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Prevalence and molecular characterization of circoviruses, astroviruses and hepadnaviruses in the avifauna of Kopački rit Nature Park, Croatia

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ABSTRACT

Wild birds harbour a plethora of viruses but the research into them has mainly focused on those possessing zoonotic potential or causing significant economic losses in poultry, while others, such as astroviruses (AstVs), circoviruses (CV) and hepadnaviruses (HBV), remain neglected. In this study, 35 dead wild birds belonging to at least 21 species from five orders, collected in Kopački rit Nature Park were tested for the presence of AstVs, CV and HBV using PCR with subsequent nucleotide sequencing. A positive result was found in 18 birds. AstVs were found in three passerine birds, CV was detected in 11 birds belonging to Accipitriformes, Anseriformes, Charadriiformes, Passeriformes or Piciformes orders while HBV was found in four birds belonging to either Anseriformes or Passeriformes. More than one virus was found in a single species (AstV and CV) although in two separate birds. Phylogenetic analysis revealed divergent AstVs somewhat similar to different unclassified AstVs detected in undetermined birds. One AstV shared the highest homology with a bat AstV (98.77%). All CV found in the study resemble avian CV, but none of them clusters in the phylogenetic tree with any avian or avian-like CV sequence. All four HBV sequences detected in this study cluster within the duck HBV group, indicating unusual interspecies transmission to cohabitating passerines. The results of this study represent an important contribution to knowledge about the prevalence, ecology and epidemiology of AstVs, CV and HBV in different wild bird species, indicating the need for further studies, including whole genome sequencing of the detected viruses.

Key words: wild birds; astroviruses; circoviruses; hepadnaviruses

Introduction

The main focus on viral infections in wild birds is usually on avian influenza (AI) and West Nile virus (WNV), primarily due to the high zoonotic potential of these two viruses (DAVIS et al., 2006.; MOSTAFA et al., 2018) and significant economic losses in the poultry industry due to AI outbreaks (MARTINS, 2012.; PAUL et al., 2019). Nevertheless, there is a vast majority of other viruses infecting free living birds and domestic poultry. Only a few studies have reported the detection of novel viruses, although increased knowledge about viruses circulating in wild birds is important (FULLER et al., 2012; BODEWES and KUIKEN, 2018). It is known that astroviruses (AstV),

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circoviruses (CV) and hepadnaviruses (HBV) play a role in the pathology of domestic poultry, but their role in wild birds is poorly understood and there are scarce reports on these viruses in avian wildlife. Avian AstVs (AAstV) belong to the genus *Avastrovirus* which, along with *Mamastrovirus,* make up the two viruses in the *Astroviridae* family (ANON, 2020). They are small, 27 to 30 nm in diameter, non-enveloped RNA viruses with a star-like surface morphology (COOK and MYINT, 1995.; KOCI et al., 2000). AstVs in avian species commonly cause mild gastroenteritis, particularly in younger birds, but more severe diseases have been described in poultry, with substantial economic losses (PANTIN-JACKWOOD et al., 2012). Occasionally, other pathological conditions have been reported, such as nephritis or hepatitis (IMADA et al., 2000; TODD et al., 2009). Most of the reports on AastVs are from poultry studies but there is a paucity of information from avian wildlife. Nevertheless, AstVs have been detected in different wild bird species (DONATO and VIJAYKRISHNA, 2017; FERNÁNDEZ-CORREA et al., 2019). CVs (family *Circoviridae*) are also small (approximately 20-25 nm in diameter) viruses, with a spherical outline and circular single-stranded DNA (HUGHES and PIONTKIVSKA, 2008). The best characterized CV in avian fauna is the beak and feather disease virus (BFDV), typically causing abnormal feathering and beak deformities in psittacine birds (RITCHIE et al., 1989; TODD, 2000). However, CVs have been detected in various domestic, captive and free living bird species (TODD, 2000; WOODS and LATIMER, 2000; HATTERMANN et al., 2003) recently with an increasing number of reports in avian wildlife (STEWART et al., 2006; HALAMI et al., 2008; WHITE et al., 2016; MORANDINI et al., 2019; LEVY et al., 2020). Nevertheless, the pathogenicity and/or role of certain novel avian wildlife CVs in the disease remain unclear (BODEWES and KUIKEN, 2018.). Hepadnaviruses (family *Hepadnaviridae)*, also known as hepatitis B viruses, are another group of small viruses which possess double stranded DNA. They are further divided into several genera including *Avihepadnavirus*, which infect birds. HBVs display strong hepatotropism (BIĐIN et al., 2014) and are characterized by a very narrow host range (FUNK et al., 2007). Avian hepadnaviruses were found to infect a variety of domestic and wild birds, primarily aquatic birds (SCHAEFER, 2007), but occasionally also others, such as a parrot (PIASECKI, et al., 2012) and a ratite (JO et al., 2017). These reports are generally related to diseased birds with the direct evidence of avian HBV, yet some studies on endogenous viral elements indicate that numerous other bird species may harbour or might have been harbouring HBV (GILBERT and FESCHOTTE, 2010; SUH et al., 2013; CUI et al., 2014).

