Review

2A and 2A-like Sequences: Distribution in Different Virus Species and Applications in Biotechnology

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Abstract: 2A is an oligopeptide sequence that mediates a ribosome “skipping” effect and can mediate a co-translation cleavage of polyproteins. These sequences are widely distributed from insect to mammalian viruses and could act by accelerating adaptive capacity. These sequences have been used in many heterologous co-expression systems because they are versatile tools for cleaving proteins of biotechnological interest. In this work, we review and update the occurrence of 2A/2A-like sequences in different groups of viruses by screening the sequences available in the National Center for Biotechnology Information database. Interestingly, we reported the occurrence of 2A-like for the first time in 69 sequences. Among these, 62 corresponded to positive single-stranded RNA species, six to double stranded RNA viruses, and one to a negative-sense single-stranded RNA virus. The importance of these sequences for viral evolution and their potential in biotechnological applications are also discussed.

Keywords: 2A peptide; double-stranded RNA virus; positive-sense single-stranded RNA virus; Totiviridae; Picornaviridae

1. Introduction

2A and 2A-like sequences are oligopeptides with approximately 18–25 amino acids and can mediate a co-translation “cleavage” of polyproteins in eukaryotic cells. The “core” sequence at the C-terminus of 2A, together with the N-terminal proline of the downstream protein, contains the canonical motif—(G/H)1D2(V/I)3E4X5N6P7G8P9—involved in a ribosome “skipping” effect during translation, which separates two proteins without needing a protease [1,2].

The 2A cleavage occurs between the G8 site at the upstream protein (P1) and the P9 site at the downstream protein (P2). During amino acid insertion into the protein, the 2A sequence can cause a structural modification at the ribosome peptidyl-transferase center (PTC), making the ribosome “skip” the proline codon. It inhibits the formation of a glycin-proline peptide bond because of the hydrolysis of the peptidyl (2A)-tRNAGly ester linkage, releasing the polypeptide from the translational complex [3,4]. In this way, the first amino acid, proline, of the downstream encoded protein, is specified by the third codon in the sequence of P7G8P9, and the C-terminal amino acid of the upstream encoded protein is a glycine encoded by the second codon in that sequence [5,6]. This ribosome “skipping” effect is also referred to as “Stop-Carry On” or “StopGo” translation [6]. Thus, the ribosome activity does not depend on structural elements within the mRNA but a peptide sequence, differentiating this mechanism from the other forms of non-canonical mRNA processing. Because of this activity, the 2A and 2A-like sequences can be named CHYSELs (cis-acting hydrolase elements) [7].

Originally, the term “2A” was assigned to define a specific region of the genome of the foot-and-mouth disease virus (FMDV), a positive-sense single-stranded RNA (pssRNA) virus and member of the Picornaviridae family [1,4,8–10]. Similar sequences discovered in...
other viruses were named “2A-like.” These sequences have been described in other Picornaviridae, such as Equine rhinitis A virus and Porcine teschovirus-1, in other viruses of the Dicistroviridae and Iflaviridae families [2], and even in the infectious myonecrosis virus (IMNV), a double-stranded RNA (dsRNA) virus belonging to the Totiviridae family [11].

From these first discoveries, the 2A and 2A-like proteolytic cleavage activities have been demonstrated in several eukaryotic systems in vitro and in vivo [2,12]. Because of their mechanism of action, some authors also refer to 2A and 2A-like peptides as cis-acting hydrolase elements [7,13].

In 2017, Yang et al. reviewed the 2A sequence structures and functions of Picornaviridae members [14]. The latest works analyzing 2A and 2A-like sequences, including viruses from other families, were conducted by Luke et al. in 2008, 2009, and 2014 and by Luke and Ryan in 2013 [2,15–17]. With advances in sequencing technology, in recent years, there has been a significant increase in the number of viral sequences added to the National Center for Biotechnology Information (NCBI) database. Therefore, the goal of this article was to introduce a new screening of 2A and 2A-like sequences in viral genomes available from the NCBI database to revise the principal 2A and 2A-like sequences, describe their occurrence in different viral families, and discuss their potential applications in biotechnology.

2. Materials and Methods

The sequences used in this study were obtained from the viral databank (https://www.ncbi.nlm.nih.gov/genome/viruses/, accessed on 9 January 2021). To find 2A/2A-like sequences, the viral genomes were aligned against some of the 2A/2A-like classical motifs (GDVEENPGP; GDVESNPGP; HDIETNPGP; GDVELNPGP; GDIELNPGP; GDIESNPGP; HDVEMNPGP) using the Blastp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 9 January 2021) and the non-redundant protein sequences database (nr) only including viruses (taxid:10239). Search parameters were set to return a maximum of 500 sequences for each query. Repeated viral sequences were excluded from the analysis.

An active search was performed on the publication linked to the sequence annotation in the NCBI database to identify whether the sequences found had already been reported in the literature after the initial screening. If no report was found, an active search was performed using the Google Scholar search tool, with each respective virus name plus the word “2A” as keywords. If no articles reported the presence of 2A/2A-like in the query virus, we considered this finding novel.

