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Short communication

Identification, molecular and biological characterization of two novel secovirids in wild grass species in Belgium

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ABSTRACT

High throughput sequencing was performed on virion-associated nucleic acids (VANA) from a pool of fifty asymptomatic rough bluegrasses (*Poa trivialis* L.) collected in a Belgian grazed pasture. Bioinformatics analyses produced some contigs presenting similarities with secovirid genomes, in particular nepoviruses and waikaviruses. Three distinct positive-sense single-stranded RNAs including 5' and 3' UTR were reconstructed and they represented two novel viruses infecting rough bluegrass, for which the provisional names poaceae Liege nepovirus A (PoLNVA, 7298 nt for RNA1 and 4263 nt for RNA2) and poaceae Liege virus 1 (PoLV1, 11,623 nt) were proposed. Compared to other *Secoviridae* members, the highest amino acid identity reached 90.7 % and 66.7 % between PoLNVA and nepoviruses for the Pro-Pol and CP regions respectively, while PoLV1 presented the highest amino acid identity with waikaviruses but with lower identities, *i.e.* 41.2 % for Pro-Pol and 25.8 % for CP regions, far below the ICTV demarcation criteria for novel secovirid. Based on sequence identity and phylogenetic analyses, PoLNVA was proposed to belong to the genus *Nepovirus* and PoLV1 as an unclassified secovirids. Detection of the two novel viruses was confirmed in high prevalence in rough bluegrass and ten other wild *Poaceae* species (*Agropyron repens, Agrostis capillaris, Apera spica-venti, Anthoxanthum odoratum, Cynosorus cristatus, Festuca rubra, Holcus lanatus, Lolium perenne, Phleum bertolini* and *Phleum pratense*) by RT-PCR and Sanger sequencing, revealing a diverse host range within *Poaceae* for these novel secovirids. Seed transmission was evaluated and confirmed for PoLNVA.

Poaceae-based communities, whether they are cultivated or wild species, are of primary ecological and economical importance throughout the world. Cereals (such as wheat, maize, rice, sorghum and millets) directly or indirectly provide more than 50 % of calories consumed by humans worldwide (Awika, 2011). *Poaceae* also represents core species of global grasslands, pastures and many wetlands, and contributors to diverse forest and woodland understories. More than 208 different virus species were found to infect members of the *Poaceae* family, with at least 74 virus species for cereals (Lapierre, 2004) causing losses reaching 47 % for barley yellow dwarf virus (BYDV, *Luteovirus, Luteoviridae*) alone, one of the most damaging viruses in the world (Ordon et al., 2009). Virus infection has also been examined in wild *Poaceae* growing in pastures and grasslands, for instance in Australia, New Zealand and the USA (Guy, 2014; Ingwell and Bosque-Pérez, 2015; Jones, 2013). Indeed, viruses can affect pasture productivity and thus decrease feeding for livestock. Infection of English ryegrass (*Lolium perenne* L.), a major species in pastures of Western Europe, by lolium latent virus (LLV, *Potexvirus, Alphaflexiviridae*) can lead to a reduction of nearly 50 % in plant dry matter (Lapierre, 2004). The study reported here focuses on *Poa trivialis* L., a widespread species in pastures and grasslands which can be considered as a weed in cereal fields. Plants belonging to the *Poa* genus are known to be host to at least 24 virus species but little attention was paid to *Poa trivialis*, with only one virus, the ubiquitous BYDV, recorded on this wild grass species (Eweida and Rydén, 1984).

The application of high throughput sequencing (HTS) has the potential to determine the global virus diversity existing in plant communities and ecosystems, and to identify virus exchanges between wild and cultivated reservoirs. Understanding this exchange is of most importance for viruses infecting *Poaceae* since they are found to infect

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both cereals and wild species. In addition, the use of HTS allows for the detection of novel viruses in grass plants, as demonstrated by Kraberger et al. (2017) who found novel mastreviruses in wild rice.