The Kopački rit nature park is a habitat to numerous migratory bird species. It is located in the Baranja region in Eastern Croatia, on the Danube and Drava river confluence. The Park represents the best-preserved inland wetland that is part of the remaining Danube river floodplain. Thus, it is a significant area for bird nesting, wintering and resting on the Danube migration route, especially for water fowl. To date, about 300 bird species have been recorded, of which more than 140 are nesting birds (MIKUŠKA et al., 2002.). The aim of this study is to gain an insight into the prevalence and molecular characteristics of the astroviruses, circoviruses and hepadnaviruses in the nature park bird fauna, and to contribute to the knowledge of these neglected viruses in avian wildlife.

Materials and methods

Samples. A total of 35 bird carcasses, belonging to at least 21 species from five orders, were collected and tested for the presence of AstV, CV and HBV (Table 1). Most of the birds were passerine. A pool of organs (intestines, spleen and kidney) from each carcass was homogenized with the addition of approximately a 5 to 10 times higher volume of PBS. The homogenates were centrifuged at 2,000 g for 20 minutes and the supernatants were used for nucleic acid extraction.

Molecular methods and phylogenetic analysis. Viral RNA and DNA were extracted from the homogenized tissue supernatants using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany).

AstV RNA was detected by one-step RT-PCR, targeting the open reading frame (ORF) 1b

Table 1. The number of bird carcasses, by species and order, collected and tested within the study (n=35). Detection of astroviruses (n=3), circoviruses (n=11) and hepadnaviruses (n=4) is summarized in the last column.

Order	Species	No.of birds	Virus detected
Accipitriformes	Eurasian sparrowhawk (Accipiter nisus)	1	-
	White-tailed eagle (Haliaeetus albicilla)	3	CV
Anseriformes	Goosander (Mergus merganser)	1	CV
	Mute swan (Cygnus olor)	1	HBV
Charadriiformes	Black-headed gull (Larus ridibundus)	1	CV
Passeriformes	Barn swallow (Hirundo rustica)	1	CV
	Black redstart (Phoenicurus ochruros)	1	HBV
	Common blackbird (Turdus merula)	4	CV
	Common firecrest (Regulus ignicapillus)	1	-
	Eurasian nuthatch (Sitta europaea)	2	HBV
	Eurasian tree sparrow (Passer montanus)	2	CV
	European robin (Eritachus rubecula)	3	AstV
	Goldcrest (Regulus regulus)	3	CV
	Great grey shrike (Lanius excubitor)	1	AstV
	Great tit (Parus major)	2	AstV, CV*
	Marsh tit (Parus palustris)	1	-
	Tit (Parus sp.)	1	-
	Trush (Turdus sp.)	1	-
	White wagtail (Motacilla alba)	1	CV
	Yellow wagtail (Motacilla flava)	1	-
	Yellowhammer (Emberiza citrinella)	1	HBV
Piciformes	Great spotted woodpecker (Dendrocopos major)	2	CV**

CV – circovirus; HBV-hepadnavirus; AstV – astrovirus
*each virus found in a separate bird, **virus found in both birds