3. Results and Discussion

3.1. 2A/2A-Like Distribution on Viruses

Table 1 shows the principal 2A or 2A-like motifs that had their self-cleavage efficiencies tested in vitro, confirming that these sequences are widely distributed among the pssRNA and dsRNA viruses, ranging from insect to mammalian viruses. Luke et al. were the first to report this wide distribution and identified motifs similar to those found in the FMDV [2].

| Virus                              | Family             | Motif         | Cleavage Efficiency | References |
|------------------------------------|--------------------|---------------|---------------------|------------|
| Euprosterna elaeasa virus (EeV)    | Alphatetraviridae  | GDVEENPGP     | ~99%                | [2,18]     |
| Providence virus (PrV)             | Alphatetraviridae  | GDVESNPGP     | ~99%                | [2]        |
| Providence virus (PrV)             | Alphatetraviridae  | GDIEKNPGP     | ~94%                | [2]        |
| Providence virus (PrV)             | Alphatetraviridae  | GDVEKNPGP     | ~96%                | [2]        |
| Thosea asigna virus (TaV)           | Alphatetraviridae  | GDVEENPGP     | ~99%                | [1]        |
| Acute bee paralysis virus (ABPV)   | Dicistroviridae    | GDVETNPGP     | ~94%                | [1,2]      |
| Cricket paralysis virus (CrPV)     | Dicistroviridae    | GDVESNPGP     | ~90%                | [1,2]      |
| Drosophila C virus (DCV)           | Dicistroviridae    | GDVETNPGP     | ~95%                | [1]        |
| Ectropis oblique picorna-like virus (EoPV) | Iflaviridae   | GDVESNPGP     | ~99%                | [2,19]     |
| Ectropis oblique picorna-like virus (EoPV) | Iflaviridae   | GDIESNPGP     | ~99%                | [2,19]     |
| Infectious flacherie virus (IFV)   | Iflaviridae        | AGIESNPGP     | ~99%                | [1,2]      |
| Perina nuda picorna-like virus (PnPV) | Iflaviridae   | GDVESNPGP     | ~99%                | [2,20]     |
Table 1. Cont.

| Virus | Family | Motif | Cleavage Efficiency | References |
|-------|--------|-------|---------------------|------------|
| Perina nuda picorna-like virus (PnPV) | Iflaviridae | GDIESNPGP | ~99% | [2,20] |
| Encephalomyocarditis virus (EMCV) | Picornaviridae | HDIETNPGP | ~91% | [1,6] |
| Equine rhinitis A virus (ERAV) | Picornaviridae | GDVESNPGP | ~99% | [1,21] |
| Equine rhinitis B virus (ERBV-1) | Picornaviridae | GDVELNPGP | ~99% | [2,22] |
| Foot-and-mouth disease virus (FMDV) | Picornaviridae | GDVESNPGP | ~99% | [8,10] |
| Ljungan virus (LV) | Picornaviridae | GDVETNPGP | ~99% | [2,23] |
| Porcine teschovirus 1 (PTV-1) | Picornaviridae | GDVETNPGP | ~94% | [1,24] |
| Saffold virus (SAF-V) | Picornaviridae | HDVETNPGP | ~99% | [2,25] |
| Théler’s murine encephalomyelitis virus (TMEV) | Picornaviridae | HDVESNPGP | ~99% | [10] |
| Bombyx mori reoviridae 1 (BmCPV-1) | Reoviridae | GDIESNPGP | ~99% | [2,26] |
| Human reoviridae C (HurV-C) | Reoviridae | GDIELNPGP | ~82% | [2] |
| New adult diarrhea virus (ADRV-N) | Reoviridae | ECIESNPGP | ~97% | [2,27] |
| Operophtera brumata reoviridae 18 (OpbuCPV-18) | Reoviridae | GDVESNPGP | ~99% | [2] |
| Porcine reoviridae A (Porv-C) | Reoviridae | GDVELNPGP | ~89% | [1,2] |
| Infectious myonecrosis virus (IMNV) | Totiviridae | GDVESNPGP | ~99% | [2,11] |
| Infectious myonecrosis virus (IMNV) | Totiviridae | GDVEENPGP | ~99% | [2,11] |

The search for these motifs in the viral genomes available in the NCBI database revealed 69 sequences containing 2A-like motifs that had not been identified. Among these, 62 corresponded to pssRNA, six to dsRNA, and one to a negative-sense single-stranded RNA (nssRNA) virus. Additionally, 2A-like motifs, previously described in 102 sequences, were confirmed. All 2A/2A-like motifs and their respective species resulting from the search are described in Tables 2 and 3.