In this study, fifty rough bluegrass plants (*Poa trivialis* L.) were collected in 2018 in a grazed pasture from Antheit (50°33′19.3″N 5°14′14.1″E) within the Natural Park of Burdinale-Mehaigne (Province of Liège, Belgium) in order to investigate their infection status. No foliar symptoms were observed on the plants. Plant material (leaves and stems) was frozen in liquid nitrogen and then stored at −20 °C. The fifty *P. trivialis* plants were then pooled and virus particle enrichment was performed by virion-asociated nucleic acids extraction (VANA), adapted from (Palanga et al., 2016) and (François et al., 2018). First, 200 mg of frozen tissues from each individual plant were pooled into a filtered bag (*i.e.* 10 g for fifty plants) and then ground in 70 mL of Hanks' buffered salt solution (HBSS, composed of 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.1 g glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$), using a tissue homogenizer. The homogenised plant extract was transferred into a 50 mL Falcon conical and centrifuged at 3200 *g* for 5 min. The supernatant was further centrifuged at 8200 *g* for 3 min and then filtrated through a 0.45 µm sterile syringe filter. The supernatant (25 mL) was transferred into an ultracentrifuge tube. Next, a sucrose cushion, made of 3 mL of 30 % sucrose in 0.2 M potassium phosphate pH 7.0, was deposited at the bottom of the tube. The extract was then centrifuged at 148,000 *g* for 2.5 h at 4 °C using a SW48 rotor (Beckman). The resulting pellet was resuspended in 1.5 mL of HBSS and kept overnight at 4 °C in 1.5 mL of HBSS. From a 200 µL resuspension, unencapsidated nucleic acids were digested with 15 U of bovine pancreas DNase I (Euromedex, France) and 1.9 U of bovine pancreas RNase A (Euromedex, France) and incubated at 37 °C for 90 min. Encapsidated nucleic acids were then extracted using a PureLink Viral RNA/DNA Mini Kit (ThermoFischer Scientific, Belgium) following the manufacturer's protocol.

Library preparation followed the protocol of Palanga et al. (2016). Briefly, viral cDNA synthesis was synthesized using dodecamers and Superscript III (Invitrogen, USA), followed by priming and extension by Klenow polymerase, and then the cDNA was amplified with multiplex identifier (MID) linker. The DNA product obtained from the sample was cleaned using the Nucleospin Gel and PCR clean up (Macherey-Nagel, Germany) following the manufacturer's protocol. An Illumina library was then prepared using NEBNext Ultra II DNA library prep kit (New England BioLabs, US). Sequencing was performed on Nextseq 500 sequencing platform with 2 × 150 nt sequencing reads. A total of 666, 080 reads were obtained and analysed using Geneious Prime 2019.2.1 software (http://www.geneious.com). After quality control, cleaning reads, trimming MID linkers and removing duplicates, 590,084 unique reads were *de novo* assembled using SPAdes (Bankevich et al., 2012) and 1371 contigs longer than 150 nt were generated. BLASTx analysis performed on NCBI non-redundant database (Altschul et al., 1997) revealed that 28 contigs presented similarities with secovirids: maize chlorotic dwarf virus (MCDV, GenBank accession U67839), tomato black ring virus (TBRV, GenBank accessions AY157993 and AY157994) and beet ringspot virus (BRSV, GenBank accessions D00322 and X04062). *De novo* assembly (Geneious assembler, medium sensitivity) and mapping of deduplicated reads (Geneious mapper, medium-low sensitivity, 10 % mismatches allowed) generated three large contigs likely representing the almost complete genomes of two novel viruses: one novel species close to members of the the genus *Nepovirus* (RNA1 segment of 7087 nt and a mean read depth of 591; RNA2 segment of 4056 nt and a read depth of 99) with the proposed name poaceae Liege nepovirus A (PoLNVA); and one novel species close to members of the genus *Waikavirus* (10,843 nt and a mean read depth of 35) with the proposed name poaceae Liege virus 1 (PoLV1). Further confirmation performed by BLASTx searches on the NCBI non-redundant database resulted in 77.8 % and 60.3 % identities (over 96 % and 92 % coverage) with BRSV (GenBank accessions D00322 for RNA1 and X04062 for RNA2, respectively) for PoLNVA, and 29.8 % identity (over 81 % coverage) with *Brassica napus RNA virus 1* (BRNV1, GenBank accession MH844554) for PoLV1. The three genomic RNA segments ends were completed by Rapid Amplification of Complementary DNA Ends (RACE) in duplicates using the kit SMARTer® RACE 5'/3' (Clontech) and Gene Specific Primers (GSPs, see Table S1) according to manufacturer's instructions, and then Sanger sequenced in both directions by Macrogen (The Netherlands).