according to TODD et al. (2009). A SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Waltham, Massachusetts) and degenerate-based primers (forward: GAYTGGACIMGITAYGAYGGIACIATICC; reverse: YTTIACCCACATICCRAA) were used to amplify a fragment of approximately 434 nucleotides. The same primers were used for nucleotide sequencing of detected AstV. CV DNA was detected by nested PCR according to LI et al. (2010) targeting rep gene. GoTaq Green Mastermix (Promega Madison, Wisconsin) and degenerated primers CV-F1 (GGIAYICCICAYYTICARGG), CV-R1 (AWCCAICCRTARAARTCRTC), CV-F2 (GGIAYICCICAYYTICARGGITT), and CV-R2 (TGYTGYTCRTAICCRTCCCACCA) were used to amplify a fragment of approximately 400 bp. The primers CV-F2 and CV-R2 were used for sequencing of the PCR products obtained. HBV DNA was detected by a new method developed in this study. The extracted viral DNA was amplified by PCR with GoTaq Green Mastermix, and the following primers were used at 1μM final concentration, forward CATGCTCATTTGAAAGCTTATG and reverse TCCTAGCAGGTAATTTATTTCC, targeting 5-end of the virus genome. Cycling

conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 40 s, 51°C for 40 s, and 72°C for 20s; and 72°C for 5 min. The expected amplified fragment was 428 bp. The same primers were used for nucleotide sequencing.

PCR products for all three viruses were separated on 1% agarose gel and visualized with ethidium bromide. Specific bands were excised and purified with a QIAquick purification kit (Qiagen, Germantown, Maryland). Purified PCR products were Sanger sequenced in both directions by Humanizing Genomics, Macrogen Inc. After sequencing, the raw nucleotide sequences were assembled and the primer sequences were trimmed off. Genotyping and phylogenetic grouping of the obtained sequences were based on comparison with strains retrieved from the GenBank, and obtained using BLAST algorithm (http://www.ncbi.nlm.nih.gov). Neighbour-joining analysis was conducted and the evolutionary analyses were performed using MEGA7 (KUMAR et al., 2016).

Results and discussion

The results of AstV, CV and HBV detection in wild birds are presented in Table 1. Phylogenetic analyses of the detected viruses are presented in Figs 1, 2 and 3, respectively.

The emergence and re-emergence of viruses harboured in wild birds that cause significant economic losses in the poultry industry, or pose a zoonotic threat, such as AI and WNV, have shadowed a plethora of other viruses that may gain important economic or zoonotic importance in the future. Therefore, the prevalence was studied of AstV, CV and HBV in 35 dead wild birds in Kopački rit Nature Park. A positive result was found in 18 birds, most of them being CV (11), followed by HBV (4) and AstV (3). Detection of more than one virus in a breed was only found in the great tit (AstV and CV), although in two separate animals.

According to the current ICTV taxonomy (ANON, 2020) the genus *Avastrovirus* comprises three species: *Avastrovirus 1*, represented by the commonly known Turkey astrovirus-1 and other astroviruses detected in chickens, ducks, guineafowl and geese; *Astrovirus 2*, by Avian nephritis viruses -1 and -2 which are found mainly in chicken, but occasionally in turkeys, pigeons and ducks; and *Astrovirus 3* involving Turkey astrovirus-2 and Turkey astrovirus-3 and Duck astrovirus-1. This classification is based on AstVs from domestic poultry, but recent studies on avian wildlife have discovered divergent AAstV, hence, the clades *Astrovirus 4* and *Astrovirus 5* were proposed (FERNÁNDEZ-CORREA et al., 2019). Unfortunately, no *Astrovirus 5* sequences are available and only tentative capsid gene sequences for *Astrovirus 4* are available, thus our results for AstV could not be compared with these two tentative clades. In addition WILLE at al. (2018) found novel AAstV in waders in Australia, and termed them Neva, Carnarvon and Blencathra viruses. Our AstV sequences from European robins and great grey shrike are somewhat similar to the Blencathra virus although each of the two sequences has shown more similarity to different unclassified AstVs detected in anal swabs from undetermined birds. Similarly, our sequence from great tit clusters with Carnarvon virus shares the highest homology (98.77%) with AstV detected in a bat (Figure 1). The high diversity of novel AAstVs detected in wild birds, also detected in this study, requires redefinition of the AAstV classification with inclusion of additional *Avastrovirus* species.

The genus *Circovirus* comprises numerous species detected in mammals and birds, as well as two tick associated species (ANON, 2020). In this study we detected 11 CVs in ten wild bird species from five orders (Table 1). Most of them are closely related with each other, while a divergent CV nucleotide sequence was found in the white wagtail. Nevertheless, although all of them resemble avian CV, none of them clusters in the phylogenetic tree with any avian or avian-like CV sequence (Figure 2). The closest sequence to all CV sequences found in this study was an avian-like tick associated CV detected in *Ixodes scapularis*. This tick is commonly known as the deer tick, but is also known to parasitize migratory birds (OGDEN et al., 2008). Since knowledge on CVs in ticks is scarce, it is possible that ticks that feed on birds may be involved in CV transmission among birds. This

could explain the closely related CV nucleotide sequences found in different bird species in the same habitat. However, the finding of two different genetic lineages in this study, one comprising the majority of the CV sequences, and the other shaped by the Wagtail CV sequence, requires additional studies, including whole genome sequencing, for a better understanding of the CV epidemiology in wild birds.