Table 2. Positive-sense single-stranded RNA virus containing 2A-like motifs.

| Accession Number | Virus | 2A Motif | Taxon |
|------------------|-------|----------|-------|
| YP_003620399.1   | Providence virus—2A1 | GDVEKNPGP | Carmotetraviridae |
| YP_003620399.1   | Providence virus—2A2 | GDVESNPGP | Carmotetraviridae |
| YP_003620399.1   | Providence virus—2A3 | GDIEKNPGP | Carmotetraviridae |
| NP_066241.1      | Acute bee paralysis virus | GDVETNPGP | Dicistroviridae |
| NP_066241.1      | Anopheles C virus | GDVELNPGP | Dicistroviridae |
| NP_647481.1      | Cricket paralysis virus | GDVESNPGP | Dicistroviridae |
| NP_044945.1      | Drosophila C virus | GDVESNPGP | Dicistroviridae |
| AMO03208.1       | Empeyrat virus | GDVELNPGP | Dicistroviridae |
| YP_008888535.1   | Formica exsecta virus 1 | GDIESNPGP | Dicistroviridae |
| YP_009221981.1   | Goose dicistrovirus | GDVELNPGP | Dicistroviridae |
| AS863246.1       | Israeli acute paralysis virus | GDVEENPGP | Dicistroviridae |
| NP_851403.1      | Kashmir bee virus | GDIELNPGP | Dicistroviridae |
| YP_009011065.1   | Fusarium graminearum hypovirus 1 | GDVEKNPGP | Hypoviridae |
| YP_009361829.1   | Diamond back moth iflavivirus—2A1 | GDVESNPGP | Iflaviridae |
| YP_009361829.1   | Diamond back moth iflavivirus—2A2 | GDVESNPGP | Iflaviridae |
| NP_919029.1      | Ectropis obliqua picorna-like virus—2A1 | GDIESNPGP | Iflaviridae |
| NP_919029.1      | Ectropis obliqua picorna-like virus—2A2 | GDIESNPGP | Iflaviridae |
| NP_277061.1      | Perina nuda virus—2A1 | GDVESNPGP | Iflaviridae |
| YP_009010984.1   | Spodoptera exigua iflaviruses 2 | GDVESNPGP | Iflaviridae |
| NP_573542.1      | Euprosterna clausa virus | GDVEENPGP | Permutotetraviridae |
| AAC97195.1       | Thosea asigna virus | GDVEENPGP | Permutotetraviridae |
| AXF38648.1       | Aristeirus sp.—2A1 | GDVESNPGP | Picornaviridae |
| AXF38648.1       | Aristeirus sp.—2A2 | GDVESNPGP | Picornaviridae |
| AXF38648.1       | Aristeirus sp.—2A3 | GDVESNPGP | Picornaviridae |
| AXF38648.1       | Aristeirus sp.—2A4 | GDVESNPGP | Picornaviridae |
| AUX16868.1       | Aristeirus AVE052/AsV | GDIEENPGP | Picornaviridae |
### Table 2. Cont.