The *Secoviridae* family contains nine genera (*Comovirus, Fabavirus, Nepovirus, Sequivirus, Waikavirus, Cheravirus, Sadwavirus, Stralarivirus* and *Torradovirus*) presenting similar structural and functional characteristics. Their non-enveloped particles are icosahedral, with a diameter of 25−30 nm and empty particles are found in virus preparations (Sanfaçon, 2015). The secovirid genome consists of one or two molecules of linear positive-sense ssRNA, with a length of 10–12 kb for monopartite genomes, while bipartite genomes present a larger RNA of 6−8 kb (RNA1) and a smaller one of 4−7 kb (RNA2). Each RNA usually encodes a single and large polyprotein, but potential additional open reading frames (ORF) have been identified in torradoviruses and waikaviruses (Sanfaçon, 2015; Thompson et al., 2017). Consistently with the current knowledge, the complete PoLNVA genome sequence was 7298 nt for RNA1 (including 157 nt 5' UTR and 295 nt 3'UTR) and 4263 nt for RNA2 (including 196 nt 5' UTR and 287 nt 3'UTR) (GenBank accession no. MW289235 and MW289236). The complete PoLV1 genome sequence was 11,623 nt (including 664 nt 5'UTR and 534 nt 3'UTR, GenBank accession no. MW289237). Unique open reading frames (ORFs) encoding large polyproteins were identified for each sequence (Fig. 1), using the NCBI ORF finder (https://www.ncbi.nlm.

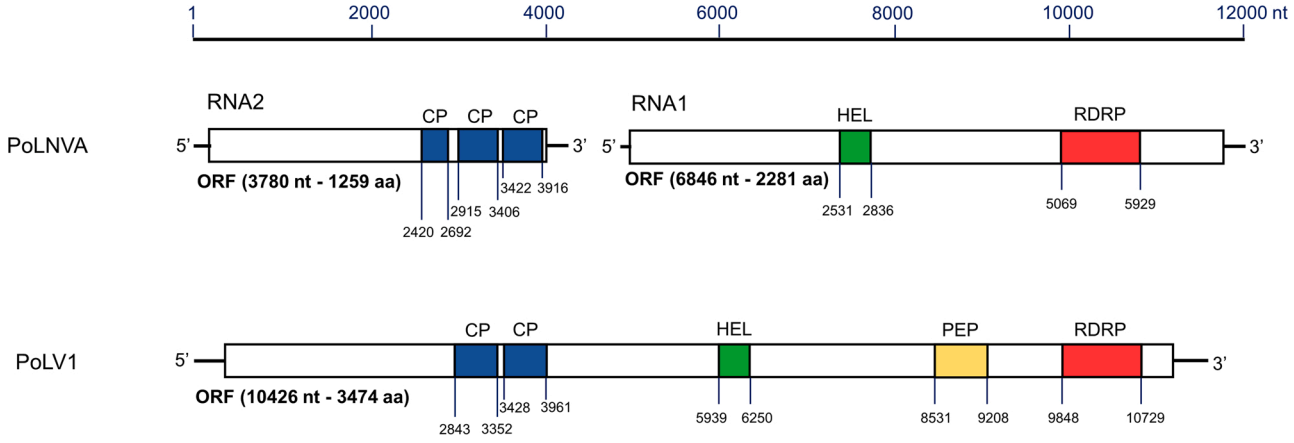

Fig. 1. Genome organization of the two putative novel secovirids (PoLNVA and PoLV1) and position of conserved protein domains along the genome (HEL for RNA helicase, RDRP for RNA-dependent RNA polymerase, CP for coat protein and PEP for peptidase).

nih.gov/orffinder/). The protein domains of this large ORF were identified and annotated using the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/) (Fig. 1). The different domains were identified in the same order as other viruses belonging to the *Secoviridae*, with putative capsid protein (CP) domains (pfam03689, pfam03391 and pfam03688 for PoLNVA; pfam12264 and cd00205 for PoLV1), an RNA helicase domain (pfam00910), a peptidase domain (pfam12381), and an RNA-dependent RNA polymerase domain (cd01699).

