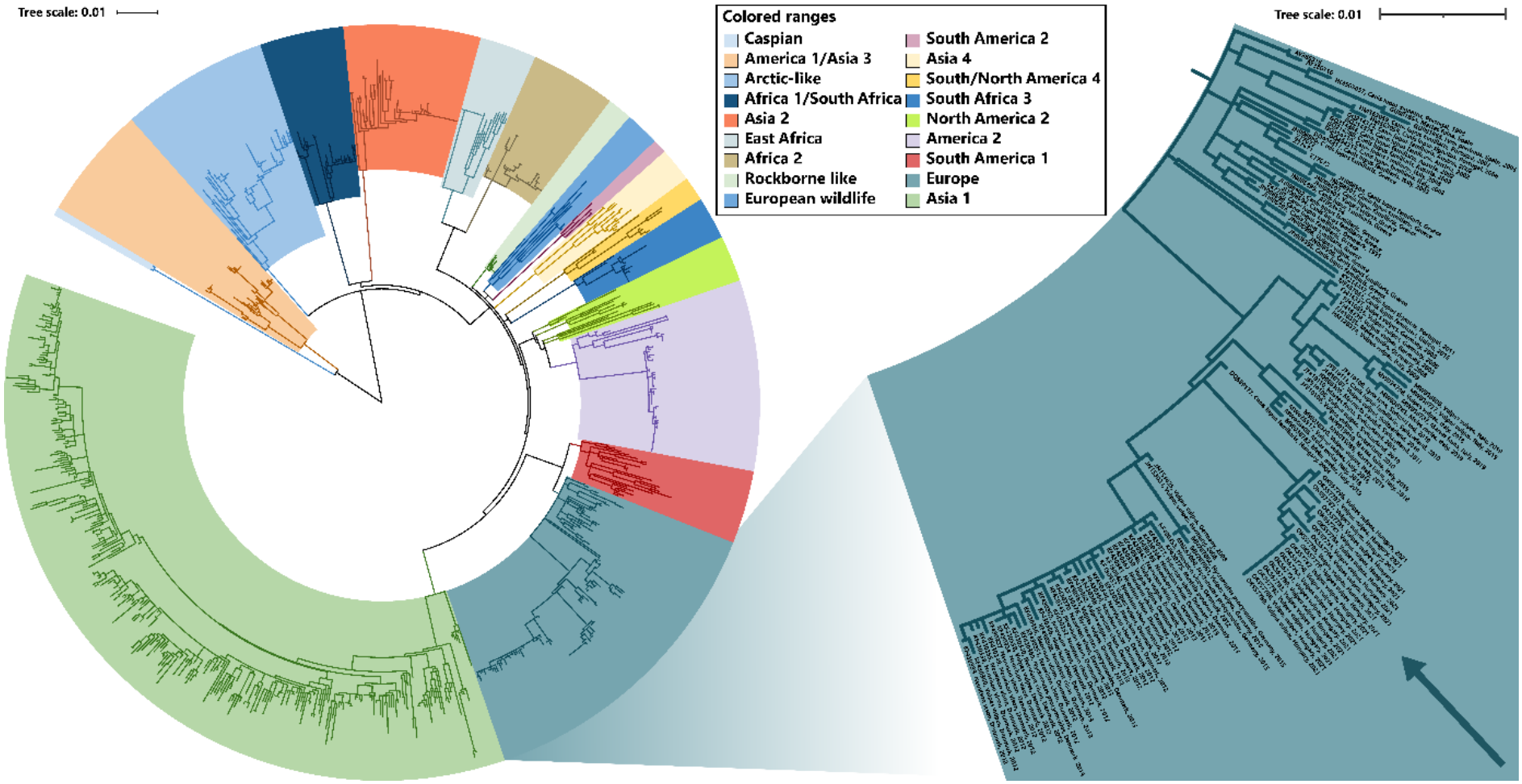


**Figure 11.** Maximum Likelihood phylogenetic tree based on 846 complete Hemagglutinin (H) nucleotide sequences. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root the phylogenetic tree. The Europe lineage of interest is highlighted in light blue (Lanszki et al., 2022).

### *4.3. Eurasian otters from Hungary*

#### *4.3.1. RT-PCR screening*

Eurasian otter samples were collected from all nineteen counties throughout Hungary. From these, canine distemper virus RNA was identified by real-time RT-PCR screening in 2/339 samples. Both originated from western Hungary; one was collected in 2006 and the other in 2010. The first infected animal was a young (4–5 months old) male in poor body condition (K = 0.80) (Kruuk, 2006) and was found to be deceased due to natural infection, on the edge of a marshland (Kis-Balaton). The second was an adult male in good body condition (K = 1.19), found as road-killed near a river (Rába). Consequently, only 2 of the 339 samples were found to be CDV-positive, although this minimum case number does not exclude the possibility of the presence of CDV in the other samples, since we had no data on the viral RNA degradation in these samples.

#### *4.3.2. Sequencing results*

Based on the real-time RT-PCR results, the viral genomic copy numbers were calculated as follows: ~1,021,000 copies/µL of the sample from 2006 and <10 copies/µL from the sample from 2010. The complete genome nucleotide sequence was determined for the sample from 2006 using Oxford Nanopore sequencing technology (Figure 12). To our knowledge, these are the first two CDV genome sequences from the Eurasian otter. The full-length Hemagglutinin nucleotide sequence of the other sample was obtained with specific PCRs and Sanger sequencing. These sequence data were submitted to the GenBank databases (accession numbers OM811640 and OM811639).

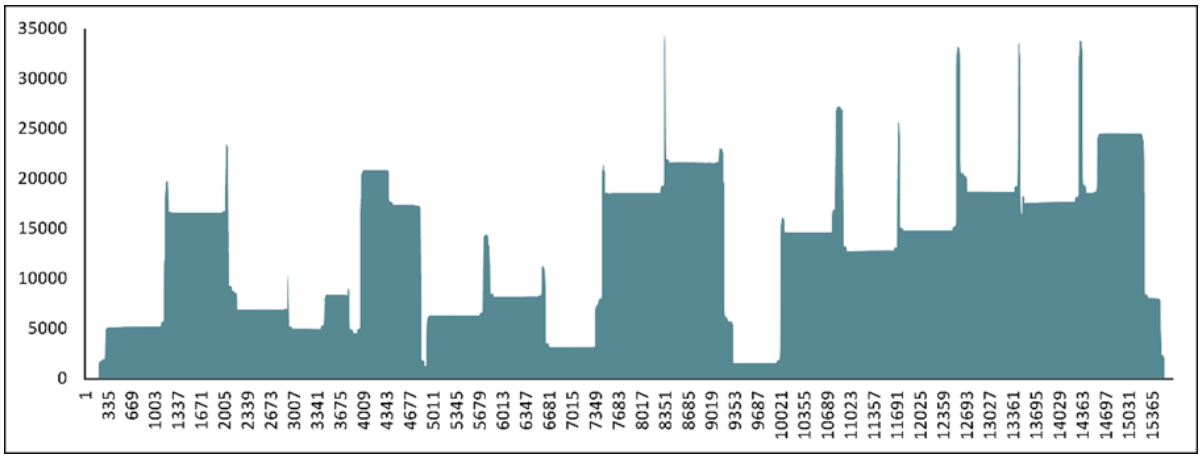


**Figure 12.** Visualization of sequencing coverage via the amplicon-based sequencing method for the sample from 2006, with all primer sets (1000 and 2000 bp). The horizontal scale represents the genomic position, while the vertical scale displays the coverage values of the sequencing reaction (Lanszki et al., 2022).

### *4.3.3. Phylogenetic analysis*

Based on the phylogenetic analysis of the hemagglutinin gene sequences, the two CDV sequences in our study belong to the Europe lineage. The complete-genomic-sequence-based analysis confirmed this observation (Figure 13). Both sequences clustered in a separate cluster among the other Europe sequences, separately from other clusters described for the other species, such as foxes. More studies are needed to reveal the presence and understand the risk of cross-species transmission events between otters and other carnivores. Our data revealed the presence of a distinct strain that was detected in two different years within the same region of Hungary. This finding suggests the existence of previously unknown diversity among these animals, highlighting the need for further exploration and investigation into the genetic variations of CDV in this species.