| Accession Number | Virus | 2A Motif | Taxon |
|------------------|-------|----------|-------|
| YP_009345900.1   | Bat crohivirus | GDIESNPGP | Picornaviridae |
| YP_006607894.1   | Bluegill picornavirus—2A | GDVESNPGP | Picornaviridae |
| YP_006792625.1   | Bovine hungarovirus 1 | GDVELNPGP | Picornaviridae |
| YP_009116874.1   | Bovine picornavirus | GDIESNPGP | Picornaviridae |
| YP_009352243.1   | Bovine rhinovirus 1 | GDVETNPGP | Picornaviridae |
| QEQ92497.1       | Burpengary virus | GDVEQNPGP | Picornaviridae |
| YP_009345900.1   | Crohivirus | GDIESNPGP | Picornaviridae |
| YP_006607894.1   | Crohivirus B | GDVESNPGP | Picornaviridae |
| QMI57967.1       | Chestnut teal aalivirus | GDVEENPGP | Picornaviridae |
| YP_002956074.1   | Cosavirus A | GDIESNPGP | Picornaviridae |
| YP_002956076.1   | Cosavirus D | GDGETNPQ | Picornaviridae |
| YP_009345900.1   | Crohivirus | GDIESNPGP | Picornaviridae |
| YP_009345900.1   | Crohivirus B | GDIESNPGP | Picornaviridae |
| YP_009026377.1   | Duck picornavirus GL/12—2A | GDVEENPGP | Picornaviridae |
| YP_009026377.1   | Duck picornavirus GL/12—2A | GDVEENPGP | Picornaviridae |
| AAA43035.1       | Encephalomyocarditis virus | HDIETNPQ | Picornaviridae |
| AKE44318.1       | Encephalomyocarditis virus | HDVETNPQ | Picornaviridae |
| AGU38152.1       | Encephalomyocarditis virus | HDVELNPG | Picornaviridae |
| AFO66759.1       | Encephalomyocarditis virus type 2 | HDVETNPQ | Picornaviridae |
| NP_653077.1      | Equine rhinitis B virus 1 | GDVELNPG | Picornaviridae |
| ANJ20934.1       | Equine rhinitis B virus 2 | GDVESNPG | Picornaviridae |
| ANJ20932.1       | Erbovirus A | GDVESNPG | Picornaviridae |
| ANJ20933.1       | Erbovirus A | GDVESNPG | Picornaviridae |
| YP_009243853.1   | Falcon picornavirus—2A | GDVEENPGP | Picornaviridae |
| YP_009243853.1   | Falcon picornavirus—2A | GDVEENPGP | Picornaviridae |
| AYL36968.1       | Fathead minnow picornavirus—2A | GDVESNPG | Picornaviridae |
| AAT17107.2       | Feline hantavirus | GDVEENPGP | Picornaviridae |
| AAT01791.1       | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AAT1791.1        | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AAT1791.1        | Foot-and-mouth disease virus—type SAT 1 | GDVESNPG | Picornaviridae |
| AAE48748.1       | Foot-and-mouth disease virus—type SAT 2 | GDVESNPG | Picornaviridae |
| AAT1795.1        | Foot-and-mouth disease virus—type SAT 3 | GDVESNPG | Picornaviridae |
| AIB08613.1       | Genet fecal theilovirus | HDVEMNPQ | Picornaviridae |
| YP_009026376.1   | Human cosavirus | HDIETNPQ | Picornaviridae |
| AFJ0437.1        | Human cosavirus A20 | GDVESNPG | Picornaviridae |
| YP_009351367.1   | Human cosavirus B | HDIETNPQ | Picornaviridae |
| AFJ71567.2       | Human hantavirus | GDVEENPGP | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 1 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 2 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 3 | GDVESNPG | Picornaviridae |
| YP_009026376.1   | Human cosavirus | HDIETNPQ | Picornaviridae |
| AAT01719.1       | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AFM56034.1       | Foot-and-mouth disease virus—type O | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 1 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 2 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 3 | GDVESNPG | Picornaviridae |
| AIB08613.1       | Genet fecal theilovirus | HDVEMNPQ | Picornaviridae |
| YP_009026376.1   | Human cosavirus | HDIETNPQ | Picornaviridae |
| AFJ0437.1        | Human cosavirus A20 | GDVESNPG | Picornaviridae |
| YP_009351367.1   | Human cosavirus B | HDIETNPQ | Picornaviridae |
| AFJ71567.2       | Human hantavirus | GDVEENPGP | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 1 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 2 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 3 | GDVESNPG | Picornaviridae |
| AIB08613.1       | Genet fecal theilovirus | HDVEMNPQ | Picornaviridae |
| YP_009026376.1   | Human cosavirus | HDIETNPQ | Picornaviridae |
| AAT01719.1       | Foot-and-mouth disease virus—type A | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 1 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 2 | GDVESNPG | Picornaviridae |
| AAT17978.1       | Foot-and-mouth disease virus—type SAT 3 | GDVESNPG | Picornaviridae |
| AIB08613.1       | Genet fecal theilovirus | HDVEMNPQ | Picornaviridae |
Table 2. Cont.