In order to clarify the taxonomic status of these novel species within the *Secoviridae* family, sequence comparisons and phylogenetic analyses were carried out for the amino acid sequence spanning the conserved CG-GDD motif of the Pro-Pol domain; a species demarcation criteria of the ICTV (Thompson et al., 2017), and for the amino-acid sequence of the conserved CP region. The full list of the accessions used in the analyses are listed in Tables S2–S4. Multiple alignments were performed with the MUSCLE algorithm using default parameters and eight iterations from which maximum likelihood trees were calculated and bootstrapped one thousand times with MEGA-X software. The analysis of the conserved CG-GDD domain showed that PoLNVA was related to the genus *Nepovirus* with TBRV and BRSV being the closest relatives (90.7 % and 87.2 % amino acid identity respectively, see Table S5). Similarities between PoLNVA, TBRV and BRSV were also observed in the maximum likelihood tree (Fig. 2). However, examination of the PoLNVA coat protein region with other nepoviruses showed lower percentages of amino acid identity (with a maximum of 66.9 % with BRSV and 56.8 % with TBRV, see Table S6 and Fig. 3A). The ICTV genomic criteria to demarcate species within the *Secoviridae* family are less than 75 % identity for the coat protein amino acid sequence, and less than 80 % identity for the conserved Pro-Pol region amino acid sequence. It appeared that PoLNVA could be considered as a novel virus based on its CP region but not for the Pro-Pol region. This situation is similar to BRSV and TBRV that are closely related in the Pro-Pol sequence (89 % amino acid identity) but are much more divergent in the CP sequence (62 % amino acid identity) and thus represent two distinct species. As both criteria do not need to be simultaneously met, PoLNVA could be classified as a new virus species. Moreover, PoLNVA also differed in host range compared to other members of the *Nepovirus* subgroup B. The detection of this virus taxa within *Poaceae* would contribute to its status of novel species.

On the other hand, the virus taxa closest to PoLV1 seemed to be members of the genera *Waikavirus* and then *Sequivirus* for both Pro-Pol and coat protein regions but PoLV1 formed a distinct branch between the two genera (Figs. 2 and 3B). In addition, the percentages of amino acid identities between PoLV1 and *Waikavirus* were quite low (41.2 % amino acid identity with MCDV for Pro-Pol and 25.81 % with BRNV1 for the coat protein), and even lower with *Sequivirus* (27.3 % and 15.6 % amino acid identity with *Carrot necrotic dieback virus* for Pro-Pol and coat protein regions respectively) (see Table S7). PoLV1 presented the same genome organization as *Waikavirus* (same monocistronic genome, with similar length, coding for the expected conserved protein domain in the same order as representative members, see Fig. S1), but low levels of identity in both Pro-Pol and CP region could not suggest PoLV1 as a *Waikavirus* and define it as an unclassified virus within the *Secoviridae* family.

Sequence integrity was supported for both novel viruses by the large coverage depth observed in HTS results. Regarding the very low identity levels of PoLV1 compared to references, the contiguous sequence was further confirmed by overlapping RT-PCR (list of primers used in Table S1) and Sanger sequencing (GenBank accession MW701433). The sequence was 96 % identical to the HTS contig and presented same organization in terms of ORFs and protein domains. In addition, recombination analysis was performed for both novel virus species using RDP4 software with RDP method and default parameters (Martin et al., 2015) to support the classification results, and did not reveal any recombination events (unpresented results).