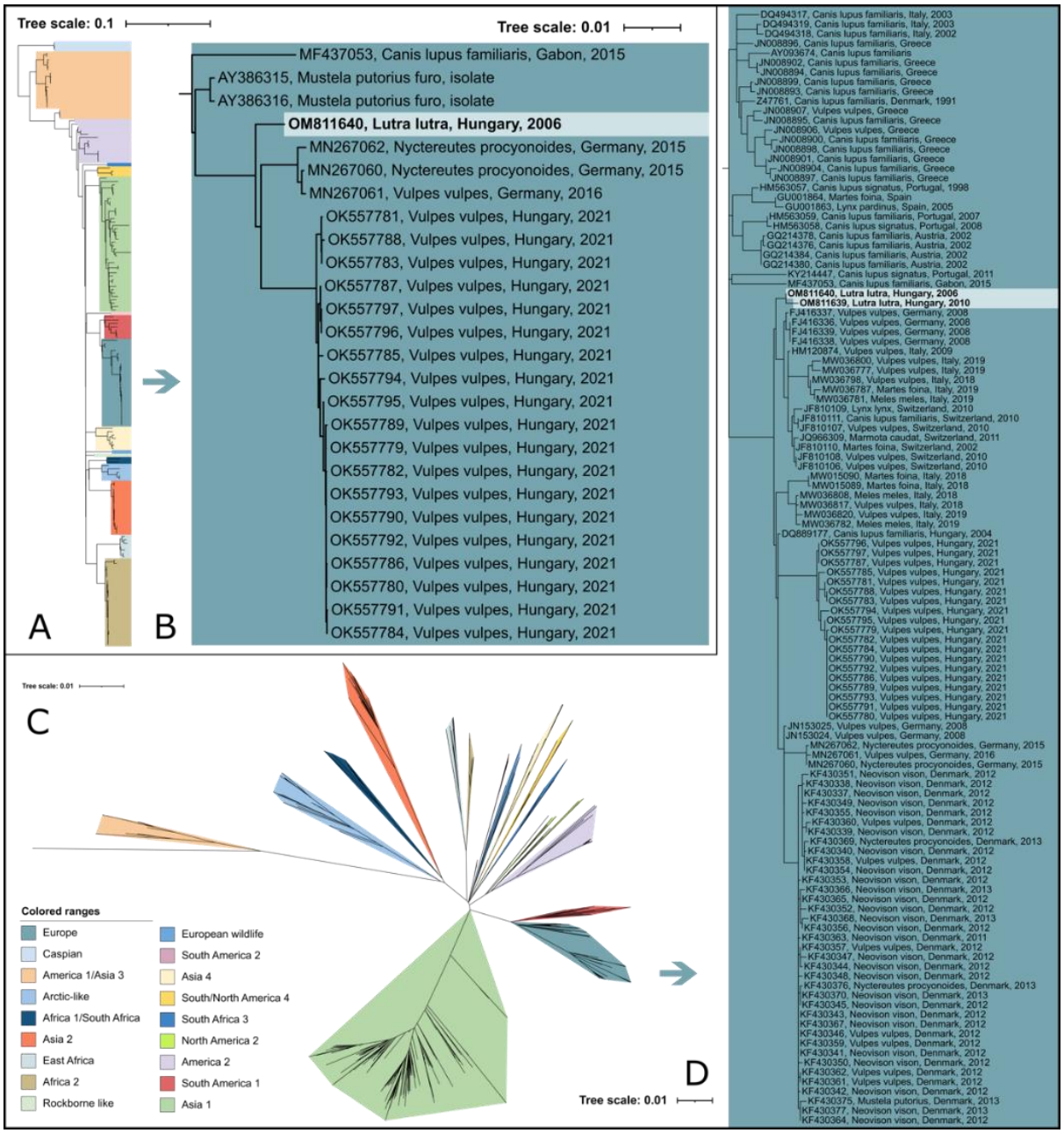


**Figure 13.** (**A**) Maximum-likelihood phylogenetic tree based on 180 CDV complete-genome nucleotide sequences. (**B**) The Europe lineage of interest is highlighted in blue color. (**C**) Maximum-likelihood phylogenetic tree based on 843 complete hemagglutinin (H) nucleotide sequences. (**D**) The Europe lineage of interest is highlighted in blue. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root both phylogenetic trees (Lanszki et al., 2022).

### *4.4. Terrestrial mustelids from Hungary*

#### *4.4.1. RT-PCR screening*

Canine distemper virus RNA was detected in three out of the six investigated species: 2 positives out of 64 steppe polecats, 1 positive out of 36 European polecats and 2 positives out of 36 stone martens (Table 4).

**Table 4.** Sample numbers of mustelids tested for canine distemper virus in Hungary (Total/Positive). The years 1998, 1999, 2001, 2013, 2014 and 2015 are not included in the table, there was no sample from these years (Lanszki et al., 2022).

| | Steppe polecat | European polecat | Stone marten | Pine marten | Least weasel | Stoat |
|---|---|---|---|---|---|---|
| 1997 | - | - | 1 | - | - | - |
| // | | | | | | |
| 2000 | - | - | - | - | 1 | - |
| // | | | | | | |
| 2002 | - | - | - | - | 2 | - |
| 2003 | - | - | 1 | - | - | - |
| 2004 | - | - | 4 | - | - | - |
| 2005 | - | 1 | - | - | 1 | - |
| 2006 | - | - | 4 | - | 1 | 1 |
| 2007 | - | 1 | 8/1 | - | 1 | - |
| 2008 | 2 | 1 | 1 | - | 2 | - |
| 2009 | - | 1 | 2 | - | - | - |
| 2010 | 1 | - | - | - | - | - |
| // | | | | | | |
| 2012 | - | - | 1 | - | - | - |
| 2014 | - | - | - | 2 | - | - |
| 2016 | 2 | - | - | - | - | 1 |
| 2017 | 3 | 3 | - | - | 1 | - |
| 2018 | 10/1 | 5 | 2 | 2 | - | 1 |
| 2019 | 8 | 11/1 | 1 | 2 | 1 | 1 |
| 2020 | 12 | 4 | 5/1 | 5 | - | - |
| 2021 | 18/1 | 7 | 5 | 5 | - | 2 |
| 2022 | 8 | 2 | 1 | 2 | - | - |
| **Total/Positive** | **64/2** | **36/1** | **36/2** | **18/0** | **10/0** | **6/0** |

Samples screened from 18 pine martens, 10 least weasels and 6 stoats were negative. The European polecat detected in 2019 and the stone marten in 2020 originated from Western Hungary, both steppe polecats (collected in 2018 and 2021) originated in Eastern Hungary, and the stone marten (sampled in 2017) was collected in Southern Hungary (Figure 14).

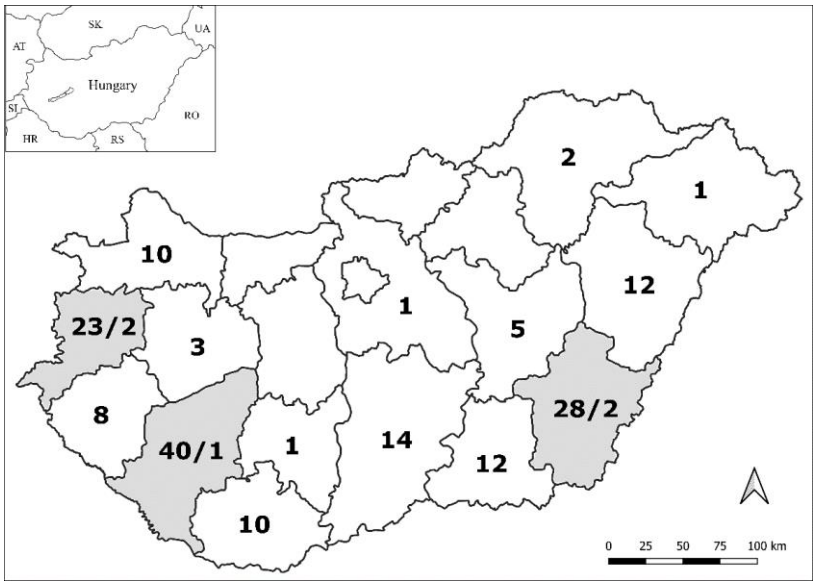


**Figure 14.** Regional distribution of the sample numbers, in Hungary (Total/Positive by county) (Lanszki et al., 2022).

Two CDV test positive animals (a stone marten and a steppe polecat) showed signs of bites on their bodies, which indicates combat with another carnivore (Table 5). As the sample collection efforts were not evenly distributed during the study period, CDV prevalence could not be estimated.

**Table 5.** Summary data of CDV-positive mustelids collected in Hungary (Lanszki et al., 2022).

| Species | Stone marten | Steppe polecat | European polecat | Stone marten | Steppe polecat |
|---|---|---|---|---|---|
| Date of finding | 2007 | 07.12.2018 | 26.03.2019 | 01.12.2020 | 02.05.2021 |
| Tissue | spleen | spleen | lung | spleen | spleen |
| Age category | juvenile | adult | adult | juvenile | adult |
| Sex | female | male | female | male | male |
| Cause of death | road-killed | road-killed | road-killed | road-killed | road-killed |
| County (settlement) | Somogy | Békés (Battonya) | Vas (Bozsok) | Vas (Felsőjánosfa) | Békés (Nagybánhegyes) |
| Body condition | poor | good | average | average | good |
| Other details | - | - | - | bite on the body | bite on the body |
| RT-PCR Ct value | 34.60 | 38.16 | 25.61 | 24.11 | 46.56 |
| Number of multiplex PCR cycles during sequencing protocol | 35 | 35 | 27 | 26 | 35 |
| Mean sequencing coverage of the targeted region (reads) | 11994.3 | 3587 | 17555.3 | 2280 | 102.4 |
| Accession Number | OP209188 | OP209186 | OP209187 | OP209189 | OP209185 |

*4.4.2. Sequencing and phylogenetic analysis*

Complete genomes were successfully retrieved from all positive samples. Sequences were deposited in GenBank (accession numbers OP209185-OP209189). Based on the phylogenetic analysis of complete genomes, all these sequences belong to the Europe lineage (Figure 15). The Hemagglutinin gene sequence-based analysis confirmed this result (Figure 15).

Sequences are dispersed among two clusters within the Europe lineage, and both clusters are composed of sequences from Hungary. Based on complete genomes (Figure 15), one cluster contains only mustelid sequences, whereas one steppe polecat sample was grouped with red fox samples in a separate clade. Based on the H gene phylogenetic tree, both steppe polecat samples (OP209186) grouped with red fox samples on a distant clade (Figure 16).