| Accession Number | Virus 2A Motif Taxon | Taxon               |
|------------------|----------------------|---------------------|
| SNQ28005.1       | Pasivirus A           | GDEIEQNPgp Picornaviridae |
| APA29021.1       | Picornaviridae sp. rodent | GDVELNPGP Picornaviridae |
| ADN52625.1       | Porcine encephalomyocarditis virus | HDIETNPGP Picornaviridae |
| AAK12398.1       | Porcine teschovirus 1 | GDVEENPGP Picornaviridae |
| AAK12413.1       | Porcine teschovirus 10 | GDVEENPGP Picornaviridae |
| AAK12390.1       | Porcine teschovirus 11 | GDVEENPGP Picornaviridae |
| AAK12381.1       | Porcine teschovirus 2 | GDVEENPGP Picornaviridae |
| AAK12382.1       | Porcine teschovirus 3 | GDVEENPGP Picornaviridae |
| AGB67759.1       | Porcine teschovirus 4 | GDVEENPGP Picornaviridae |
| ACT66681.1       | Porcine teschovirus 5 | GDVEENPGP Picornaviridae |
| AAK12409.1       | Porcine teschovirus 6 | GDVEENPGP Picornaviridae |
| AAK12386.1       | Porcine teschovirus 7 | GDVEENPGP Picornaviridae |
| AAK12388.1       | Porcine teschovirus 9 | GDVEENPGP Picornaviridae |
| QHX40840.1       | Porcine teschovirus 22 | GDIEENPGP Picornaviridae |
| ACD67870.1       | Rat theilovirus 1     | GDVETNPGP Picornaviridae |
| ACD67870.1       | Rat theilovirus 2     | GDVETNPGP Picornaviridae |
| ACO92353.1       | Saffold virus         | GDVETNPGP Picornaviridae |
| YP_001210296.2   | Saffold virus         | GDVETNPGP Picornaviridae |
| APZ85840.1       | Senecavirus A         | GDVETNPGP Picornaviridae |
| AHW37724.1       | Sikhote-Alin virus    | GDVETNPGP Picornaviridae |
| AUK47911.1       | Swine parvovirus SPaV1/US/17-508161A60467-1/2001 | GDVEENPGP Picornaviridae |
| BAU11153.1       | Swine picornavirus    | GDVEENPGP Picornaviridae |
| NP_653143.1      | Teschovirus A         | GDVETNPGP Picornaviridae |
| ACG55799.1       | Theiler’s encephalomyelitis virus | GDVETNPGP Picornaviridae |
| BAC58035.1       | Theiler’s-like virus of rats | GDVETNPGP Picornaviridae |
| AIY68187.1       | Toroise picornavirus  | GDVEQNPg Picornaviridae |
| AIY68186.1       | Toroise picornavirus  | GDVEQNPg Picornaviridae |
| ACG55801.1       | Vulpus human encephalomyelitis virus | GDVETNPGP Picornaviridae |
| AVM87411.1       | Yili teratoscincus roborowskii picornavirus 2 | GDVEQNPg Picornaviridae |
| YP_009329817.1   | Binatke RNA virus G1 | GDVETNPGP Unassigned Dicistroviridae |
| QNL09596.1       | Clinch dicistro-like virus 2—2A | GDVETNPGP Unassigned Dicistroviridae |
| QJ52079.1        | Dicistroviridae sp.   | GDVEMNPg Unassigned Dicistroviridae |
| AYQ66681.1       | Drosophila kikkawai virus 1 | GDVEMNPg Unassigned Dicistroviridae |
| YP_009336571.1   | Hubei dipta virus 1   | GDVEMNPg Unassigned Dicistroviridae |
| YP_009336583.1   | Hubei picorna-like virus 16 | GDVEMNPg Unassigned Dicistroviridae |
| YP_009336585.1   | Hubei picorna-like virus 17 | GDVEMNPg Unassigned Dicistroviridae |
| QKF95572.1       | Lebiluzia anandria dicistrovirus | GDIEENPGP Unassigned Dicistroviridae |
| AXA52579.1       | Linepithema humile virus 1 | GDIEPNP Unassigned Dicistroviridae |
| QIU8054.2        | Phenacoccus solenopsis virus | GDIEPNP Unassigned Dicistroviridae |
| YP_009336473.1   | Wenzling crustacean virus 3 | GDVEMNPg Unassigned Dicistroviridae |
| YP_009333180.1   | Wenzling picorna-like virus 2 | GDVEMNPg Unassigned Dicistroviridae |
| YP_009342327.1   | Wihan insect virus 11 | GDIEANP Unassigned Dicistroviridae |
| YP_009329857.1   | Beihai hepe-like virus 4 | GDIESNP Unassigned Hepeviridae |
| QDY81493.1       | Bipolaris oryzae hypovirus 1 | GDVEANP Unassigned Hypoviridae |
| YP_009337372.1   | Hubei picorna-like virus 43 | GDIESNP Unassigned Hypoviridae |
| QKN89050.1       | Iflaviridae sp.—2A | GDVSNP Unassigned Ifracviridae |
| QK77896.1        | Perth bee virus 3 | GDVETNPGP Unassigned Ifracviridae |
| YP_009336821.1   | Wenzhou picorna-like virus 49 | GDVEMNPg Unassigned Ifracviridae |
| AVM87450.1       | Guangdong spotted longbarbel catfish picornavirus—2A1 | GDVESNP Unassigned Picornavirales |
| AVM87450.1       | Guangdong spotted longbarbel catfish picornavirus—2A2 | GDVEMNPg Unassigned Picornavirales |
| AVM87450.1       | Guangdong spotted longbarbel catfish picornavirus—2A3 | GDVEMNPg Unassigned Picornavirales |
| ASC92543.1       | Picornavirales Q_5K_DV_036 | GDVEANP Unassigned Picornavirales |
### Table 2. Cont.

