Short Communication

Genetic characterization of a novel picornavirus distantly related to the marine mammal-infecting aquamaviruses in a long-distance migrant bird species, European roller (*Coracias garrulus*)

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Despite the continuously growing number of known avian picornaviruses (family *Picornaviridae*), knowledge of their genetic diversity in wild birds, especially in long-distance migrant species is very limited. In this study, we report the presence of a novel picornavirus identified from one of 18 analysed faecal samples of an Afro-Palearctic migrant bird, the European roller (*Coracias garrulus* L., 1758), which is distantly related to the marine-mammal-infecting seal aquamavirus A1 (genus *Aquamavirus*). The phylogenetic analyses and the low sequence identity (P1 26.3 %, P2 25.8 % and P3 28.4 %) suggest that this picornavirus could be the founding member of a novel picornavirus genus that we have provisionally named 'Kunsagivirus', with 'Greplavirus A' (strain roller/SZAL6-KuV/2011/HUN, GenBank accession no. KC935379) as the candidate type species.

Picornaviruses (family *Picornaviridae*) are small, non-enveloped viruses with single-stranded, positive-sense genomic RNA. In general, the 7.2–9.1 kb polyadenylated picornaviral genome consists predominantly of a single polyprotein coding region flanked by highly structured 5′ and 3′ untranslated regions (UTRs), although substantial divergence from the common genome organization have been observed recently (Woo *et al.*, 2012). The viral polyprotein is co- and post-translationally processed into multiple capsid monomers: VP0 (sometimes cleaved to VP4 and VP2), VP3 and VP1, and non-structural proteins: 2A, 2B, 2C, 3A, $3B^{VPg}$, $3C^{pro}$ and $3D^{pol}$, and the presence of a leader (L) protein upstream of the capsid proteins is also observable in some picornaviruses (Racaniello, 2007; Boros *et al.*, 2012a).

The family *Picornaviridae* consists of 37 species grouped into 17 officially recognized genera (*Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Megrivirus*, *Parechovirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus*); and, currently 28 (but a rapidly increasing number) candidate species (Knowles *et al.*, 2012; Adams *et al.*, 2013; http://www.picornaviridae.com).

Free-living birds are effective hosts and dispersers of different viruses such as Newcastle disease virus (family *Paramyxoviridae*), Japanese encephalitis virus (family *Flaviviridae*) and avian influenza virus (family *Orthomyxoviridae*) that are potentially hazardous to livestock, poultry and even humans (Leighton & Heckert, 2007; McLean & Ubico, 2007; Stallknecht *et al.*, 2007). Despite studies predominantly related to the human threat of avian-borne viruses, knowledge of avian picornaviruses, especially viruses in wild birds are still limited. Of the 16 so far described avian picornaviruses, only duck hepatitis A virus (genus *Avihepatovirus*) from mallard ducks (*Anas platyrhynchos*), turdivirus 1 (unassigned species), turdivirus 2 and 3 (unassigned species) from dead birds of the family *Turdidae* and pigeon picornavirus A and B (unassigned species) from feral pigeons (*Columba livia*) are thought to infect wild birds (Knowles *et al.*, 2012; Gough & Wallis, 1986; Woo *et al.*, 2010; Kofstad & Jonassen, 2011).

The GenBank/EMBL/DDBJ accession number for the study sequence is KC935379.

One supplementary table is available with the online version of this paper.

Analysis of avian picornaviruses in free-living migratory birds is particularly important because these birds are easily capable of travelling long distances, even across continents, potentially transmitting avian-borne picornaviruses to new animal populations.

The European roller (*Coracias garrulus* L., 1758 of the family *Coraciidae*) is an Afro-Palearctic migrant (long-distance migrants that breed in Europe, including Hungary, and winter in sub-Saharan Africa) bird species living mainly in loose nomadic associations and sometimes forming large flocks containing hundreds of individuals (Fry, 2001). Due to the continuous decrease in population size, this bird species is now considered to be globally 'near threatened' by the International Union for Conservation of Nature, and is on their Red List of Threatened Species.

This is the first report of the presence of a novel picornavirus identified in a long-distance migrant bird species and distantly related to the marine-mammal-infecting seal aquamavirus A1 (SeAV-A1, genus *Aquamavirus*). Here, we proposed it as the prototype species in a novel genus in the family *Picornaviridae*.