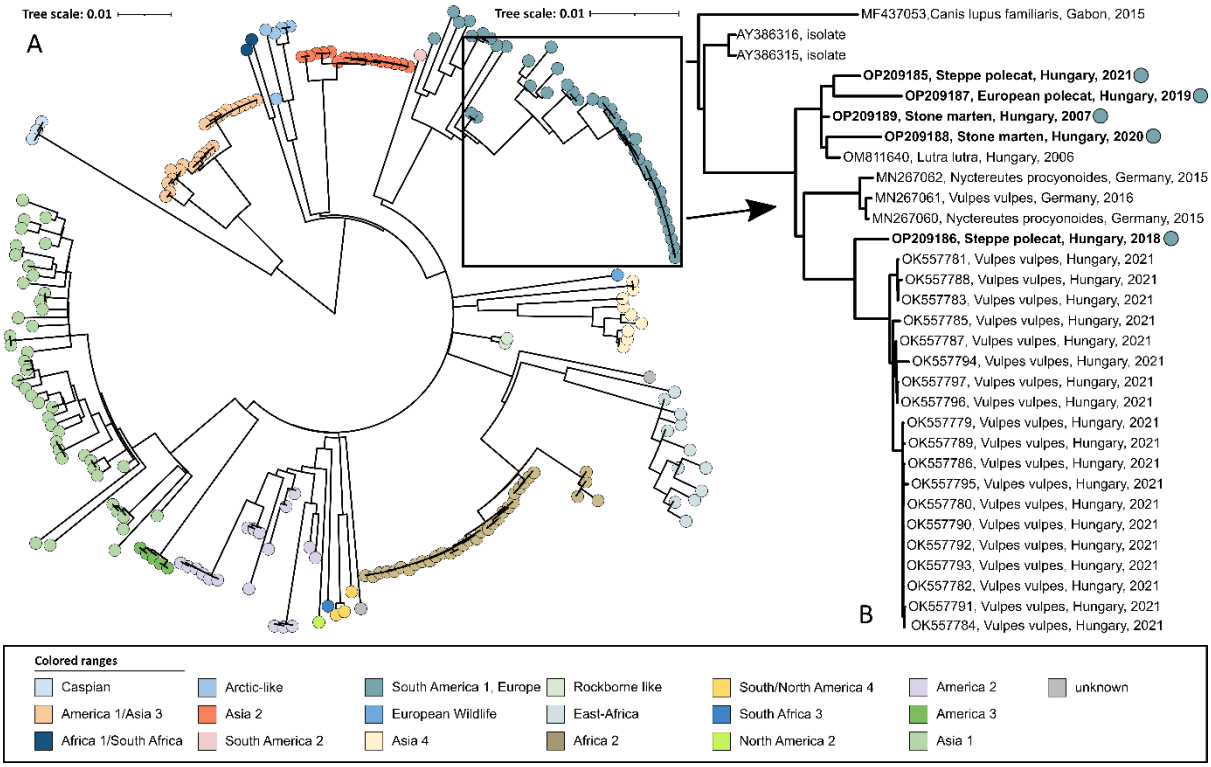


**Figure 15** (A) Maximum likelihood phylogenetic tree based on 221 CDV complete genomes. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root the phylogenetic tree. The Europe lineage of interest is highlighted in blue. (B) Expanded portion of Europe lineage. Dots represent sequences obtained in this study (Lanszki et al., 2022).

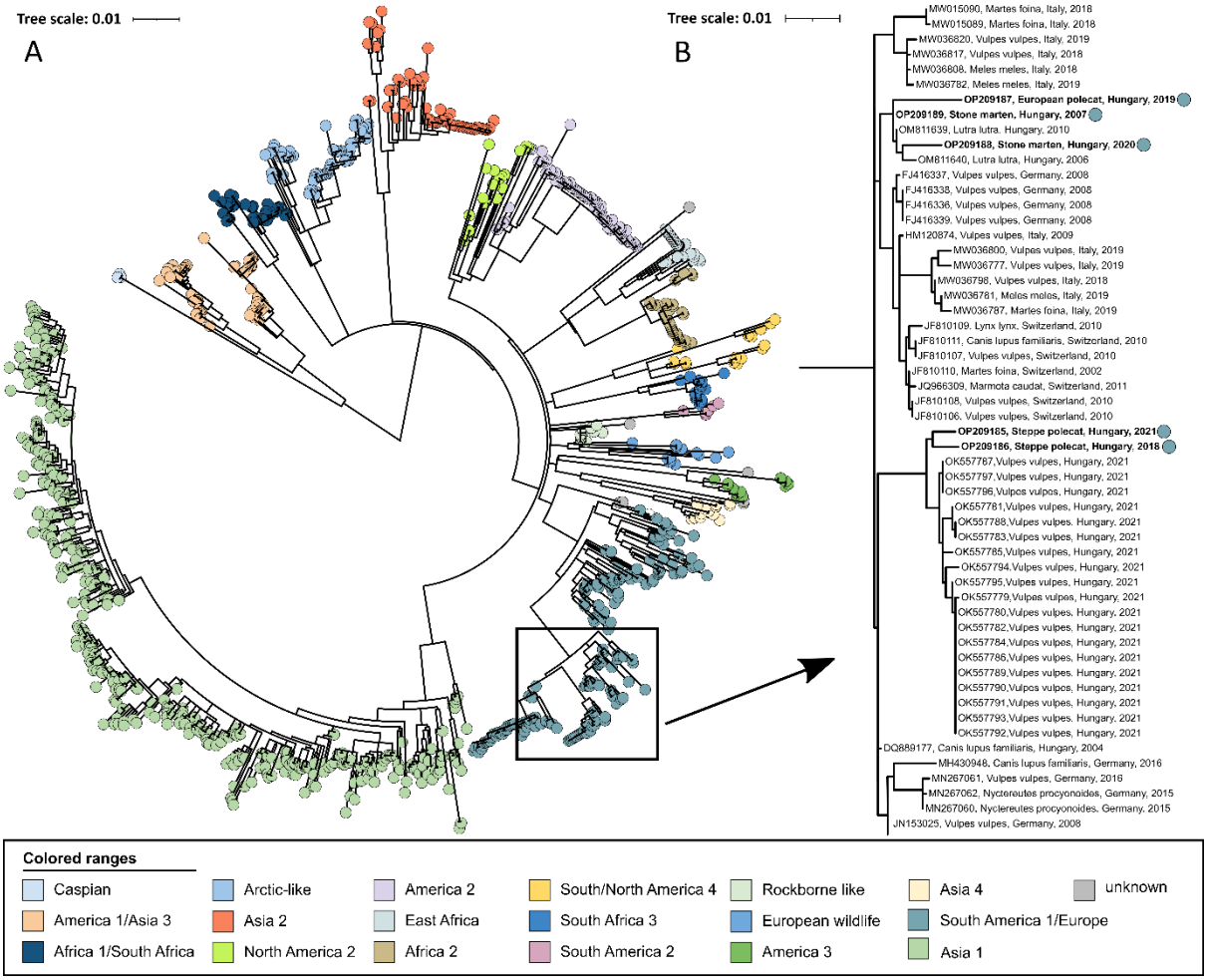


**Figure 16.** (A) Maximum Likelihood phylogenetic tree based on 969 complete Hemagglutinin (H) nucleotide sequences. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root the phylogenetic tree. The Europe lineage of interest is highlighted in blue. (B) Expanded portion of Europe/ America 1 lineage. Dots represent sequences obtained in this study (Lanszki et al., 2022).

### *4.4.3. Recombination analysis*

The distinct clustering pattern of OP209185 from a steppe polecat on the phylogenetic trees (Figure 15 and 16) indicates a recombination event in association with the Hemagglutinin genomic region. The SimPlot analysis confirmed the recombination of the Hemagglutinin gene with a closely related, Europe lineage strain. Also, it confirmed multiple additional recombination points in the genome (Figure 17).

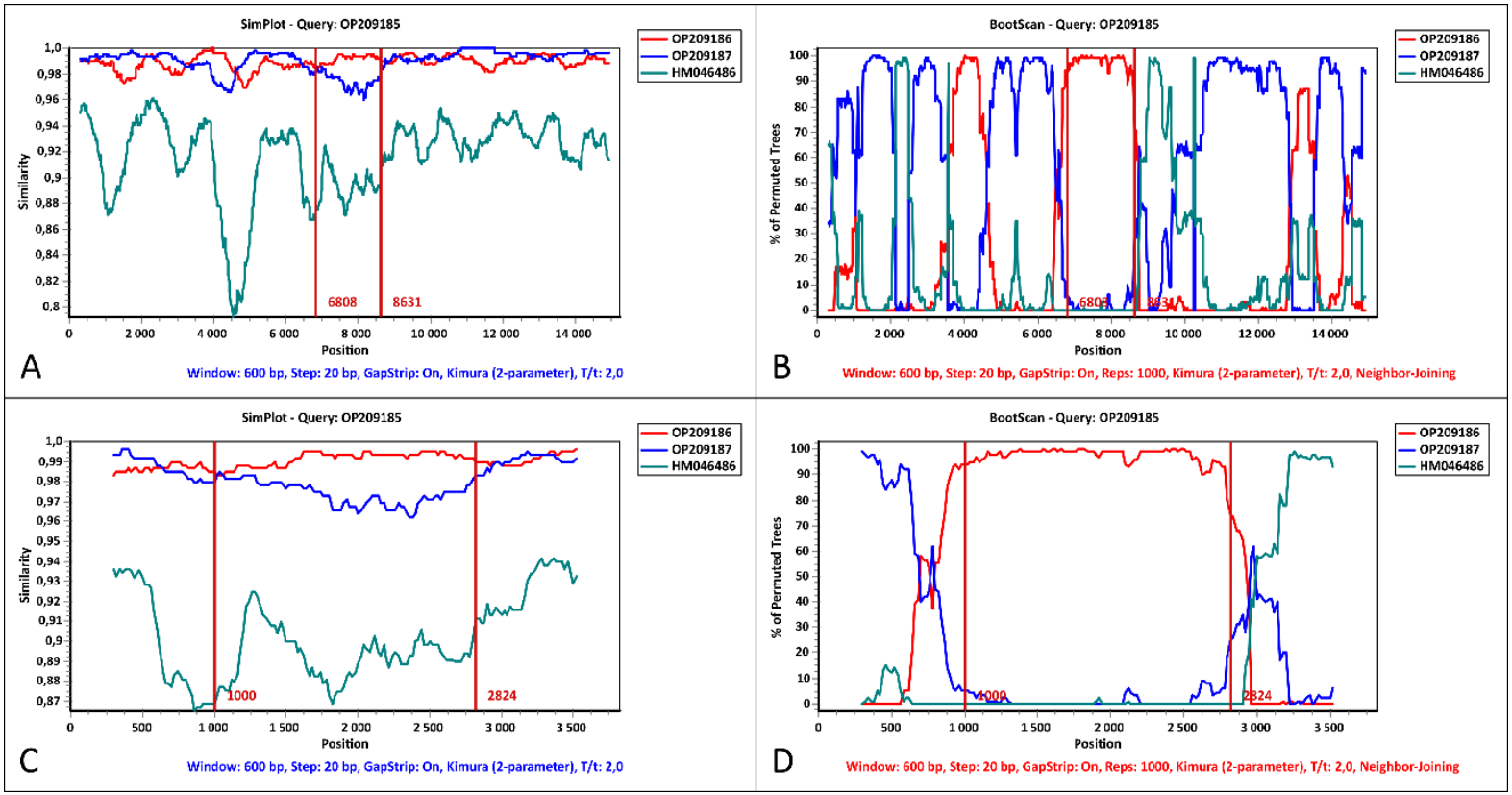


**Figure 17.** Recombination analysis of the *Canine distemper virus* (OP209185): (A) Similarity Plot analysis of the complete genome sequences of OP209185 (steppe polecat, Hungary, 2021) and its putative parents OP209186 (steppe polecat, Hungary, 2018) and OP209187 (European polecat, Hungary, 2019). The OP209185 was used as the query. (B) Boot Scan analysis of OP209185 and its parent sequences. (C) Similarity Plot analysis of the H gene sequences of OP209185 and its putative parents. (D) Boot Scan analysis of OP209185 and its parent sequences in the H gene. A CDV isolate, HM046486 (Caspian lineage), was used as an outgroup in all analyses. The red vertical line represents the H gene segment region. The y-axis indicates the percentage of identity with a window size of 600 bp and a step size of 20 bp. The comparison was performed using 50% consensus sequences with 1000 bootstrap replicates (Lanszki et al., 2022).

