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)

Ákos Boros,¹ Tamás Kiss,² Orsolya Kiss,³ Péter Pankovics,¹ Beatrix Kapusinszky,⁴ Eric Delwart⁴,⁵ and Gábor Reuter¹

¹Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary
²Hungarian Ornithological and Nature Conservation Society, Budapest, Hungary
³Ecology Department, Szeged University, Közép fasor 52, Szeged, Hungary
⁴Blood Systems Research Institute, San Francisco, CA, USA
⁵University of California San Francisco, San Francisco, CA, USA

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-Palaearctic 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, 3BVPg, 3Cpro and 3Dpol, 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, Dicippivirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Megivirus, 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; 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).
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 Picorniridae.

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 Mezőseg, 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 of viral-particle associated nucleic acids and 454 GS FLX random reverse transcriptase-PCR (RT-PCR) amplification to viral metagenomics analysis using sequence independent samples (one from each breeding territory) were subjected of viral-particle associated nucleic acids and 454 GS FLX random reverse transcriptase-PCR (RT-PCR) amplification to viral metagenomics analysis using sequence independent...

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 P protein or the maturation cleavage of VP0 similar to the members of genus Aquamavirus and other avian picornaviruses such as avihepat-, 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 (GxSxS/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 the P2 region revealed the presence of an aphthovirus-like ‘ribosome-skipping’ motif (DxExNPG383/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 aviparvo- and avivirus). The analysis of the P2 region revealed the presence of an aphthovirus-like ‘ribosome-skipping’ motif (DxExNPG383/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).

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The proteolytic cleavage site mapping strongly suggested the release of a single, 24 aa 3BVPg 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%).
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 3D 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

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**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 identical amino acids 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. (d) Predicted secondary RNA structure of the 5′-UTR type IV 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).
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.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type species</th>
<th>GenBank accession no.</th>
<th>Genome size (nt)</th>
<th>G+C content (mol%)</th>
<th>IRES type</th>
<th>Roller/SZAL6-KuV/2011/HUN (KC935379) pairwise amino acid identity (%)</th>
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<tbody>
<tr>
<td>Aphthovirus</td>
<td>Foot-and-mouth disease virus</td>
<td>AF274010</td>
<td>8115</td>
<td>54.08</td>
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<td>EU142040</td>
<td>6718</td>
<td>43.85</td>
<td>Type IVB</td>
<td>26.3 25.8 28.4 34.7 34.9</td>
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<tr>
<td>Avihepatovirus</td>
<td>Duck hepatitis A virus</td>
<td>DQ249299</td>
<td>7687</td>
<td>43.23</td>
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<td>26.3 19.2 22.9 30.3 30.2</td>
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<td>Cardiovirus</td>
<td>Encephalomyocarditis virus</td>
<td>M81861</td>
<td>7835</td>
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<td>15.1 15.5 19.6 18.9 22.2</td>
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<td>Cosavirus</td>
<td>Cosavirus A</td>
<td>FJ438902</td>
<td>7632</td>
<td>43.75</td>
<td>Type II</td>
<td>16.0 17.0 21.6 21.4 25.3</td>
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<td>46.35</td>
<td>Type I</td>
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<td>Equine rhinitis B virus</td>
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<td>Hepatitis A virus</td>
<td>M14707</td>
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<td>7590</td>
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<td>Type II</td>
<td>20.7 19.7 24.3 26.7 30.5</td>
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<td>Type IVB</td>
<td>15.7 15.5 19.9 18.9 24.3</td>
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<td>Seneca Valley virus</td>
<td>DQ641257</td>
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<td>51.62</td>
<td>Type IVA</td>
<td>14.5 16.1 19.4 21.3 24.3</td>
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<td>Teschovirus</td>
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<td>AJ011380</td>
<td>7117</td>
<td>44.83</td>
<td>Type IVB</td>
<td>14.1 16.2 20.1 21.6 24.1</td>
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<td>Tremovirus</td>
<td>Avian encephalomyelitis virus</td>
<td>AJ225173</td>
<td>7055</td>
<td>44.88</td>
<td>Type IVA</td>
<td>10.3 13.7 17.1 19.0 21.5</td>
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<td>‘Avisivirus’</td>
<td>‘Turkey avisivirus’</td>
<td>KC465954</td>
<td>7532</td>
<td>44.97</td>
<td>Type II</td>
<td>20.1 18.4 23.0 26.1 27.6</td>
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<td>‘Pasivirus’</td>
<td>‘Swine pasivirus’</td>
<td>JQ316470</td>
<td>6916</td>
<td>43.20</td>
<td>Undefined</td>
<td>19.4 20.1 23.1 29.1 26.7</td>
</tr>
</tbody>
</table>
Fig. 2. Phylogenetic relationships between roller/SZAL6-KuV/2011/HUN (indicated in bold and with an arrow), representative members of the 17 picornaviruses 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 IIIId G loop, but GpG instead of CpG dinucleotide pairing in the IIIIf (Fig. 1d) (Hellen & de Brayne, 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 3Dpol primers (Szal6-AqV-3DGen-R and Szal6-AqV-3DGen-F; Table S1) were designed based on the 3Dpol sequences of roller/SZAL6-KuV/2011/HUN and aquamavirus 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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