| Accession Number | Virus | 2A Motif | Taxon |
|------------------|-------|----------|-------|
| ASG92538.1       | Picornavirales Q_sR_OV_042 | GDIEENPGP | Unassigned Picornavirales |
| ATY47693.1       | Picornavirales sp. | GDVEENPGP | Unassigned Picornavirales |
| ATY47707.1       | Picornavirales sp. | GDVELNPGP | Unassigned Picornavirales |
| AWK02666.1       | Rhinolophus sinesis picornavirus | GDIEENPGP | Unassigned Picornavirales |
| QQP18688.1       | Soybean thrips picorna-like virus | GDVEENPGP | Unassigned Picornavirales |
| AWK02669.1       | Suncus murinus picornavirus | GDVETNPGP | Unassigned Picornavirales |
| AWK77886.1       | Victoria bee virus 1 | GDVETNPGP | Unassigned Picornavirales |
| AWK77887.1       | Victoria bee virus 2 | GDIEINPGP | Unassigned Picornavirales |
| AVM87443.1       | Wollong thamnaconus septentrionalis picornavirus | GDVETNPGP | Unassigned Picornavirales |
| AVM87419.1       | Western African lungfish picornavirus | GDVEENPGP | Unassigned Picornavirales |
| AVM87438.1       | Wollan carp picornavirus—2A1 | GDVESNPGP | Unassigned Picornavirales |
| AVM87439.1       | Wollan carp picornavirus—2A2 | GDVESNPGP | Unassigned Picornavirales |
| ANN02882.1       | Bovine rhinitis B virus 5 | GDVETNPGP | Unassigned Picornaviridae |
| AQP40272.1       | Human cosavirus (Cosavirus-zj-1) | GDVEENPGP | Unassigned Picornaviridae |
| AWG94399.1       | Human cosavirus E/D | GDVEENPGP | Unassigned Picornaviridae |
| AVX29481.1       | Marmot cardiovirus | HDVETNP6 | Unassigned Picornaviridae |
| AWK02672.1       | Niviventer confucianus hunnivirus | GDVELNPGP | Unassigned Picornaviridae |
| AVM71450.1       | Parechovirus-like virus | GDVEONPGP | Unassigned Picornaviridae |
| QBBH68005.1      | Parechovirus sp. QAPp32 | GDVEENPGP | Unassigned Picornaviridae |
| QKE55061.1       | Picornaviridae sp. | GDIEENPGP | Unassigned Picornaviridae |
| QKE55028.1       | Picornaviridae sp.—2A1 | GDVEENPGP | Unassigned Picornaviridae |
| QIM74091.1       | Picornaviridae sp.—2A2 | GDVEENPGP | Unassigned Picornaviridae |
| QIM74092.1       | Picornaviridae sp.—2A3 | GDVEENPGP | Unassigned Picornaviridae |
| YP_009336671.1   | Wenzhou picorna-like virus 48—2A1 | GDVETNPGP | Unassigned dsRNA |
| YP_009336671.2   | Wenzhou picorna-like virus 48—2A2 | GDVETNPGP | Unassigned dsRNA |
| YP_009336671.3   | Wenzhou picorna-like virus 48—2A3 | GDVETNPGP | Unassigned dsRNA |

Underlined names correspond to sequences that had no 2A sequence described before this study.

### Table 3. Double-stranded RNA viruses identified in this study containing 2A-like motifs.

| Accession Number | Virus | 2A Motif | Taxon |
|------------------|-------|----------|-------|
| AAU88188.1       | Adult diarrhea virus | ECIESNPGP | Reoviridae |
| BAR20437.1       | Bombyx mori cypovirus 1 | GDIESNPGP | Reoviridae |
| BA07973.1        | Bovine rotavirus C | GDVELNPGP | Reoviridae |
| AAO32344.1       | Dendrolimus punctatus cypovirus 1 | GDVEENPGP | Reoviridae |
| BAU80889.1       | Human rotavirus C | GDIELNPGP | Reoviridae |
| AAK73524.1       | Lymantria dispar cypovirus 1 | GDVEENPGP | Reoviridae |
| AKB17215.1       | Operophthera brunata cypovirus 18 | GDVETNPGP | Reoviridae |
| BAV34356.1       | Porcine rotavirus C | GDVELNPGP | Reoviridae |
| QBO122264.1      | Porcine rotavirus H | GDVELNPGP | Reoviridae |
| AQX34666.1       | Ratotavirus | GDVEENPGP | Reoviridae |
| CCC33025.1       | Fusarium poae mycovirus 2 | GDVEENPGP | Unassigned dsRNA |
| YP_00927910.1    | Fusarium poae mycovirus 2 | GDVEENPGP | Unassigned dsRNA |
| YP_009182156.1   | Penicillium aurantiogriseum asp-foetidas like virus 1 | GDVEENPGP | Unassigned dsRNA |
| YP_009342431.1   | Wuhan insect virus 31—2A1 | GDVELNPGP | Unassigned dsRNA |
| YP_00934933.1    | Armigeres sublatus | GDVEENPGP | Unassigned Totiviridae |
Table 3. Cont.

| Accession Number | Virus                                                | 2A Motif     | Taxon                      |
|------------------|------------------------------------------------------|--------------|---------------------------|
| YP_009256208.1   | Golden shiner totivirus                              | GDIESNPGP    | Unassigned Totiviridae    |
| AIC34742.2       | Penaeid shrimp infectious myonecrosis virus—2A<sub>1</sub> | GDIESNPGP    | Unassigned Totiviridae    |
| YP_009337085.1   | Penaeid shrimp infectious myonecrosis virus—2A<sub>2</sub> | GDVEENPGP    | Unassigned Totiviridae    |
| YP_009333269.1   | Wenling toti-like virus 2                           | GDIEPNPGP    | Unassigned Totiviridae    |
|                  | Wenling toti-like virus 1                           | GDVEMNPGP    | Unassigned Totiviridae    |

Underlined names correspond to new findings.