*4.5 Red foxes and domestic dogs from Italy*

*4.5.1. Sequencing and phylogenetic analysis*

Based on the complete genome phylogenetic analysis, 7 sequences belong to Europe lineage, and 14 sequences belong to Arctic-like lineage (Table 6, Figure 18). The sequences belonging to the 3 red foxes were all grouped into the Europe lineage.

**Table 6.** Summary data on CDV-positive samples from Italy.

| Sample ID | Year | Species | Lineage |
|---|---|---|---|
| 199/05 | 2005 | Dog | Europe |
| 224/05 | 2005 | Dog | Europe |
| 152/06 | 2006 | Dog | Arctic-like |
| 364/06 | 2006 | Dog | Europe |
| 290/08 | 2008 | Dog | Europe |
| 350/09 | 2009 | Red fox | Europe |
| 403/09 | 2009 | Red fox | Europe |
| 417/09 | 2009 | Dog | Arctic-like |
| 145/10 | 2010 | Red fox | Europe |
| 202/10 | 2010 | Dog | Arctic-like |
| 97/11 | 2011 | Dog | Arctic-like |
| 201/13 | 2013 | Dog | Arctic-like |
| 338/13 | 2013 | Dog | Arctic-like |
| 359/13 | 2013 | Dog | Arctic-like |
| 259/14 | 2014 | Dog | Arctic-like |
| 24/15 | 2015 | Dog | Arctic-like |
| 310/15 | 2015 | Dog | Arctic-like |
| 99/16 | 2016 | Dog | Arctic-like |
| 190/16 | 2016 | Dog | Arctic-like |
| 856/17 | 2017 | Dog | Arctic-like |
| 592/18 | 2018 | Dog | Arctic-like |
| 412/19 | 2019 | Dog | Arctic-like |
| 425/19 | 2019 | Dog | Arctic-like |

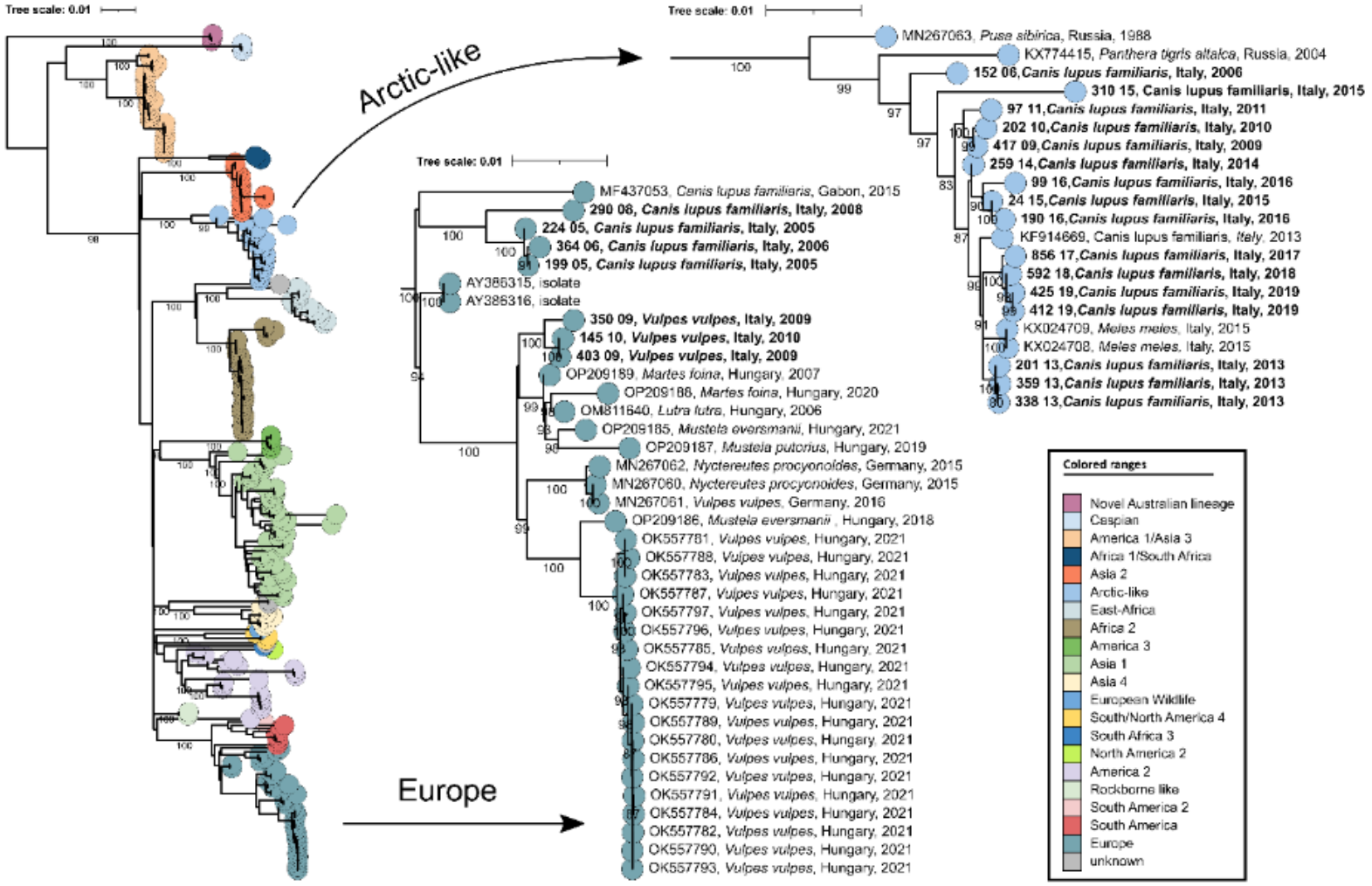


**Figure 18.** Maximum Likelihood phylogenetic tree based on 256 CDV complete genomes, using the Randomized Axelerated Maximum Likelihood (RAxML) tool. Phocine distemper virus (PDV) (GenBank accession number: KY629928) was used as an outgroup to root the phylogenetic tree. Arrows indicate expanded portion of Arctic-like and Europe lineages. The novel Italian sequences are shown in bold.

*4.6. Stray dogs from Bangladesh*

*4.6.1. RT-PCR screening*

Overall, 3.9% (10/257) of sampled animals were positive for CDV by RT-PCR. We detected CDV RNA only in the samples collected in Rajshahi city where 7.4% (10/135) of the swab samples were positive. During the sampling activity all positive dogs were observed as asymptomatic.

*4.6.2. Sequencing and Sequences Analysis*

During complete genome sequencing, we managed to obtain 4 complete and 5 partial sequences (Table 7). The coverage of one of the positive samples was too low for generating a consensus sequence. Similarly, to previous studies we observed a correlation of low-coverage or partial genome sequences with higher qRT-PCR Ct values. Based on the GenBank BLASTn search, the 4 complete genome sequences depicted the highest nucleotide similarity (96.57%, 96.58%, 96.57%, 96.55%) with a representative sequence of the MK037459 identified previously from Asiatic lions (*Panthera leo persica*) which from India (Mourya et al., 2019).

**Table 7.** Summary data of CDV positive samples. Number of multiplex PCR (mPCR) cycles during amplicon-based sequencing protocol.

| Sample ID | RT-PCR Ct value | mPCR cycle number | Sequencing results |
|---|---|---|---|
| 68 | 37,51 | 35 | partial H, L genes |
| 74 | 41,65 | 35 | partial L gene |
| 84 | 35,94 | 35 | partial H, L genes |
| 90 | 44,01 | 35 | - |
| 103 | 36,24 | 35 | partial N, P, L genes and full length M gene |
| 107 | 32,33 | 35 | full length targeted region |
| 114 | 27,52 | 30 | full length targeted region |
| 120 | 28,27 | 30 | full length targeted region |
| 128 | 37,98 | 35 | partial genome with gaps in F, H, L genes |
| 129 | 35,4 | 35 | full length targeted region |

## 5. Discussion

### *5.1. CDV in a clinical case study from Hungary*

We presented clinical and the viral genetic aspects regarding a 17 month long persistent CDV infection in a sheltered dog which was symptomatic for a month with characteristic symptoms of acute CDV infection. This was followed by an asymptomatic phase with persistent CDV-positive PCR tests for a prolonged period. The animal was quarantined at a veterinary clinic, and during this period we investigated the CDV RNA presence in the urine samples. Urine samples were previously described in other studies as the most suitable diagnostic material for CDV diagnostics (Elia et al., 2015; Saito et al., 2006; Shin et al., 2004). A clinical and molecular investigation from Switzerland was presented detailing how long dogs endure the viral RNA. Distinctly, the canine distemper virus infection was tested in a group of rescue dogs transported from Hungary to Switzerland, in which 11 of 13 dogs tested positive, and the virus was detected for a period up to four months before all dogs became PCR negative. The CDV isolates belonged to the Arctic-like lineage. The study highlighted the risks of unvaccinated dogs or dogs with incomplete vaccination (Willi et al., 2015). According to several authors, the virus can be excreted up to 60 to 90 days following infection, although shorter shedding periods are more typical (Greene, 2012). Viral shedding in feces may continue for up to 3–4 months; however, it usually resolves after 1–2 weeks (Sykes, 2013). There are few studies related to the rate of survival of infection. In an Australian study, 10 out of 13 dogs perished or were euthanized, and three out of 13 dogs recovered between 2006 and 2014. Admittedly, dogs represented a high (77%) mortality rate (Wyllie et al., 2016). Moreover, In Italy, three out of 10 dogs recovered between 2015–2016, leading to a mortality rate of 70%. Additionally, the CDV isolates were associated to the Arctic-like lineage. Out of the 10 positive dogs, two were vaccinated, five were not vaccinated and three had an unknown vaccination status. The genetic heterogeneity of field and vaccine strains may lead to vaccinated canine infections, as was previously discussed (Mira et al., 2018). However, we do not know the vaccine history of the animal in this study; thus, we present genetic data about the circulation of the CDV strain in Hungary (Mira et al., 2018).