To confirm the presence and prevalence of these two putative novel secovirids, total RNA was extracted from the same tissues as used for VANA high throughput sequencing with the protocol described in Oñate-Sánchez and Vicente-Carbajosa (2008). The host ranges of the viruses were also explored by testing other *Poaceae* species present at the same site (see floristic inventories in Table 1). In total, 268 samples belonging to 13 different *Poaceae* species were tested for both novel viruses. No obvious viral symptoms were observed in the plants. Virus detection was performed by RT-PCR with specific primer pairs designed on consensus viral genomic sequences (where read mapping presented conserved nucleotides stretches without any single nucleotide polymorphisms). For PoLNVA: PoLNVA-F1 (5'-GGAACAGTATTT TGATGCGC-3') and PoLNVA-R1 (5'- CTTTTAGAGAATATCCCCTCC-3') amplifying a 378 bp fragment (6652 to 7030 nt) of the end of the RdRp domain; and PoLNVA-F2 (5'- TGTGTCGGGAAATAAACTACAAGCA-3'), PoLNVA-R2 (5'-GCAAAAGAGCCAAACTGGAATGGTA-3') amplifying a 369 bp (3411 to 3780) of the CP domain; and for PoLV1: PoLV1-F (5'-ACCCTCAAGTTCTTTCCACTT-3') and PoLV1-R (5'-ACTCCCTCTC-CAGTATTGAA-3'), amplifying a 356 bp fragment (3227 to 3583 nt) of the PoLV1 CP domain. Both viruses were screened for on RNA extracts by reverse transcription using random hexamers and Tetro RT enzyme (Bioline, Belgium), and PCR amplification of cDNA by Mango Taq polymerase (Bioline, Belgium). Thermal cycling corresponded to: 94 °C for 4 min, 35 cycles at 94 °C for 45 s, 58 °C/63 °C/56 °C (for PoLNVA-RdRp, PoLNVA-CP or PoLV1-CP, respectively) for 1 min and 72 °C for 30 s, with a final 72 °C extension for 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer stained with GelRed® Nucleic Acid Gel Stain (Biotium) and visualized under UV light. RT-PCR analyses confirmed the presence of the two putative novel secovirids in rough bluegrass tissues, with prevalence of 74 and 38 % for PoLNVA and PoLV1 respectively (see Table 1). Ten other *Poaceae* species were found infected by these viruses, including perennial and annual species (*e.g. Apera spica-venti*), but also both structuring species (*e.g. Lolium perenne, Holcus lanatus*) and low-occurrence species such as *Anthoxanthum odoratum* (Table 1). Highest PoLV1 prevalence was found in rough bluegrass (38 % prevalence), while highest PoLNVA prevalence was found in meadow soft grass (*H. lanatus* – 89 % prevalence) and ryegrass (*L. perenne* – 86 % prevalence). The sequence of the *P. trivialis* isolates was confirmed by Sanger sequencing (Macrogen, The Netherlands) obtained from PCR products cleaned by Nucleospin Gel and PCR Clean Up (Macherey-Nagel) and deposited in GenBank (MW289238 and MW289239 for PoLNVA RdRp region (RNA1); MW289243 and MW289244 for PoLNVA CP region (RNA2); MW289245 and MW289246 for PoLV1 CP region). Nucleotide identity between the original HTS derived sequence and these Sanger derived sequences ranged from 92.3–92.9% for PoLNVA-RdRp, 96.0 % and 97.3 % for PoLNVA-CP, and from 94.7%–96.4% for the PoLV1-CP.

Possible modes of virus transmission were investigated for the novel nepovirus PoLNVA. Nepoviruses are mostly nematode vectored and/or transmitted by seeds or pollen (Rowhani et al., 2017), but they can exceptionally be transmitted by mites (*e.g.* blackcurrant reversion virus) (Susi, 2004). The seed transmission was examined here in ryegrass that presented a very high virus prevalence. Thirty ryegrass plants were randomly collected from the same studied plot in April 2019 and transplanted into greenhouse. Leaf samples were tested by RT-PCR to detect PoLNVA. Seeds were collected from the ears of infected and non-infected plants and a total of 119 seeds (72 from infected and 47 from non-infected parent plants) were sown in sterile soil and pots in October 2019. Growth conditions were 18 °C with 16:8 h day:night. After two months, leaves were collected and tested by RT-PCR, targeting the PoLNVA RdRp region. The virus was detected in six seedlings, all belonging to the same parent plant. Thirteen seeds from this parent were sown in total, meaning that virus transmission by seeds approximated 50 % for this parent plant, or 8.33 % when considering the total seed number sown from infected parents (*i.e.* 6/72 seeds). PoLNVA infection was not detected in seedlings from non-infected parents, nor in healthy