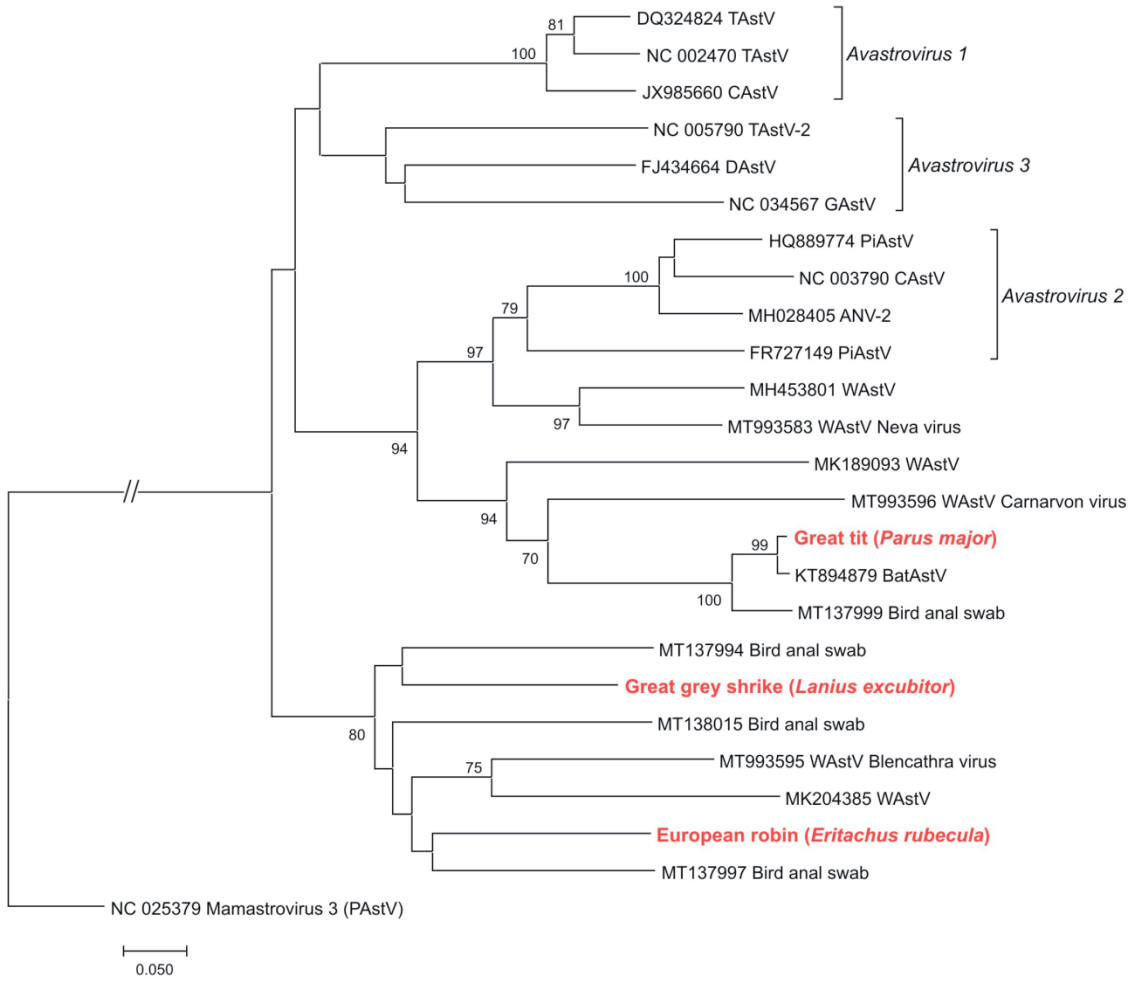

Fig 1. Phylogenetic neighbour-joining analysis based on alignment of the partial (230 nucleotides) ORF 1b sequences of avian and avian-like astroviruses (AstV).