There is a considerable lack of knowledge regarding the effect of other infections on CDV pathogenesis. CDV co-infection or superinfection cases with other pathogens have been previously reported, for instance, with viruses, canine adenovirus type 2, canine influenza virus, canine parainfluenza virus (Chvala et al., 2007; Hao et al., 2019) and bacteria: *Bordetella bronchiseptica*, *Mycoplasma cynos* (Chvala et al., 2007; Demeter et al., 2010). There are further

reports with vector-borne infections and co-infections with CDV, such as Babesia spp. and *Leishmania infantum* (Willi et al., 2015). *Dirofilaria immitis* is considered an endemic parasite with increasing relevance in Hungary. The first confirmed autochthonous canine heartworm case was detected in 2007 (Jacsó et al., 2009). Nonetheless, we have limited information regarding co-infection with CDV and no data on the potential cross activity of non-specific immune response and the effect of parasitic infection in the course in reference to CDV infection, if any.

The Arctic-like lineage was initially detected among the susceptible population of the Arctic ecosystem in the 1980s (Bolt et al., 1997; Osterhaus et al., 1988, 1989; Visser et al., 1990). This lineage was isolated from a Siberian seal in 1987–1988 from Lake Baikal in Russia (Mamaev et al., 1995). Since then, the presence of Arctic-like lineage has been reported throughout many European countries including Italy, Hungary, Switzerland, Austria and other regions from North America, Iran and China (Demeter et al., 2009; Kapil et al., 2008; Martella et al., 2006; Mira et al., 2018; Monne et al., 2011; Namroodi et al., 2015; Ricci et al., 2021; Willi et al., 2015; Zhao et al., 2010). This highlights the dominance and veterinary health relevance in certain geographic regions of the Arctic-like lineage. The Arctic-like lineage is a widespread, relatively common strain across Europe among both domestic and wild animals, based on the available published literature. In Austria, from 2002 to 2007, 14 dogs, one badger and one stone marten were diagnosed with CDV. Three of the 14 dogs survived and were released to home care. Four different lineages were detected during the period of investigation, including one seven-year-old dog that was sample vaccinated for two and a half years with the Arctic lineage from 2003; it was euthanized (Benetka et al., 2011). In Southern Italy, nine CDV strains were sequenced between 2000 and 2004. Four CDV strains from dogs belonged to the Europe strains, two CDV strains from dogs belonged to the Arctic lineage (one was vaccinated, the other was not) and one CDV strain from a fox belonged to the Europe lineage. It was assumed that the uncontrolled trading of dogs likely introduced the Arctic-like strain into Italy from Eastern Europe or Northern Asia, and that strain steadily diffused and spread across the canine population (Martella et al., 2006). At nearly the same time, 21 sequences belonging to the Arctic-like lineage were identified in north-eastern Italy, between 2000 and 2008, whereas five were related to the Europe lineage from domestic dogs (Monne et al., 2011). In another Italian study, diagnoses of 20 Apennine wolves with the CDV virus and their subsequent demise were reported in 2013. Only four of the 20 infected wolves underwent H gene sequencing and a subsequent phylogenetic classification to the Arctic lineage. This was the first report regarding CDV Arctic lineage epidemics among the wild population in Europe (Di Sabatino et al., 2014).

Following that, two badgers sampled from a rural area in Italy in 2015 were reported to be CDV-positive with PCR. The CDV isolates were lethal in badgers and belonged to the Arctic-lineage based on the H protein. Since the badgers came from the same area as the Apennine wolves, these results likely suggest that this lineage is becoming endemic in the wildlife population of the Abruzzo region, Italy (Di Sabatino et al., 2016). In particular, between 2015 and 2016, the Arctic-like lineage was still detectable throughout Italy. The infection data from recent years supports the theory that the introduction of the Arctic-like lineage was carried by dogs imported from Eastern European countries and suggests the possibility of a potential common origin (Mira et al., 2018).

Examining the Hemagglutinin gene gives us the opportunity to identify the specific CDV lineage and it's a widely used method for genetic classification. However, using the complete genome gives valuable additional opportunity to gain insight into more detailed evolutionary patterns (such as unique mutations or recombination patterns). Therefore, the complete genome analysis is a more preferable tool than H gene analysis.

*5.2. CDV in red foxes from Hungary*

We present the genomic sequencing and phylogenetic analysis of 19 complete genomes of CDV strains from red foxes during an epizootic event in Hungary, in 2021. We provided novel, complete genomic sequence data and showed the reliability of NGS sequencing in genomic epidemiological studies which may support rapid response actions during future epizootic situations. A total of 21 of the 24 foxes were positive for CDV with one-step RT-PCR. As reported by the animal rescue center, the 3 negative animals died with similar symptoms as the other 21, so it is conceivable that it was not possible to detect viral RNA in the collected swab samples due to viral clearance (Kim et al., 2006; Shabbir et al., 2011; Shin et al., 2004). In addition to the observed symptomatic animals, there may have been more undetected cases in the region. Notably, a limitation of our study is the lack of source information about the investigated animals. However, the phylogenetic relatedness and the elevated case number as experienced by the rescue center supports the idea of a more widespread epizootic event. Based on the phylogenetic analysis the sequences from the foxes belonged to the Europe lineage and showed the greatest similarity with an H gene of CDV which was detected in a Hungarian dog sample from 2004 in the same area (Demeter et al., 2007).

Across Europe, episodes of canine distemper outbreaks in non-dog host species with Europe lineage have been reported earlier. In Germany, numerous wild red foxes exhibited neurological signs suggestive of canine distemper and several badgers were found dead. After

H gene of CDV sequences were analyzed from five foxes and one badger were confirmed with Europe lineage from 2008 (Sekulin et al., 2011). In Italy, similar to the current epidemic in Hungary, at least 30 foxes with altered behavior were seen near human habitations and facilities in 2009. Most foxes were juveniles during the epizootic event. Then the presence of the Europe lineage in three infected foxes was confirmed by H gene sequencing (Martella et al., 2010). In Switzerland, numerous wild carnivores, including red foxes, Eurasian badgers, stone and pine martens, and one Eurasian lynx (*Lynx lynx*) were found with CDV lesions between 2009 and 2010. The first 50 animals were confirmed as CDV positive. This outbreak was detected in a large spectrum of affected species, with high morbidity and mortality, especially among red foxes and badgers (Origgi et al., 2012). In Denmark, a major outbreak of canine distemper virus was detected in farmed American minks from mink farms and a high number of species such as foxes, raccoon dogs (*Nyctereutes procyonoides*), and wild ferret (European polecat), between 2012 and 2013 (Trebbien et al., 2014). The Europe lineage of CDV in wildlife has continued to be reported from nearby countries, first in Italy from wild animals, mainly foxes and badgers, between 2006 and 2009 (Monne et al., 2011), thereafter in Germany from raccoons from 2015 and fox from 2016 (Jo et al., 2019), and recently in Northern Italy from foxes, badgers, and stone martens between 2018 and 2019 (Trogu et al., 2021). Based on these epizootic events, it can be assumed that this lineage will be present among European wild animals, with recurring outbreaks in the future.

Understanding the evolution of enzootic strains and the transmission risk from wildlife to domestic animals are highly important to mitigate the effect of spillover events on household animals. During the last years, several studies recognized the importance of providing genetic data (Li et al., 2014; Maganga et al., 2018; Nikolin et al., 2017; Young et al., 2021). Host jump events from wildlife to domestic animals were supposedly connected to substitutions at the amino acid positions 530 and 549 in the signaling lymphocytic activation molecule SLAM binding region. It was hypothesized in multiple studies that the substitutions at the residues G/E 530 to R/D/N and Y549H may have a crucial role in the inter-species transmission from domestic dogs to non-dog hosts (McCarthy et al., 2007). In contrast, the current study reports that all epizootic sequences from red foxes presented the 530G and 549Y, at the amino acid level. In this term, other studies support our current observations, since the CDV Hemagglutinin gene sequences of red foxes in Germany, Denmark and Italy contain a 549H and a 549Y amino acid, indicating that both versions were found in red foxes (Martella et al., 2010; McCarthy et al., 2007; Sekulin et al., 2011; Trebbien et al., 2014). Based on the data available so far, it needs

to be reconsidered whether these amino acid substitutions and constellations correspond to the host or not.

The importance of sequencing data to better understand CDV evolution is increasingly recognized in other studies as well. Apart from the limitation of our study, namely the lack of different CDV lineages to extensively verify the method, we present a novel NGS-based sequencing performance to aid future studies. We designed the method to be applicable for sequencing multiple genetic lineages. Next-generation sequencing methods were previously used in relation to CDV research. In a study, CDV infection was identified in a dog that was imported to Italy from Cuba. CDV was detected and isolated from the infected brain tissue. Subsequently, this isolate was subjected for non-targeted Next-Generation Sequencing using the MinION Nanopore technology (Peserico et al., 2019). Another recent study presented complete genomic data which was acquired by Sanger sequencing method. These studies well represent the increasing need for rapid and specific genomic data generation (da Costa et al., 2021). Using the amplicon-based NGS sequencing technology is not unique in epidemic situations, but it was fairly used in veterinary health-related events to date. We highlight the importance of similar methods to aid future investigations of epizootic events or even supporting surveillance efforts. In addition, as presented on the phylogenetic analysis, there is a significant lack of genetic sequence information about enzootic and non-enzootic CDV strains. However, we designed the application to be specific for several genetic lineages, the NGS workflow of the current study needs to be tested on other genetic lineages of CDV as well in the future.