Faecal samples from artificial nests occupied by healthy breeding pairs and nestlings of European rollers were collected from two different Hungarian breeding territories of the Great Hungarian Plain (Dorozsma-Majsai homokhát, n=14; Borsodi Mezőség, n=4) in July 2011 during the regular bird ringing process. Samples were collected by qualified ornithologists with valid permission (Permit No. of the National Inspectorate For Environment, Nature and Water: 14/1368-5/2011). Two randomly selected faecal samples (one from each breeding territory) were subjected to viral metagenomics analysis using sequence independent random reverse transcriptase-PCR (RT-PCR) amplification of viral-particle associated nucleic acids and 454 GS FLX technology, as described previously (Kapoor *et al.*, 2008a; Victoria *et al.*, 2009). To determine the complete picornavirus genome 5′/3′ RACE, RT-PCR amplification and dye-terminator sequencing were used as described previously (Boros *et al.*, 2011, Boros *et al.*, 2012b).

Five sequence contigs (Fig. 1a) originated from one of the sample (SZAL6) covering 26.8 % of a picornaviral genome related to SeAV-A1 (GenBank accession no. EU142040) as the closest match using BLASTX served as templates for virus-specific primer design (Table S1, available at JGV online). The 7272 nt RNA genome of the picornavirus strain roller/SZAL6-KuV/2011/HUN (GenBank accession no. KC935379) was predicted to possess a similar genome organization to SeAV-A1 : 5′UTR-P1(VP0-VP3-VP1)-P2(2A1-2A2-2B-2C)-P3(3A-3B-3C-3D)-3′UTR (Fig. 1). The G+C content (53.01mol %) of the entire genome is one of the highest among picornaviruses and significantly different from SeAV-A1 (Table 1).

The complete P1 (2349 nt; 783 aa), P2 (1881 nt; 627 aa) and P3 (2517 nt; 838 aa) regions showed low amino acid sequence identity to SeAV-A1 (GenBank accession no. EU142040) (Table 1). The identity calculations were performed by BioEdit software (version 7.1.3.0) (Hall, 1999) using the pairwise alignments generated by CLUSTAL_X software (version 2.0.3). The potential proteolytic cleavage sites of roller/SZAL6-KuV/2011/HUN were mapped based on (i) the aa alignment with the two SeAV-A1 sequences: HO.02.21 (GenBank accession no. EU142040) and Holland/88 (N. J. Knowles, Pirbright Institute, personal communication, 2012) (ii) and the NetPicoRNA predictions (Blom *et al.*, 1996). The predicted cleavage sites and the length of different genome regions are shown in Fig. 1(a).

The analysis of the P1 region did not support the presence of L protein or the maturation cleavage of VP0 similar to the members of genus *Aquamavirus* and other avian picornaviruses such as avihepato-, avisi-, galli-, megri- and turdiviruses (Tseng *et al.*, 2007; Boros *et al.*, 2013; Boros *et al.*, 2012a; Honkavuori *et al.*, 2011; Woo *et al.*, 2010). No potential myristoylation motif (GxxxS/T, where x is a non-conserved amino acid) was recognizable at the N-terminal end of the viral polyprotein, which suggests that, similar to the aquamaviruses and parechoviruses, myristoylation of VP0 may not occur (Kapoor *et al.*, 2008b).

The analysis of P2 region revealed the presence of an aphthovirus-like 'ribosome-skipping' motif ($DxExNPG_{838}$/P) similar to SeAV-A1, leading to the release of a 55 aa 2A1 protein. The C-terminal 22 aa residues of roller/SZAL6-KuV/2011/HUN 2A1, which could be the core site of 'ribosomal skipping' (Ryan *et al.*, 1991), shows 59 % amino acid identity to the 29 aa 2A1 of SeAV-A1 (EU142040). The N-terminal part (33 aa) of the 2A1 protein showed no significant sequence identity to any of the known picornaviral 2A sequences (Fig. 1b). The proteolytic cleavage site analysis revealed the presence of a second, 165 aa 2A protein that showed only 11 % amino acid identity to the 100 aa 2A2 of SeAV-A1 and did not possess any of the known picornaviral 2A characteristic motifs (e.g. catalytic sites of trypsin proteases, H-box/NC-motifs or the GxGxxGKS motifs of NTP-binding sites of 2As of avihepato- and avisiviruses) (Tseng *et al.*, 2007; Boros *et al.*, 2013). A Conserved Domain Database (CDD) search (Marchler-Bauer *et al.*, 2011) identified some functional sites of protein kinases (CDD-ID: cd05094) and adenylate forming domains (CDD-ID: cl17068) in the 2A2 of roller/SZAL6-KuV/2011/HUN, although with a relatively high E-values (Fig. 1b). The 2C protein – similar to the other picornaviruses – falls into the class III helicases and all three functional motifs (A–C) were identifiable (Fig. 1a) (Hales *et al.*, 2008).