3.2. pssRNA Viruses

Here, we registered 62 new 2A-like notifications in pssRNA viruses, as presented in Table 2 (underlined). The positions in each respective genome are shown in Figure 1.
Figure 1. Cont.
Figure 1. Cont.
Figure 1. Schematic representation of positive-sense single-strand RNA virus sequences. Schematic representations of pssRNA virus sequences showing the location of each respective 2A-like (yellow rectangles). The nucleotide positions and size of each predicted polypeptide are represented by the numbers below and above the bars, respectively. The annotations of each viral sequence were included according to the NCBI. The nucleotide and protein accession numbers are presented forward and above each scheme, respectively. Representations of each genome are not in scale. This figure is presented in four parts.

In most pssRNA viruses, 2A/2A-like segments are used in primary polypeptide processing. The pssRNA viruses commonly possess one 2A/2A-like sequence, but some viruses have two, three, or even four motifs (Table 2). Many of them are members of the order Picornavirales, such as Picornaviridae, Dicistroviridae, and Iflaviridae. Currently, the Picornaviridae family has 63 assigned genera [28], but 2A/2A-like sequences have been found in viruses assigned or tentatively assigned to 15 genera: Aphthovirus, Avihepatovirus, Cardiovirus, Cosavirus, Crohivirus, Erbovirus, Hunninivirus, Limnipvirus, Mistchivir, Mosavirus, Parechovirus, Subtivirus, Senecavirus, Teschoivirus, and Torchivirus.
the upstream polyprotein (P1) until it is removed by secondary proteinase cleavage [8,9]. However, in parechoviruses, the 2A-like region has no protease or protease-like activity, and its apparent function is to alter host cell metabolism because it possesses a high homology to cellular protein H-rev107 that regulates cell proliferation (H-box 2A) [29].

In insect Iflaviruses, the 2A-like sequence separates the capsid and replicative protein domains. The Dicistroviridae family is composed of the Aparavirus, Cripavirus, and Triatovirus genera, in which the 2A-like sequences occur at the N-terminal region of the replicative protein open reading frame (ORF) [2,14].

Members of the Permutotetraviridae and Carmotetraviridae families (previously Tetraviridae), *Thosea asigna* virus and *Euprosterna elaeasa* virus, encode a 2A-like sequence at the N-terminus of the structural ORF [1]. The *Providence* virus has three 2A-like sequences, 2A2 and 2A3, located in the capsid protein precursor (VCAP), and 2A1 at the N-terminus of the p130 ORF, which encodes the viral replicase [30].

### 3.3. dsRNA Viruses

Among the dsRNA viruses, 2A-like sequences not yet reported were found in six species. The new 2A-like sequences are underlined in Table 3, and their localization inside the genome is schematized in Figure 2.

![Figure 2. Schematic representation of double-stranded RNA virus sequences. Schematic representations of dsRNA virus sequences showing the location of each respective 2A-like (yellow rectangles). The nucleotide positions and size of each predicted polypeptide are represented by the numbers below and above the bars, respectively. The annotations of each viral sequence were made according to the information available at the NCBI. The nucleotide and protein accession numbers are located forward and above each scheme, respectively. Representations of each genome are not in scale.](image-url)

In double-stranded viruses, 2A-like sequences are present in two families: Totiviridae and Reoviridae. In Totiviridae, 2A-like sequences are distributed in all representatives of the IMNV-like group [31]. These viruses predominantly infect arthropods, such as penaeid shrimp [32], mosquitoes [33,34], and the fruit fly *Drosophila melanogaster* [35], except for the golden shiner *Tetirivirus* that infects the fish *Notemigonus crysoleucas* [36]. The genome of IMNV-like viruses is composed of two ORFs, and the 2A-like sequences separate an RNA-binding protein of other putative proteins in ORF1 [37].

In the *Reoviridae* family, 2A-like sequences are found in cypoviruses and rotaviruses with 2A-like sequences in one of the segments encoding a non-structural protein. In *Operophtera brumata cypovirus 18* and *Bombyx mori cypovirus 1*, 2A-like sequences occur within segment 5. In type C rotaviruses, 2A-like sequences link the ssRNA-binding protein NSP3 to dsRNA-binding protein (dsRBP). In porcine and human rotavirus C, the 2A-like
sequences are present at segment 6, although in the adult diarrhea virus, the sequence appears in segment 5 [1,2]. All cypoviruses and rotaviruses possess only one 2A-like sequence (Table 3).

3.4. nssRNA Virus

Surprisingly, one 2A-like motif (GDIEQNPGP) was found in a tentatively assigned virus of the Bunyaviridae family (Accession number: APG79245.1). This motif is located in the RNA-dependent RNA polymerase (RdRp) sequence (Figure 3). This is the first report of a 2A-like sequence in a nssRNA virus.