From a nature conservation point of view, it is of paramount importance to learn more about diseases in animal species susceptible to CDV infection and prepare or aid mitigation efforts during epizootic events. Foxes' social behavior during the reproductive season and the dispersion of juvenile animals can play a major role in epizootic CDV amplification and diffusion in a wide geographic range, as discussed before (Martella et al., 2010; Trogu et al., 2021). Considering that the red fox is one of the most common wild carnivorous species in the world, interactions with dogs and other carnivores can be frequent, that it can potentially transmit the virus. CDV is known to easily cross species barriers and is able to infect different animal species. Notably, to better understand recurring epizootics of enzootic CDV strains needs the perspective of OneHealth concept. We need to better understand environmental and animal behavioral factors, among many others.

In the case of human outbreaks (such as the recent COVID-19 pandemic), the development and use of rapid, sensitive and specific NGS-based genomic epidemiological tools is very common, especially the use of amplicon sequencing-based approaches. Following this trend, using similar methods is getting more common in animal disease surveillance as well. Based on our current knowledge this is the first NGS-based amplicon sequencing method for CDV.

*5.3. CDV in Eurasian otter from Hungary*

Although the Eurasian otter is a well-studied "key species" of aquatic habitats in ecological and zoological terms (Kruuk, 2006), there is a considerable lack of knowledge of the microbiological context across its distribution range. Aleutian mink disease parvovirus, carnivore protoparvovirus 1, feline panleukopenia virus, and canine adenovirus type 1 were previously detected in the species (Mañas et al., 2001; Park et al., 2007; Viscardi et al., 2019). Regarding the *Morbillivirus* virus family, dolphin morbillivirus was detected in Eurasian otters (Padalino et al., 2019). Nevertheless, the presence of canine distemper virus was only described based on the histology among European otters (Akdesir et al., 2018; Geisel, 1979). CDV infections were reported among the members of the family Mustelidae, and the virus has been detected in several different otter species. Under zoo conditions in Belgium, CDV was observed in littermates of the Asian small-clawed otter (*Aonyx cinereus*), based on histopathology and direct immunofluorescence (De Bosschere et al., 2005). CDV was detected in a sea otter (*Enhydra lutris*) population using immunohistochemistry, RT-PCR, genetic sequencing, virus isolation ,and serology in upstate Washington, USA (Thomas et al., 2020). North American river otters (*Lontra canadensis*) were seropositive against canine distemper virus in the northern and eastern regions of the USA, implying its circulation among these animals (Kimber et al., 2000). Seemingly, CDV infections are present in a wide range of species within the *Mustelidae* family. Considering their relevance regarding their conservation biology, vaccination may provide a solution for avoiding epizootic events within otter populations. The vaccination of otters is not without precedent; it is reported and evaluated in multiple publications (Jessup et al., 2009; Peper et al., 2014).

We emphasize the importance of providing novel sequence data in accordance with recent studies, in which the complete genomic sequence data of CDV are increasingly reported. This will greatly facilitate a better understanding of both the evolution of CDV and epizootic patterns in the near future (Li et al., 2014; Maganga et al., 2018; Nikolin et al., 2017; Peserico et al., 2019; Young et al., 2021). In our study, we used a recently published pan-genotype CDV-specific amplicon-based sequencing method developed for the Oxford Nanopore Technologies

platform (Lanszki et al., 2022). A key advantage regarding this method is its ability to sequence the entire genome of CDV quickly and efficiently with multiplexed amplicons, without the necessity for in vitro isolation procedures.

The Europe lineage of CDV was detected in many wild-animal species originating in various European countries. This lineage was frequently observed among red foxes and badgers in several countries and across multiple years, such as Germany in 2008 (Sekulin et al., 2011) and Italy between 2006 and 2009 (Martella et al., 2010; Monne et al., 2011). Moreover, in Switzerland, numerous wild carnivores, including red foxes, Eurasian badgers, stone and pine martens, Eurasian lynx, and domestic dogs, were found with CDV lesions between 2009 and 2010 (Origgi et al., 2012). In Denmark, a major outbreak of CDV was detected in many carnivore species, between 2012 and 2013 (Trebbien et al., 2014). Recently, the Europe lineage of CDV was detected in Germany among raccoons from 2015, in red foxes from 2016 (Jo et al., 2019), and in Northern Italy in red foxes, badgers, and stone martens between 2018 and 2019 (Trogu et al., 2021). In addition to these studies, numerous red foxes were detected in Hungary in 2021, in association with a possible countrywide epizootic event (Lanszki et al., 2022). Apparently, CDV is a widespread and, at the same time, fairly deeply investigated pathogen among wild carnivores throughout Europe. We demonstrated the reliability of road-killed animal samples for retrospective virological examination regarding CDV genomic patterns, prevalence, and host range. The growing number of genomic data may significantly aid in comprehending and predicting future epizootic events.

The detection of CDV in Eurasian otters in Hungary provides valuable insights for their conservation. Our findings allow us to make practical recommendations to protect the species. Based on our results, we propose the implementation of CDV vaccination for otters in shelters and during conservation-related repositioning efforts.

### *5.4. CDV in terrestrial mustelids from Hungary*

We present the circulation of CDV throughout the country over several years, supporting the endemic nature of this virus among mustelids. An important finding of the current study is the detection of CDV in wild-living steppe polecats. It is a rare and protected mammal species of our region and by using retrospective virus surveillance methods (i.e. without disturbance and invasive sampling of the animals), we were able to indicate the role of these animals in CDV transmission. Steppe polecat was already a suspected host for CDV (Heptner, 1967), however, due to its rareness and elusive nature, only a few molecular biological investigations have been performed on this species without presenting viral genomic

data (Pavlacik et al., 2007). In the current study we present the first two complete CDV genomes from the steppe polecat, enriching the diversity of available CDV genomes. By revealing the presence of a recombinant CDV strain in these animals we demonstrated the importance of generating complete genomic data. This approach may ultimately lead to better understanding CDV evolution, since partial genome fragments are not suitable to understand the impact of recombination events in CDV evolution or the role of coding regions other than H. Furthermore, the presence of CDV was confirmed in two additional species in this study. The European polecat is also at risk of infection by CDV, for instance, the virus was detected with RT-PCR (qPCR) from the Asturias region of Atlantic Spain in 2021 (Oleaga et al., 2021). The stone marten is a well-known host of CDV, and in recent decades, many cases have been detected in nearby countries including such as Austria, the Czech Republic, Germany, Switzerland and Italy (Balboni et al., 2021; Benetka et al., 2011; Frölich et al., 2000; Kličková et al., 2022; Origgi et al., 2012; Pavlacik et al., 2007; Trogu et al., 2021).

According to our findings and previous literature data, CDV is present in 4 out of the 8 species of the *Mustelidae* family in our region (Demeter et al., 2007b, 2009; Lanszki et al., 2019; Lanszki et al., 2022). Considering the relevance of these animals in conservation biology, vaccination in wildlife rescue centers may be an important tool in the conservation of rare and protected mustelids (Wright et al., 2022). Since CDV vaccination safety and effectiveness data is scarce for the Eurasian otter, vaccination at rescue centers with the available attenuated CDV vaccines is not applicable widely. However, in other members of the family it is possible. For instance, the black-footed ferret, whose population has almost been extinct due to CDV infection, is a close relative of the steppe polecat. The vaccination of black-footed ferret x steppe polecat hybrids was reported as surrogates for endangered black-footed ferrets (Williams et al., 1996; Wimsatt et al., 2003). In Europe, CDV was detected in Spain in four carnivore species collected in 2020–2021, including the Eurasian badger, pine marten, European polecat and the red fox (Oleaga et al., 2021). In the Czech Republic, CDV was detected between 2012–2020 in the red fox, stone marten, raccoon, pine marten and the European badger (Kličková et al., 2022). Similar outbreaks were observed among red foxes across Europe due to this strain (Lanszki, et al., 2022; Martella et al., 2010; Nikolin et al., 2012; Sekulin et al., 2011; Trogu et al., 2021). Europe lineage was also detected in many other species such as Iberian grey wolves (*Canis lupus signatus*), an Asian marmot kept in a zoo, a stone marten, pine marten, Eurasian lynx, Iberian lynx and a domestic dog (Conceição-neto et al., 2017; Maganga et al., 2018; Meli et al., 2010; Müller et al., 2011; Origgi et al., 2012, 2013).

For effective transmission of CDV, close contact among infected and susceptible animals is necessary. Bites on two positive animals (stone marten and steppe polecat) were observed presumably as a direct indication of contact with other carnivores. Aggressive intra- and interspecific behavior are relatively common in the mustelid species, and competition for territory (Palomares & Caro, 1999), food, or mating partner may bring these animals to close-contact situations when the spread of the disease is more likely. Nonetheless, according to published literature, skin contact, feces or urine are less important means of transmission (Kiupel & Perpiñán, 2014). However, the primary method of transmission in CDV infection is theorized to be via the respiratory tract droplets (Rendon-Marin et al., 2019; Shin et al., 2022), which may have relevance under fighting conditions. More studies and observational data are necessary to better understand the natural transmission and circulation patterns of CDV.

Based on literature data, the Europe lineage of CDV, which circulates among mustelids throughout Hungary, is also present in surrounding countries (Kličková et al., 2022; Origgi et al., 2012; Sekulin et al., 2011; Trogu et al., 2021; Zhao et al., 2010). Similar to most of the CDV surveillance studies, H-gene phylogeny was a useful tool for lineage categorization. However, as a major limitation, H-gene based analysis is not adequate to reveal genome-scale recombination patterns and understand fine-scale evolutionary patterns. Based on literature data these viruses are prone to recombine in several genomic regions, most frequently in the H gene (Ke et al., 2015; Piewbang et al., 2019). We support this with our observation and presentation of multiple recombination points in our recombinant CDV strain. More complete genomic sequence data in the future can reveal a more accurate evolutionary scenario for our sequence. In addition the dispersive pattern among these two phylogenetic clades, composed by different CDV strains from other animal species raises the possibility for cross-species transmission events. This was already known from literature data as an important feature of CDV transmission. (Nikolin et al., 2017; Yuan et al., 2017).