The proteolytic cleavage site mapping strongly suggested the release of a single, 24 aa $3B^{VPg}$ that was nearly half the size of the aquamavirus 46 aa 3B (encoding two VPgs in tandem) and showed only 42 % amino acid identity to the C-terminal VPg of SeAV-A1. Interestingly, the roller/SZAL6-KuV/2011/HUN VPg showed low similarity (34 %)

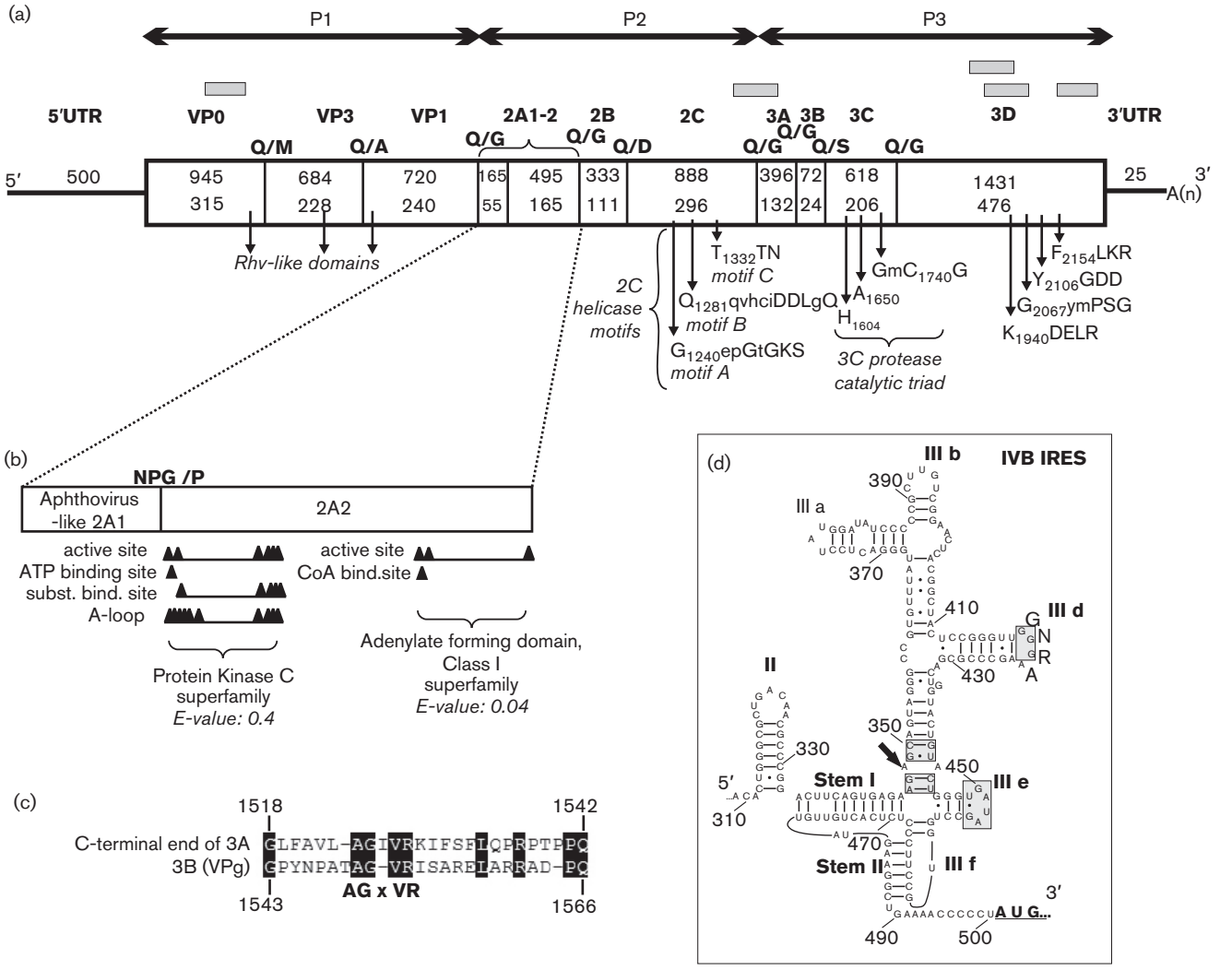

Fig. 1. (a) The genome organization, conserved picornaviral motifs and the predicted cleavage sites with the enlarged 2A genome region of roller/SZAL6-KuV/2011/HUN. Nucleotide (upper numbers) and amino acid (lower numbers) lengths are indicated in each gene box. The positions of the conserved picornaviral amino acid motifs are indicated with the first amino acid positions of the motif. The sequence contigs acquired from pyrosequencing are depicted as grey bars. (b) Functional sites of protein kinases and adenylate-forming domains of 2A2 of the study sequence identified by the CDD-search. (c) Alignment of the C-terminal end of the predicted 3A and the complete $3B^{VPg}$ of roller/SZAL6-KuV/2011/HUN. Identical amino acids are shaded. (d) Predicted secondary RNA structure of the 5′UTR type IVB IRES of the study virus. Certain parts of the IRES structure that are conserved in type IV IRES are indicated by grey shading. The location of the specific A–A mis-pair of domain III is shown by an arrow (Hellen & de Breyne, 2007).

and some conserved motifs (e.g. AGxVR) to the 25 aa peptide located at the C-terminal end of the 3A (from aa 1518 to 1542) (Fig. 1c), which suggested that the study strain originally had two VPgs, but one could have degenerated and become part of 3A.

The study sequence contains all of the conserved amino acid motifs of picornaviral 3C proteinase and 3D RNA polymerase (Fig. 1a) and showed the highest sequence identity to SeAV-A1 at the 3D region (Table 1) (Gorbalenya *et al.*, 1989).

The phylogenetic analysis was performed using the amino acid sequences of the complete P1, 2C and 3CD genome regions of roller/SZAL6-KuV/2011/HUN and the representative members of the family *Picornaviridae*. The amino acid phylogenetic trees were constructed using the neighbour-joining method based on the Jones–Taylor–Thornton matrix-based model of MEGA software (version 5.0) (Tamura *et al.*, 2011). Bootstrap values (based on 1000 replicates) for each node are shown if >50 %. All three phylogenetic trees show the consequent but distant