![Figure 3](image)

**Figure 3.** Schematic representation of a negative-sense single-strand RNA virus sequence. Schematic representations of nssRNA virus sequence showing the location of its respective 2A-like sequence (yellow rectangle). The nucleotide positions and size of the predicted polypeptide are represented by the numbers below and above the bars, respectively. The annotations of the viral sequence were made according to NCBI. The nucleotide and protein accession numbers are located forward and above the scheme, respectively. Representation of the genome are not to scale.

3.5. 2A/2A-Likes Sequences and Viral Evolution

Previous studies concerning RNA viruses and 2A-like peptides have reported that these sequences emerged independently during the evolution of viral families [2,14]. However, in a previous study [31], we showed sequences very similar to functional 2A-like sequences in some RNA viruses that could be the precursors of 2A sequences.

In particular, RNA viruses depend on the activity of RNA-dependent RNA polymerases. These enzymes have a significant error rate ($10^{-3}$ to $10^{-5}$ mutations per inserted nucleotide) because they do not have exonuclease review activity [38]. This results in a high degree of genetic heterogeneity in populations of RNA viruses, which are believed to favor adaptability to different environments and hosts [39]. Considering this, the 2A/2A-like sequences could have emerged by subsequent mutation events that ended in a cleavage function, providing the advantage of releasing more than one protein from the same ORF. Therefore, this could directly impact viral adaptation potential and viral infection mechanisms to favor their fitness in complex multicellular systems [31].

Yang et al. also suggested that picornaviruses with more complex infection mechanisms than other viruses of the same family have more than one 2A-like sequence in their genomes [14]. Taking this evidence into account, it seems that 2A/2A-like sequences may be a key element in viral genome evolution and, once acquired, its loss of function may impact virus effectiveness.

3.6. Biotechnology Applications

Various approaches have been employed to co-express multiple proteins in cells, including the use of internal ribosomal entry site (IRES) elements [40,41], dual promoter systems [42,43], and transfection of multiple vectors [44]. Each of these is associated with several limitations, such as uneven or unreliable protein expression levels, silencing of some promoters [45,46], and increased toxicity to cells (with multiple transfections) [47].

Co-expression systems, including 2A/2A-like peptides, could be an alternative strategy for expressing multiple genes under the control of a single promoter. These constructs could have the additional advantage of producing proteins at near-stoichiometric levels, unlike IRES-mediated polycistronic expression, where ribosomes are independently recruited at distinct regions with the mRNA [1,4,48,49]. This necessitates the optimization of the system by testing several combinations of promoters and/or IRES and the order of genes within the expression cassette [46]. Furthermore, IRES activity can be affected by cell type, and variable expression can be observed in the downstream coding sequence [50].
2A/2A-like sequences have been used in a range of heterologous expression systems because of their cleavage capacity. These systems include viruses [51], yeasts [52,53], fungi [54–56], insect cells [57,58], plants [59], human HTK-143 cells [9], rabbit reticulocytes [60], HeLa cells [61], CHO cells [62], HEK293 cells [63], algae [64], and other animals [65–67].

In yeasts, more than two 2A sequences have been used to co-express proteins from the same vector. As seen in [68] and [69], three proteins were produced using this strategy in S. cerevisiae. Surprisingly, up to nine proteins have been linked and successfully co-translated and separated with 2A sequences in the yeast Pichia pastoris [70].

Researchers have also attempted to use 2A for multi-gene transformation in staple crops [71,72]. They can also be used for gene fusion, as seen in tomatoes, potatoes, and others [73,74].

To construct the co-expression vectors, the 2A/2A-like sequences are usually incorporated into an adenovirus [75], adeno-associated virus (AAV) [12], retrovirus [76], lentivirus [77,78], or plasmid vector [79,80]. Many other biotechnological applications that depend on the co-expression of multiple genes use 2A/2A-like sequences, e.g., the production of antibodies and antigens that can be used in vaccine production [80–85], observation of chromatin dynamics and genome (DNA and RNA) editing in the application of cell/gene therapies [78,79,86–90], and development of optogenetic tools [91–93]. More examples of viral 2As applications can be found in [94].

4. Conclusions

In this article, we reviewed the 2A/2A-like sequence distribution of viruses and described the occurrence of these motifs in viral species where these sequences have not been previously reported. These findings need to be confirmed through in vitro tests to verify they are active 2A-like sequences.

Because of its cleavage function, the 2A/2A-like sequences appear to directly affect the complexity of the viral genome, which plays a decisive role in viral evolution. Additionally, they are excellent alternatives for developing new biotechnological tools that depend on the expression of multiple products, such as vaccines, transgenic approaches, cell/gene therapy, and optogenetic tools.

Author Contributions: Conceptualization, J.G.S.d.L. and D.C.F.L.; methodology, J.G.S.d.L.; writing—original draft preparation, J.G.S.d.L. and D.C.F.L.; writing—review and editing, J.G.S.d.L. and D.C.F.L.; visualization, J.G.S.d.L. and D.C.F.L. All authors have read and agreed to the published version of the manuscript.

Funding: We would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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