A limitation of our study is the lack of autopsy or histology data to better understand the pathogenicity of the CDV infection in these animals. Further studies are needed to discuss the pathogenic nature of these different CDV strains. However, our study highlighted the importance of genome-scale monitoring of CDV evolution, which may serve as a first step to understand genomic evolution in relation to pathogenesis. In addition to these, our study demonstrated that road-killed carcasses are a valuable source of CDV surveillance in wildlife species.

*5.5. CDV in red foxes and domestic dogs from Italy*

During our work, we were able to sequence the coding region of the genome from samples from the past, and based on the phylogenetic analysis, in the case of the dogs 4 sequences belong to Europe lineage, and 14 sequences belong to Arctic-like lineage. The sequences from 3 red foxes were all grouped into the Europe lineage. Our study aimed to fill the gap in knowledge regarding the emergence and patterns of the Arctic-lineage in Italy, particularly in the middle region. Through our research, we successfully identified the underlying patterns of CDV dispersal in mid-Italy.

The Europe lineage has been widespread in Italy for a long time, which was detected in many cases in dogs and other animals (Bianco et al., 2020). The Arctic-like lineage appeared in Italy about 20 years ago and numerous publications follow its continuous spread from the northern region of the country to the southern parts (Martella et al., 2002, 2006; Mira et al., 2018). The Arctic-like lineage was originally detected in the Arctic ecosystem in the 1980s (Bolt et al., 1997; Osterhaus et al., 1988, 1989; Visser et al., 1990). In the last decades, Arctic-like lineage was reported throughout many European countries including Italy, Hungary, Switzerland, Austria and other regions (Demeter et al., 2009; Kapil et al., 2008; Martella et al., 2006; Mira et al., 2018; Monne et al., 2011; Namroodi et al., 2015; Ricci et al., 2021; Willi et al., 2015; Zhao et al., 2010). Both lineages can affect protected and rare species such as Apennine wolves which can have significant conservation consequences (Di Sabatino et al., 2014).

Nevertheless, our research emphasized the importance of genome-scale monitoring of CDV evolution, which may serve as a first step measure to comprehend genomic evolution. Retrospective examination methods offer a valuable opportunity for the rapid evaluation of current epidemics and the ability to infer future spread patterns and potential host species. These methods aid in understanding the virus and play a crucial role in preparing for future epidemics.

*5.6. CDV in stray dogs from Bangladesh*

In this study, we utilized RT-PCR and Oxford nanopore sequencing techniques to confirm the presence of canine distemper virus in Bangladesh. To the best of our knowledge, these are the first CDV sequence data obtained from the country. The research was conducted in collaboration with local experts, with a focus on knowledge transfer and improving the

diagnosis of CDV in stray dogs. Additionally, the study demonstrated the practicality of operating a transportable field laboratory during sample collection and processing.

Based on available literature data, CDV is a common disease among stray dogs in Bangladesh. However, the genetic characteristics are unknown of these circulating strains. In Bangladesh, CDV presented with 1.61% prevalence were observed based on clinical diseases and/or clinical conditions in pet dogs from Dhaka in 2009 (Tarafder & Samad, 2010). In a case-control study, the prevalence of CDV was ascertained 5.69% of pet dogs presented in Sylhet, between 2013 and 2014, based on clinical conditions (Singh et al., 2015). In further investigation, the prevalence of CDV was observed in Dhaka in 2013. In this study, the prevalence of CDV in male dogs was 16.67%, in female dogs was 21.79%, as well as, in male cats 6.52 and in female cats 5.78 based on clinical conditions (Hossain & Kayesh, 2014). Between 2015 and 2016, prevalence of the CDV was 5.23% in pet dogs diagnosed based on clinical examination, past disease history and different laboratory methods, in Dhaka (Sultana et al., 2016). Between 2013 and 2015, 3 CDV cases were observed in dogs, from Chittagong based on clinical problems (Rahman et al., 2017). Between 2016 and 2017, CDV prevalence was estimated in pet dogs, based on clinical diseases and conditions in Chittagong (Yadav et al., 2017).

In 2010, 2 out of 5 apparently healthy golden jackals (*Canis aureus*) were positive using CDV specific RT-PCR, from Mymensingh. In this regard, the authors raised its importance, specific RT-PCR for the detection of CDV can significantly contribute to the diagnosis of the virus. Furthermore, to learn about the epidemiology of CDV, it is necessary to sequence the genome of the virus and test a large number of samples (Yousuf et al., 2014). Canine distemper virus monitoring is crucial, especially in regions with low vaccination rates that are several dogs affected. Identifying illnesses based on symptoms alone can be challenging but using rapid tests or PCR systems can facilitate the identification process.

Based on the H gene and complete genome India-1/Asia-5 lineage was detected, which was previously described in India (Bhatt et al., 2019). India-1/Asia-5 lineage was detected such as in dogs, Asiatic Lions, and leopards from India (Mourya et al., 2019). Thanks to the sequence analysis, this is the first knowledge that this lineage of CDV is present in Bangladesh.

Many regions, particularly in developing countries, face challenges in implementing effective vaccination programs due to limited resources and awareness. As a result, outbreaks of preventable diseases such as rabies pose significant threats to public health and animal welfare. Stray dogs, in particular, suffer from harsh living conditions, malnutrition, and limited access to medical care, increasing the risk of disease transmission within the dog population

and to humans. Uncontrolled breeding due to the absence of identification and control measures further exacerbates the problem. To address these issues, international organizations and animal welfare groups are actively raising awareness, improving veterinary infrastructure, and promoting responsible pet ownership globally. Encouraging communities to adopt stray dogs, implementing sterilization programs, and providing access to vaccinations are crucial steps in tackling overpopulation and mitigating disease transmission.

Vaccination against CDV is an effective preventive measure, particularly in regions with low vaccination rates. The study highlighted the significance of regulating the stray dog population, implementing spaying and neutering programs, and ensuring vaccination coverage against the virus. Further research and surveillance efforts are necessary to monitor and control CDV not only in Bangladesh but also in other regions facing similar challenges.

The limitations of our study include the difficulty in individual-level tracking of stray dogs, as there was no opportunity for follow-up sampling. During the sampling process, there was limited opportunity for a thorough examination of the dogs. Additionally, due to the low copy number of some samples, we were only able to obtain partial genome sequences in several cases.

In this work we successfully detected and sequenced the complete genome of CDV in Bangladesh using a mobile laboratory approach with the Nanopore Sequencing method. The findings underscore the importance of monitoring and preventing the spread of CDV in regions with low vaccination coverage, ultimately contributing to the well-being of both animals and humans. This work successfully demonstrated the mobile laboratory approach as a valuable tool for understanding animal diseases in low-resource conditions.

## 6. Conclusion

In this thesis we describe the significant progress that has been made from the recognition of the knowledge and technological gaps in CDV diagnostics to the development and widespread application of novel genomic epidemiological methods. Such methods are already applied in human outbreak investigation but less common in animal health research.

Canine distemper is a serious viral disease that affects many species around the world and has notable conservational effects on certain species. Rapid and effective understanding of the long-term presence of CDV in free-living mammals is of great importance. During our investigation we highlighted the need for updated surveillance practices to better understand CDV evolution and impact on certain animal species or communities and its possible role as an effector on animal populations with conservational relevance.

Our main outcome represents the successful mobilization of this sequencing method to a low-resource environment, highlighting the advantages of analyzing complete genomic sequences to identify pathogens, recombination patterns and other genomic characteristics with significant evolutionary implications. As we provided novel genomic information of CDV from multiple continents and geographic areas, we also highlighted the importance of analyzing genome-scale data by identifying recombination patterns and epidemiological scenarios.

## 7. Summary

### *7.1 Summary in English*

Canine distemper virus is a single-stranded, negative-sense RNA virus that belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family. Several lineages are known and classified according to different hosts and geographical areas based on nucleotide sequence analysis of the Hemagglutinin gene. CDV is a prevalent viral infection worldwide that affects wild and domestic animal populations. Cross-species transmission frequently occurs, which may lead to conservation problems for each rare and endangered species or animals with agricultural relevance. It is primarily transmitted through bodily fluids such as saliva, respiratory droplets, urine, and feces. In CDV-infected dogs, the mortality rate is up to 60-80% based on literature data. Whilst in infected wild mustelids, this mortality rate is often close to 100%. Our goal was the recognition of knowledge and technological gaps in CDV diagnostics and the development and extensive utilization of novel genomic epidemiological methodologies.

First, we documented the course of infection of a domestic dog in Hungary, the dog excreted CDV RNA for 17 months. As a main conclusion of this work, we identified the lack of adequate sequencing method for CDV analysis. The widely used Hemagglutinin gene-based analysis is not sufficient to reveal genome-wide evolutionary patterns, specific mutations, or recombination events. Therefore, we developed an amplicon based NGS sequencing method for optimal genomic surveillance of all known CDV lineages. To test our method, firstly we focused on the investigation of an ongoing epizootic event among Hungarian red foxes. After the success of our investigation, we extended the surveillance activity to retrospective samples from rare and protected species, victims of road-kills from 1997-2022.

Following the successful implementation of the method in Hungarian CDV strains we extended the investigation area to Italy. We identified and sequenced 20 dog and 3 red fox positive samples from the period of 2005 and 2019. This timeframe represents the expansion time of Artic-like lineage in Italy. As a main result we added novel insights to the genomic epidemiology of CDV in mid-Italy. To further demonstrate the feasibility of mobile genomic surveillance in understanding animal infectious diseases we performed a snapshot-study among stray dogs in Bangladesh. There is no available genome information of CDV from Bangladesh, so we decided to conduct a rapid surveillance among street dogs with mobilized laboratory capacity of PCR diagnostics and sequencing. Regarding the significance of this thesis work, we

were able to demonstrate the rapid and effective deployment of our sequencing method to gain insight in the epidemiology of CDV in a distant and low-resource geographic region.