The tree is rooted with Mammastrovirus 3. GenBank accession numbers and AstV designations are indicated on the branches. Viruses sequenced in this study are marked in bold and red colour with the host bird species. *Avastrovirus* species are indicated on the right. Supporting (≥70%) bootstrap values of 1,000 replicates are displayed at the nodes. The interrupted branch, indicated by double slashes, was shortened by 50% for better graphic representation. T= turkey, C=chicken, D=duck, G=goose, Pi=pigeon, W=wader, P=pig, ANV=avian nephritis virus.

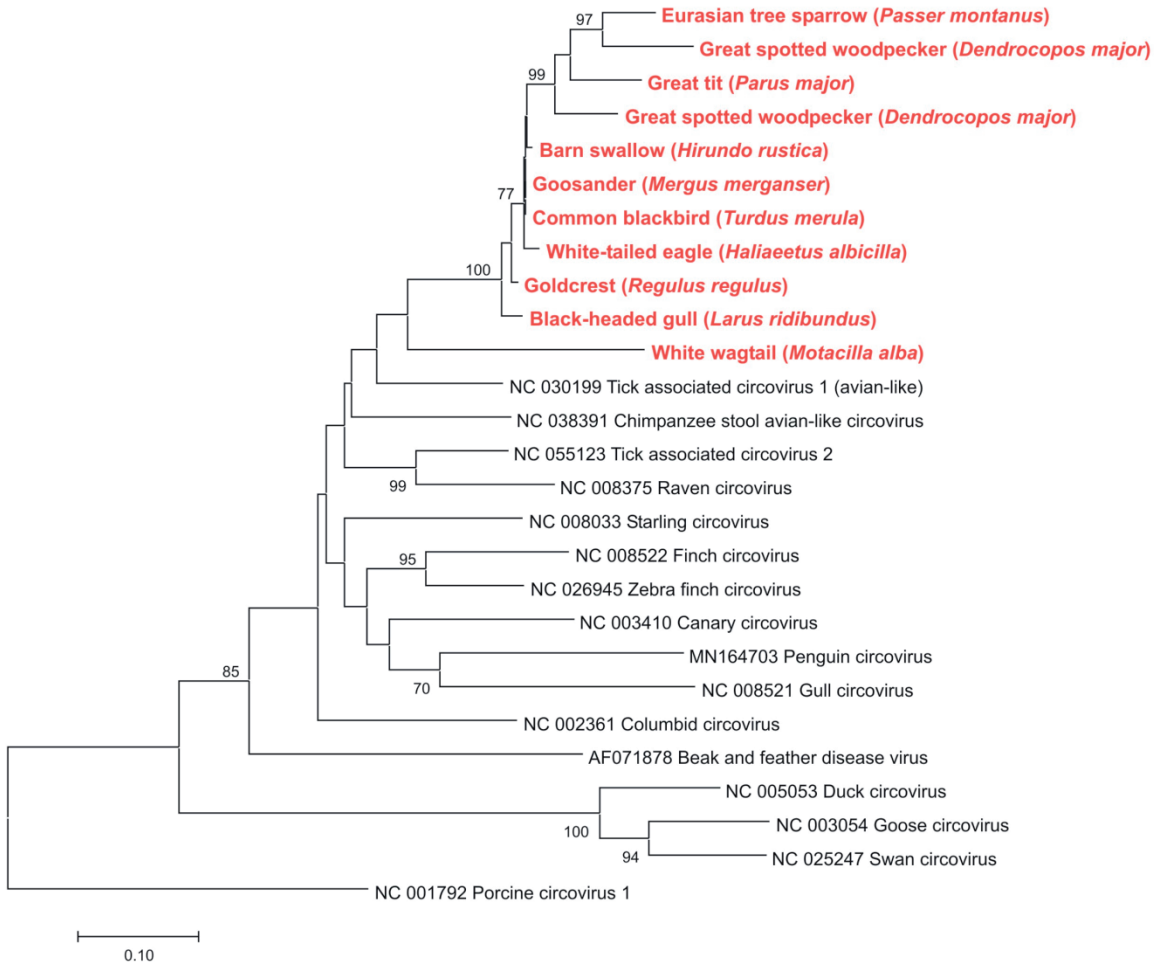

Fig 2. Phylogenetic neighbour-joining analysis based on alignment of the partial (405 nucleotides) rep gene sequences of avian and avian-like circoviruses (CV).