Table 1. Genomic features of the representative species of the 17 officially recognized and two candidate picornavirus genera and pairwise amino acid sequence identities between the P1, P2, P3, 2C and 3D proteins of roller/SZAL6-KuV/2011/HUN (KC935379) compared with those of the picornaviruses. Bold numbers indicate the highest levels of amino acid identities

Genus	Type species	Genome features				Roller/SZAL6-KuV/2011/HUN (KC935379) pairwise amino acid identity (%)				
		GenBank accession no.	Genome size (nt)	G+C content (mol%)	IRES type	P1	P2	P3	2C	3D
Aphthovirus	*Foot-and-mouth disease virus*	AF274010	8115	54.08	Type II	15.5	16.9	19.2	21.9	23.8
Aquamavirus	*Aquamavirus A*	EU142040	6718	43.85	Type IVB	**26.3**	**25.8**	**28.4**	**34.7**	**34.9**
Avihepatovirus	*Duck hepatitis A virus*	DQ249299	7687	43.23	Type IVB	20.3	19.2	22.9	30.3	30.2
Cardiovirus	*Encephalomyocarditis virus*	M81861	7835	49.47	Type II	15.1	15.5	19.6	18.9	22.2
Cosavirus	*Cosavirus A*	FJ438902	7632	43.75	Type II	16.0	17.0	21.6	21.4	25.3
Dicipivirus	*Cadicivirus A*	JN819202	8785	41.72	Undefined	13.9	15.9	19.5	20.8	24.1
Enterovirus	*Enterovirus C*	V01149	7440	46.35	Type I	14.6	14.9	19.7	20.4	24.8
Erbovirus	*Equine rhinitis B virus*	AF361253	8821	50.40	Type II	14.1	18.0	22.2	21.6	25.0
Hepatovirus	*Hepatitis A virus*	M14707	7478	37.85	Type III	11.6	16.1	16.1	20.6	18.5
Kobuvirus	*Aichivirus A*	AB010145	8251	58.91	Type VB/V	16.5	17.4	21.7	23.3	26.4
Megrivirus	*Melegrivirus A*	HM751199	9075	46.07	Type IV	12.4	16.7	16.8	23.2	21.9
Parechovirus	*Human parechovirus*	L02971	7339	39.60	Type II	21.7	18.7	22.8	27.0	28.8
	Ljungan virus	AF327920	7590	42.53	Type II	20.7	19.7	24.3	26.7	30.5
Salivirus	*Salivirus A*	GQ184145	7989	56.68	Type V	16.9	17.0	19.2	22.8	22.7
Sapelovirus	*Porcine sapelovirus*	AF406813	7491	41.04	Type IVB	15.7	15.5	19.9	18.9	24.3
Senecavirus	*Seneca Valley virus*	DQ641257	7310	51.62	Type IVA	14.5	16.1	19.4	21.3	24.3
Teschovirus	*Porcine teschovirus*	AJ011380	7117	44.83	Type IVB	14.1	16.2	20.1	21.6	24.1
Tremovirus	*Avian encephalomyelitis virus*	AJ225173	7055	44.88	Type IVA	10.3	13.7	17.1	19.0	21.5
'Avisivirus'	'Turkey avisivirus'	KC465954	7532	44.97	Type II	20.1	18.4	23.0	26.1	27.6
'Pasivirus'	'Swine pasivirus'	JQ316470	6916	43.20	Undefined	19.4	20.1	23.1	29.1	26.7