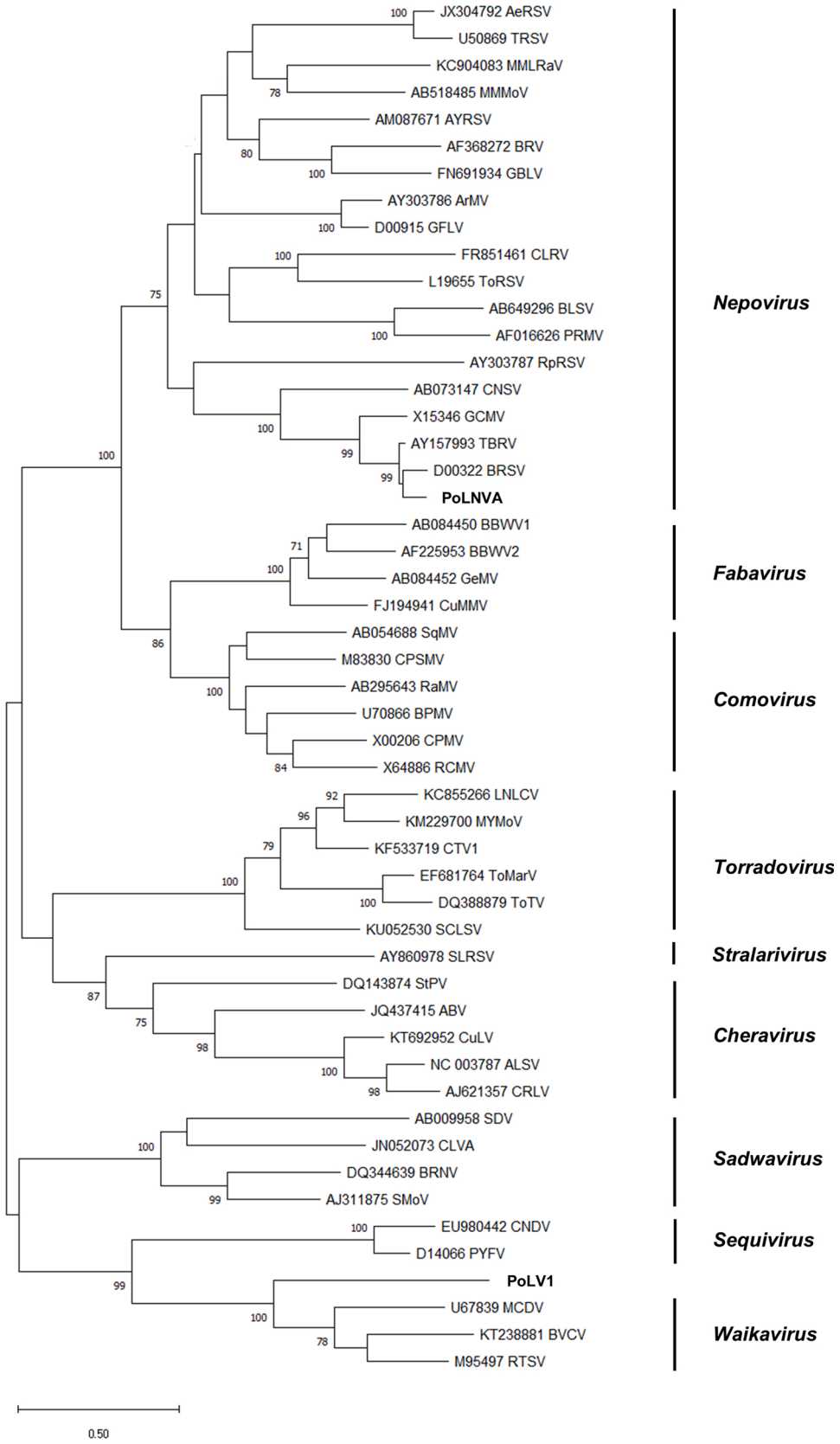

Fig. 2. Maximum likelihood inferred phylogenetic tree (Jones-Taylor-Thornton model, 1000 bootstraps, values above 70 % are shown) of the putative novel species PoLNVA and PoLV1, and members of the family *Secoviridae* based on an alignment of amino acid sequences of the conserved domains between the "CG" motif of the 3C-proteinase and the "GDD" motif of the polymerase (Pro-Pol region). Virus acronyms are described in Table S2.