The tree is rooted with Porcine circovirus 1. GenBank accession numbers and CV designations are indicated on the branches. Viruses sequenced in this study are marked in bold and red colour with the host bird species. Supporting (≥70%) bootstrap values of 1,000 replicates are displayed at the nodes.

Avian HBV are classified into three species*:* *Duck hepatitis B virus*, *Heron hepatitis B virus* and *Parrot hepatitis B virus* (ANON, 2020) and primarily infect aquatic birds (SCHAEFER, 2007). In this study we found avian HBV in four wild birds, of which only the mute swan was an aquatic bird, while the other three were passerines. This finding of HBV in passerine birds is unusual, although endogenous HBVs are present in a variety of passerines. Nevertheless, the endogenization of HBV in bird genomes occurred tens of millions of years ago, with the consequent far greater diversity of endogenous HBVs, forming diverse clades in contrast to contemporary exogenous HBV which forms a tight monophyletic group (GILBERT and FESCHOTTE, 2010; SUH et al., 2013.; CUI et al., 2014). In this study, three HBV sequences from three different passerines were identical to a duck

avihepadnavirus detected in France in 2020 (Figure 3, sequence MW176098). The sequence found in the mute swan was very similar to the duck sequence from France. All four HBV sequences detected in this study cluster within the *Duck hepatitis B virus* group (Figure 3), thus the potential detection of endogenous HBV in yellowhammer, black redstart and eurasian nuthatch can be ruled out. Since all known HBV are strongly cell type specific and have a narrow host range, restricting them to their natural host and a few closely related species (FUNK et al., 2007), this finding indicates interspecies transmission of *Duck hepatitis B virus,* within an environment abundant in water fowls, to cohabitating passerines. Similar host switching events were reported for other viruses, for example, transmission with self-limiting BFDV infection in a group of rainbow bee-eaters (*Merops ornatus*) which are birds unrelated to parrots (SARKER et al., 2015).

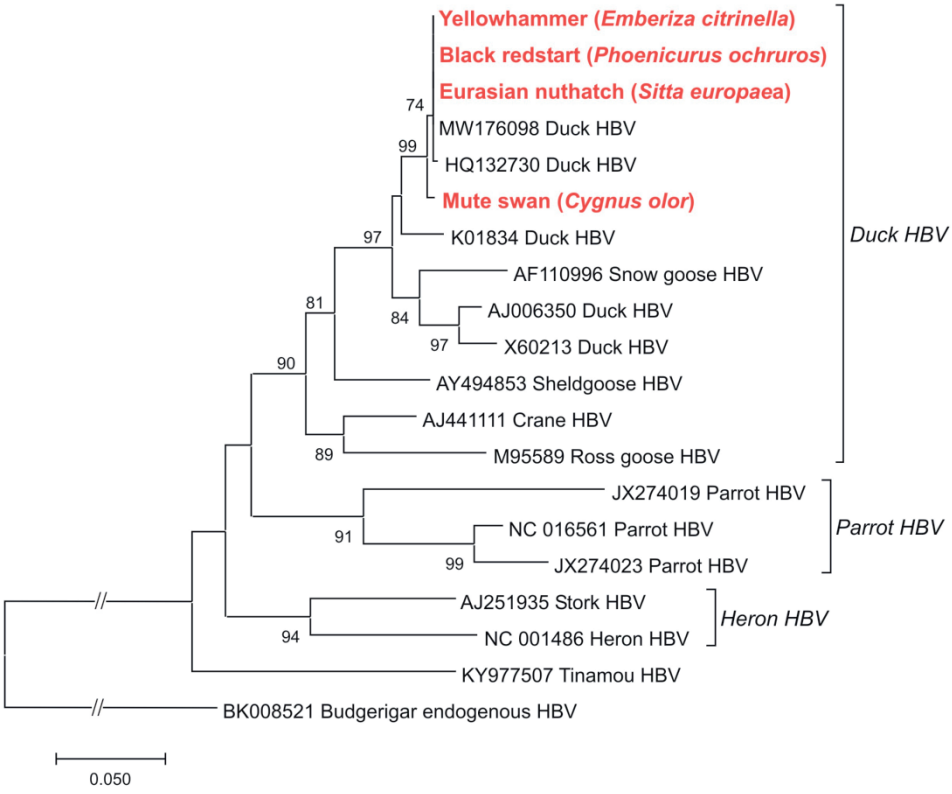

Fig 3. Phylogenetic neighbour-joining analysis based on alignment of the partial (361 nucleotides) 5' genome sequences of avihepadnaviruses.