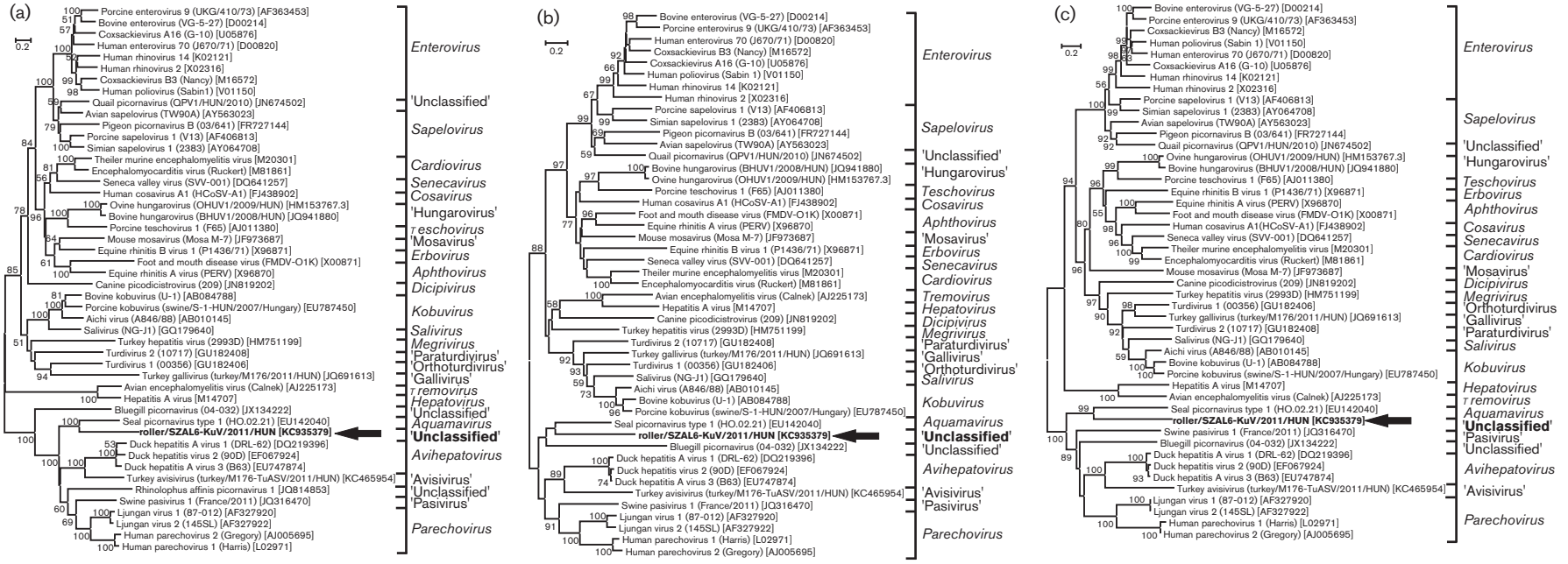

Fig. 2. Phylogenetic relationships between roller/SZAL6-KuV/2011/HUN (indicated in bold and with an arrow), representative members of the 17 picornavirus genera and unassigned picornaviruses based on amino acid sequences of the different picornavirus proteins: P1 (a), 2C (b) and 3CD (c). Bars indicate amino acid substitutions per site.

relationship of roller/SZAL6-KuV/2011/HUN to SeAV-A1 (*Aquamavirus*) (Fig. 2).