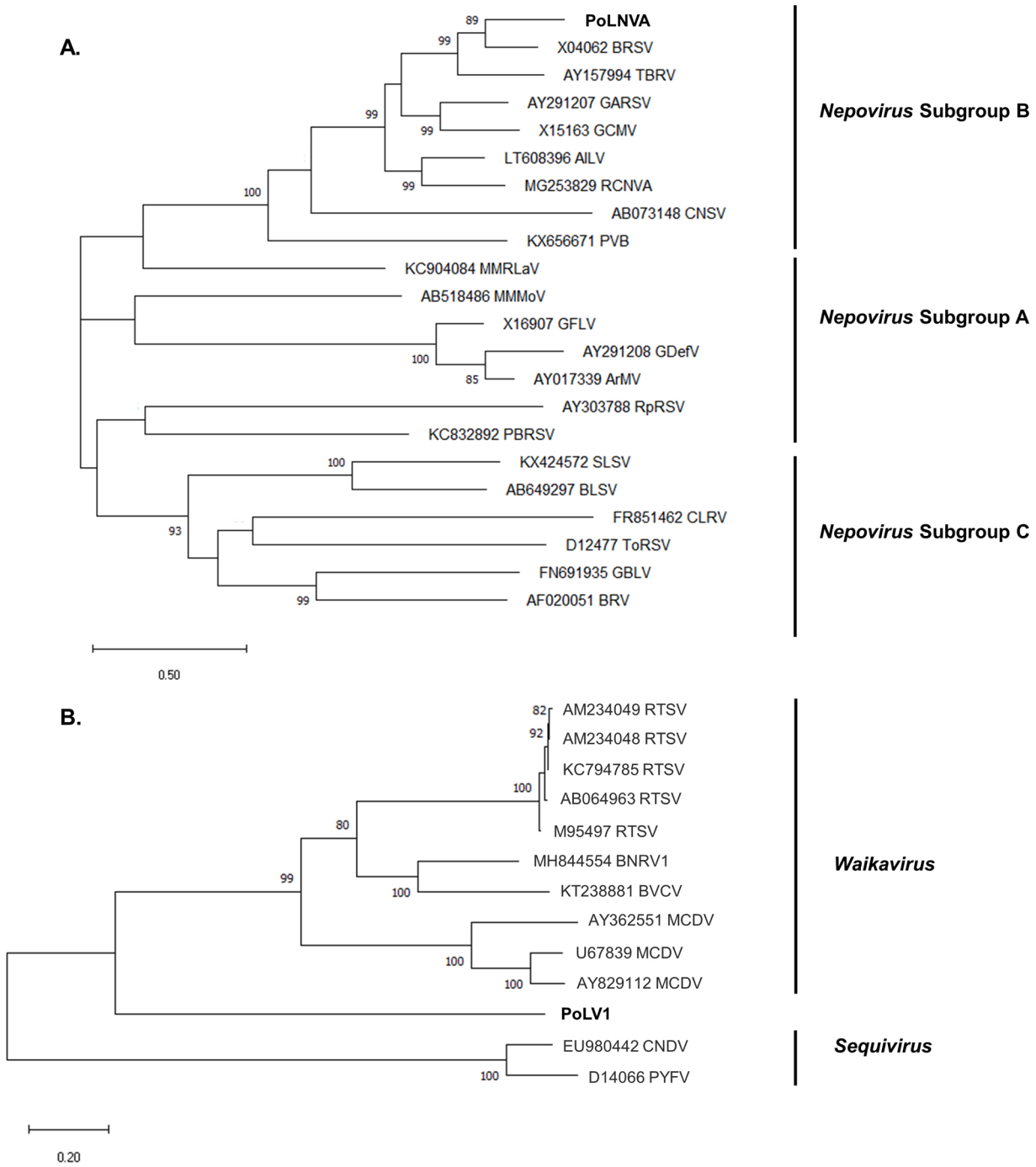

Fig. 3. Maximum likelihood inferred phylogenetic trees (Jones-Taylor-Thornton model, 1000 bootstraps, values above 70 % are shown) of the putative novel species PoLNVA and PoLV1, and members of the *Nepovirus* genus (A) or members of *Waikavirus* and *Sequivirus* genera (B), based on an alignment of amino acid sequences of the complete coat protein region. Virus acronyms are described in Tables S3 and S4.