The tree is rooted with highly divergent budgerigar endogenous hepadnavirus. GenBank accession numbers and virus designations are indicated on the branches. Viruses sequenced in this study are marked in bold and red colour with the host bird species. *Avihepadnavirus* species are indicated on the right. Supporting (≥70%) bootstrap values of 1,000 replicates are displayed at the nodes. The interrupted branches, indicated by double slashes, were shortened by 50% for better graphic representation. HBV=Hepatitis B Virus.

It is still not fully understood why certain common infections sometimes develop into emerging infectious diseases (JONES et al., 2008), although there is a hypothesis that parasites with a broad host range are more likely to switch hosts, triggering an emerging infectious disease (WOOLHOUSE and GOWTAGE-SEQUERIA, 2005). In this study, the majority of the wild bird species tested were shown to harbour at least one of the three viruses, while certain viruses were detected for the first time in a novel avian host species. CV was detected in ten wild bird species, which is approximately half of the tested species. The birds that tested positive for CV belonged to all the five orders included in the study. Contrarily, AstV was detected in only three bird species, all being passerines, but the detected AstV showed significant nucleotide divergence from each other and from other known AstVs. Interestingly, the nucleotide sequence of AstV detected in the great tit showed high similarity to the AstV detected in a bat. HBV was detected in this study in four wild bird species, however, three species belonged to Passeriformes which represents an unusual HBV finding. All four HBVs detected belonged to the *Duck hepatitis B virus* which means that the virus crossed the species barrier from Anseriformes to Passeriformes.

There has been a considerable bias in research into infectious diseases in favour of domestic birds compared to wild ones (FULLER et al., 2012), and there are evidently significant gaps in the knowledge of viral infections in wild avifauna. Therefore, the results of this study represent an important contribution to knowledge about the prevalence, ecology and epidemiology of AstV, CV and HBV in different wild bird species, indicating the need for further studies, including whole genome sequencing of detected viruses.

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SAŽETAK

Iako divlje ptice nose brojne viruse, istraživanja su uglavnom usmjerena na viruse koji posjeduju zoonotski potencijal ili uzrokuju znatne ekonomske gubitke u peradi, dok ostali, poput astrovirusa (AstV), cirkovirusa (CV) i hepadnavirusa (HBV), ostaju zanemareni. U ovom je istraživanju 35 uginulih divljih ptica, koje pripadaju najmanje 21 vrsti iz pet redova prikupljenih u Parku prirode Kopački rit, testirano na prisutnost AstV-a, CV-a i HBV-a PCR-om uz naknadno nukleotidno sekvenciranje. Pozitivan je rezultat ustanovljen u 18 ptica. AstV je pronađen u tri vrapčarke, CV je otkriven u 11 ptica koje pripadaju redovima Accipitriformes, Anseriformes, Charadriiformes, Passeriformes ili Piciformes dok je HBV pronađen u četiri ptice koje pripadaju rodovima Anseriformes ili Passeriformes. Više od jednog virusa pronađeno je u samo u jednoj vrsti (AstV i CV), iako u dvije zasebne ptice. Filogenetska analiza otkrila je divergentni AstV donekle sličan različitim neklasificiranim astrovirusima otkrivenima u neodređenim vrstama ptica. Jedan AstV dijeli najveću sličnost s AstV-om šišmiša (98,77 %). Svi cirkovirusi pronađeni u istraživanju nalikuju na ptičji CV, ali ni jedan od njih ne grupira se u filogenetskom stablu s bilo kojom sličnom sekvencijom. Sve četiri sekvencije HBV-a otkrivene u ovom istraživanju grupiraju se unutar skupine pačjih hepadnavirusa, što upućuje na neuobičajen prijenos na kohabitirajuće vrapčarke. Rezultati ovog istraživanja važan su doprinos spoznajama o prevalenciji, ekologiji i epidemiologiji AstV-a, CV-a i HBV-a u različitim vrstama divljih ptica, što upućuje na potrebu daljnjih istraživanja, uključujući sekvenciranje cijeloga genoma otkrivenih virusa.

Ključne riječi: divlje ptice; astrovirusi; circovirusi; hepadnavirusi