The results primarily draw attention to the fact that CDV has been present in domestic dog and wild-living carnivore populations in Hungary for the past two decades and provides novel sequencing method with new genomic data to better understand the evolution of these lineages. The impact of CDV on the wild-living carnivore populations is currently unclear. Still, it is noteworthy that the infected animals in our study originated from different regions of the country and years, suggesting the endemic nature of this lineage in Hungary. In the course of our work, we succeeded in sequencing complete genomes from samples from Italy, which gives us the opportunity to detect and understand epidemics in the past. We also successfully tested the method we developed on a new strain in Bangladesh, a CDV lineage that is phylogenetically well separated from the lineages found in Europe and far away.

These animals are relevant in ecosystems, viral genomics, and monitoring diseases of wildlife in terms of preparing for and treating future epidemics. Complete genomic data provides a stronger basis for understanding the evolution of the virus, compared to the widely used Hemagglutinin gene. In addition, the long-term built tissue sample collections provide a unique opportunity for retrospective pathogens examination, connecting to conservation ecological relationship analyses. Knowledge of infection events can help the conservation of certain wild species and even household animal populations.

In summary, we assessed the technological and knowledge gaps in CDV research and diagnostics and provided an alternative and modern solution to advance genomic surveillance of the virus. Following the demonstration of our method in multiple setups, we provided a significant amount of available genome sequences of CDV, approximately 53 complete genome data was generated and shared during the work, and we proved two new host species for CDV.

### *7.2 Magyar nyelvű összefoglaló*

A canine distemper virus (a szopornyicát okozó vírus), egy negatív egyszálú RNS-vírus, amely a *Paramyxoviridae* családba, azon belül a *Morbillivirus* nemzetségébe tartozik. A Hemagglutinin gén alapú genetikai csoportosítás alapján számos törzse ismert, amelyek különböző gazdaszervezetek és földrajzi területek szerint osztályozhatók. A CDV világszerte elterjedt vírusfertőzés, amely a vadon élő és háziállat populációkat egyaránt érinti. Gyakori a fajok közötti átvitel, ami a ritka és veszélyeztetett fajok esetében természetvédelmi problémákhoz vezethet. Elsősorban testnedvekkel, például nyállal, légúti cseppekkel, vizelettel és ürülékkel terjed. A vírusfertőzést követően az érintett populációkban magas halálozási arány

jellemző, am a kutyáknál akár 60-80%-os is lehet, vadon élő menyétféléknél megközelíti a 100%-ot. Munkánk során célunk volt a Magyarországon jelenleg és a múltban megtalálható szopornyica törzsek és az érintett fajok feltérképezése. Ehhez célul tűztük ki egy általánosan, minden ismert törzs esetén alkalmazható teljes genom szekvenáló módszer fejlesztését, majd a módszer tesztelését különböző olaszországi és bangladesi mintákon.

Az első vizsgálatban egy magyarországi menhelyi kutya fertőzésének lefolyását követtük nyomon. A kutya 2019 februárjától 2020 júniusáig, vagyis 17 hónapig ürítette a vírust. A munka fő következtetéseként azonosítottuk a megfelelő szekvenálási módszer hiányát a CDV hatékony vizsgálatához. A széles körben alkalmazott Hemagglutinin gén alapú elemzés nem elegendő a genomszintű evolúciós minták, specifikus mutációk vagy rekombinációs események feltárásához. Ezért kifejlesztettünk egy amplikon alapú NGS szekvenálási módszert az összes ismert CDV-vonal teljeskörű vizsgálatához. Módszerünk teszteléséhez először 2021-ben természetes fertőzés következtében elpusztult vörös rókák, valamint az 1997-2022 között boncolt további kis- és közepes testű menyétfélék mintáit vizsgáltunk, amelyek többnyire közútigázolás okozta pusztulásból származnak.

A módszer magyar CDV törzseken való sikeres alkalmazását követően a vizsgálati területet kiterjesztettük. Olaszországban 20 kutya és 3 vörös róka 2005 és 2019 közötti időszakból származó pozitív mintáján retrospektív módon teszteltük. Ennek fő eredményeként új betekintést kaphatunk a CDV genomikai epidemiológiájába Olaszország középső részén. Annak érdekében, hogy tovább demonstráljuk a mobilizálható genom szekvenálás megvalósíthatóságát az állatok fertőző betegségeinek megértésében, rövidtávú vizsgálatot végeztünk Bangladesben kóbor kutyák körében. A Bangladesből származó CDV genomjára vonatkozóan nem áll rendelkezésre információ, ezért úgy döntöttünk, hogy gyors megfigyelést végzünk utcai kutyák körében, mobilizált PCR diagnosztikai és szekvenálási laboratóriumi kapacitás segítségével. Itt bemutattuk szekvenálási módszerünk gyors és hatékony alkalmazását, hogy betekintést nyerjünk a CDV epidemiológiájába egy távoli és alacsony erőforrású földrajzi régióban.

Az eredmények elsősorban egyrészt arra hívják fel a figyelmet, hogy a CDV Magyarországon az elmúlt két évtizedben jelen volt a kutya- és a vadonélő ragadozó populációkban, továbbá, hogy ezeknek a jelenlévő törzseknek jobb megértésére az új genomadatok és a teljes genom szekvenálási módszer jól és rugalmasan alkalmazható. A CDV hatása a vadonélő ragadozópopulációkra jelenleg nem ismert. Mindazonáltal figyelemre méltó, hogy a vizsgálatunkban szereplő fertőzött állatok az ország különböző régióiból és különböző évekből származtak, ami arra utal, hogy ezek a törzsek Magyarországon endemikusnak

tekinthetők. A munkánk során sikerült olaszországi mintákból teljes genomokat szekvenálni, ami lehetőséget ad a korábbi járványok felderítésére és megértésére. A további vizsgálatok szempontjából fontos, hogy egy földrajzilag távol álló területen, Bangladesben egy, az Európában található törzsektől filogenetikailag jól elkülönülő és távol eső új szopornyica törzsön is sikeresen teszteltük az általunk fejlesztett módszert.

A vizsgált fajok vagy társállatként (kutya) fontosak számunkra, vagy a vadonélők az ökoszisztéma fontos részét képezik. A velünk élő és a vadonélő állatok betegségeinek nyomon követése kiemelt feladat a jövőbeli járványokra való felkészülés és kezelés szempontjából. Az új technikákra alapozottan felderíthető teljes genomikai adatok erősebb alapot adnak a vírus evolúciójának megértéséhez, mint az eddig széles körben használt Hemagglutinin gén vizsgálatok. A hosszú távon bővített és fenntartott szövetminta-gyűjtemények egyedülálló lehetőséget biztosítanak a betegségek retrospektív vizsgálatára, például korábbi populációdinamikai események megértéséhez. Ilyen módon a betegségökológia (disease ecology) és a konzervációökológia szakterülete szorosan összekapcsolódik. A fertőzési események ismerete segíthet bizonyos vadon élő fajok állományainak megőrzésében, és a felelős társállattartás szemléletének elmélyítésében.

Összefoglalva, felmértük a CDV-kutatás és -diagnosztika technológiai és tudásbeli hiányosságait, és alternatív és modern megoldást kínáltunk a vírus genomikai vizsgálatának előmozdítására. Módszerünk több összeállításban történő bemutatását követően jelentős mennyiségű elérhető CDV genom szekvenciát biztosítottunk, mintegy 53 teljes genom adat keletkezett és két újabb gadaszervezetett sikerült azonosítani a munka során.

## 8. Novel Scientific Results

- We observed the longest known CDV infection in a sheltered dog with general molecular diagnostic procedures.

- We developed a novel, pan-genotype CDV-specific amplicon-based next-generation sequencing method and optimized it for Oxford Nanopore sequencing platform.

- We applied this method in multiple scenarios: outbreak investigation in red foxes, retrospective surveillance of rare and protected mustelid species.

- We demonstrated the mobility and high flexibility of the method in genetically distant CDV lineages with the mobilization of the technique in Italy and Bangladesh.

- We provided a significant amount of genomic sequence data CDV, approximately 53 near-complete genome data was generated and shared on public databases.

- We provided the first CDV genomic sequence data from Bangladesh.

- We identified two new host species for CDV: Eurasian otter, steppe polecat.

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## 11. Publications

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*9.1. Publication related to the thesis topic*

**Lanszki, Z.**, Lanszki, J., Tóth, G. E., Cserkész, T., Csorba, G., Görföl, T., Csathó, A.I., Jakab, F., Kemenesi, G. (2022) Detection and sequence analysis of Canine morbillivirus in multiple species of the Mustelidae family. *BMC Veterinary Research,* 18, 450. DOI:10.1186/s12917-022-03551-7 IF: 2.600

**Lanszki, Z.**, Lanszki, J., Tóth, G.E., Zeghbib, S., Jakab, F., Kemenesi, G. (2022) Retrospective Detection and Complete Genomic Sequencing of Canine morbillivirus in Eurasian Otter (*Lutra lutra*) Using Nanopore Technology. *Viruses,* 14, 1433. DOI: 10.3390/v14071433 IF: 4.700

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*9.2. Conferences related to the thesis topic*

**Lanszki, Z.,** Lanszki, J., Tóth, G.E., Cserkész, T., Jakab, F., Kemenesi, G. *Canine Morbillivirus* detection and sequence analysis in different carnivores in the last two decades from Hungary, 8th European Congress of Virology 2023, Poland, Gdańsk, 2023

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*9.3. Publication outside the thesis topic*

Tóth, G. E., Hume, A. J., Suder, E. L., Zeghbib, S., Ábrahám, Á., **Lanszki, Z.**, Varga, Z., Tauber, Z., Földes, F., Zana, B., Scaravelli, D., Scicluna, M.T., Pereswiet-Soltan, A., Görföl, T., Terregino, C., De Benedictis, P., Garcia-Dorival, I., Alonso, C., Jakab, F., Mühlberger, E., Leopardi S., Kemenesi, G. (2023) Isolation and genome characterization of Lloviu virus from Italian Schreibers's bats. *Scientific Reports*, 13(1), 11310. DOI:10.1038/s41598-023-38364-7

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*9.4. Conferences outside the thesis topic*

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