control plants. Three amplicons from infected seedlings were Sanger sequenced and deposited in GenBank (MW289240, MW289241 and MW289240), supporting the seed transmission of PoLNVA.

In conclusion, two putative novel species of the *Secoviridae* family tentatively named poaceae Liege nepovirus A (PoLNVA) and poaceae Liege virus 1 (PoLV1) were identified in rough bluegrasses (*Poa trivialis* L.) from a grazed pasture in Belgium. Sequence and phylogenetic analyses placed PoLNVA in the genus *Nepovirus*, close to TBRV and BRSV, while PoLV1 presented similarities for genome size and protein domain structure with the genera *Waikavirus* and *Sequivirus*. However, the low levels of amino acid identity for both Pro-Pol and CP regions could define PoLV1 as an unclassified secovirid. A survey in Belgian grasslands and pastures detected PoLNVA and PoLV1 with high prevalence in rough bluegrasses and ten other wild *Poaceae* species, highlighting the large host range within *Poaceae* of these two novel secovirids. Virus transmission was also investigated and PoLNVA was found to be seed-transmitted. Nematode transmission remains to be explored for this *Nepovirus*. Agents transmitting PoLV1 are still unknown and also need investigation, starting for instance by arthropods as the closest virus taxa (*Waikavirus* and *Sequivirus*) are known to be vectored by aphids and leafhoppers (Sanfaçon, 2015). In this study, no visible viral symptoms were observed in wild plants. However, no symptoms does not mean no

Table 1
Prevalence of the two putative novel secovirids in different *Poaceae* species. The number of plants per species is representative of its abundance on the sampling site.

Poaceae species	PoLNVA Prevalence	PoLV1 prevalence
Agropyron repens L.	1/3	1/3
Agrostis capillaris L.	4/11	0/11
Apera spica-venti L.	2/3	1/3
Anthoxanthum odoratum L.	2/2	2/2
Bromus mollis L.	0/1	0/1
Cynosorus cristatus L.	11/16	1/16
Dactylis glomerata L.	0/3	0/3
Festuca rubra L.	1/3	0/3
Holcus lanatus L.	16/18	1/18
Lolium perenne L.	86/100	15/100
Poa trivialis L.	74/100	38/100
Phleum bertoloni L.	1/6	0/6
Phleum pratense L.	1/2	0/2

effect on plant fitness (survival, growth or reproduction) (Alexander et al., 2020; Prendeville et al., 2012). A series of secovirids are considered as major diseases in cereal crops, such as the maize chlorotic dwarf virus (MCDV) in maize in the USA and rice tungro spherical virus (RTSV) in rice in South and Southeast Asia (Nault et al., 1976; Thapa et al., 2012). Investigating the propagation of these two novel viruses among host reservoirs (wild and domestic) and their impact on plant fitness could thus be of great interest and should contribute to better understand the ecology of secovirids in agro-ecosystems.

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Declaration of Competing Interest

The authors report no declarations of interest.

CRediT authorship contribution statement

François J. Maclot: Writing - original draft, Methodology, Investigation, Software, Formal analysis. **Virginie Debue:** Investigation, Software. **Arnaud G. Blouin:** Investigation, Software, Validation, Writing - review & editing. **Núria Fontdevila Pareta:** Investigation, Software, Validation, Writing - review & editing. **Lucie Tamisier:** Software, Validation, Writing - review & editing. **Denis Filloux:** Software, Validation, Writing - review & editing. **Sébastien Massart:** Conceptualization, Funding acquisition, Supervision, Resources, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2021.198397.

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