The 500 nt 5′UTR was similar in length to the 5′UTR of SeAV-A1 (506 nt) and contained three terminal uracils. The classification and analysis of the internal ribosomal entry site (IRES) (from nt 309 to 503) of the study sequence was performed by the Mfold program (Zuker, 2003). The predicted secondary structure of roller/SZAL6-KuV/2011/HUN had close similarity to the type IVB IRES structures of members of the genera *Sapelovirus*, *Teschovirus* and *Aquamavirus* (Table 1); thus, it contained the conservative IIIe stem–loop with highly conserved unpaired bases and the IIId G loop, but GpG instead of CpG dinucleotide pairing in the IIIf (Fig. 1d) (Hellen & de Breyne, 2007; Kapoor *et al.*, 2008b). The 25 nt 3′UTR of the study sequence was similar in length to the 34 nt 3′UTR of SeAV-A1, the shortest among the known picornaviruses, and with a fold to a single stem–loop predicted by the Mfold program (data not shown).

Generic 3D^{pol} primers (Szal6-AqV-3DGen-R and Szal6-AqV-3DGen-F; Table S1) were designed based on the 3D^{pol} sequences of roller/SZAL6-KuV/2011/HUN and aquamaviruses for screening all of the faecal samples collected from the European rollers. No other picornaviruses were detected using this RT-PCR.

In this study, using metagenomic and RT-PCR approaches, we have reported the first complete genome sequence of a novel picornavirus (roller/SZAL6-KuV/2011/HUN) isolated from a long-distance migrant bird species, European roller, in Hungary. According to the current guidelines of the ICTV *Picornaviridae* Study Group (http://www.picornastudygroup.com/definitions/genus_definition.htm), novel picornavirus genera are defined by amino acid identities in the P1, P2 and P3 regions being less than <40, <40 and <50 %, respectively, compared with other genera (Table 1). Based on these guidelines, and the supporting phylogenetic analyses, roller/SZAL6-KuV/2011/HUN could be the founding member of a novel picornavirus genus. Given the lack of knowledge about the origin and pathogenic role of this picornavirus species, we propose to name it Greplavirus A (from the geographical name of the Great Hungarian Plain) in a novel genus 'Kunsagivirus' (from the name of the part of the Great Hungarian Plain – 'Kunság' – where the samples were collected), in the family *Picornaviridae*.

The identification of roller/SZAL6-KuV/2011/HUN from only one of the analysed faecal samples raises the possibility that the European roller is not the natural host of this virus but that it originated from another animal that was eaten. This is suspected for other enteric viruses identified using viral metagenomic approach, e.g. di-cistronic viruses from human faeces (Kapoor *et al.*, 2010) and bat guano (Li *et al.*, 2010). The European rollers consume primarily medium-sized (<35 mm) insects (Orthoptera, Coleoptera), although occasionally small vertebrates [e.g. pygmy shrews (*Soricidae*), lizards (*Lacertidae*)] may also serve as a food source (Molnar, 1998). Interestingly, we found co-infections (data not shown) of different rodent-origin/rodent-related picornaviruses (e.g. mosavirus and kobuvirus), mamastroviruses, picobirnavirus and Puumala virus (genus *Hantavirus*) with roller/SZAL6-KuV/2011/HUN in sample SZAL6 using BLASTX on the sequences of viral metagenomics. Five viruses related to rodent-borne viruses support the dietary origins of the identified group of viruses, although the relatively low detection rate of roller/SZAL6-KuV/2011/HUN does not necessarily imply an outside source of the virus. Further epidemiological studies and supporting experiments (e.g. follow-up and seroprevalence studies) on the possible hosts (e.g. rollers, pygmy shrews, lizards) should be conducted to answer this question.

The analysis of viruses in faecal samples of such endangered, migrant bird species may help identify viruses that are potentially capable of long-distance spread and transmission to other animal populations.

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