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A novel species of RNA virus associated with root lesion nematode *Pratylenchus penetrans*

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Abstract

The root lesion nematode *Pratylenchus penetrans* is a migratory species that attacks a broad range of plants. While analysing transcriptomic datasets of *P. penetrans,* we have identified a full-length genome of an unknown positive-sense single-stranded RNA virus, provisionally named root lesion nematode virus 1 (RLNV1). The 8614-nucleotide genome sequence encodes a single large polyprotein with conserved domains characteristic for the families *Picornaviridae*, *Iflaviridae* and *Secoviridae* of the order *Picornavirales*. Phylogenetic, BLAST and domain search analyses showed that RLNV1 is a novel species, most closely related to the recently identified sugar beet cyst nematode virus 1 and potato cyst nematode picorna-like virus. *In situ* hybridization with a DIG-labelled DNA probe confirmed the presence of the virus within the nematodes. A negative-strand-specific RT-PCR assay detected RLNV1 RNA in nematode total RNA samples, thus indicating that viral replication occurs in *P. penetrans*. To the best of our knowledge, RLNV1 is the first virus identified in *Pratylenchus* spp.

In recent years, several new viruses infecting plant-parasitic nematodes have been described [1–5]. Thus far, the viruses have been identified in sedentary nematode species, such as the soybean cyst nematode (SCN; *Heterodera glycines*), two potato cyst nematode (PCN) species, *Globodera pallida* and *G. rostochiensis*, clover cyst nematode (CCN; *H. trifolii*) and sugar beet cyst nematode (SBCN; *H. schachtii*). This is the first report of a novel, positive-sense single-stranded RNA virus infecting the migratory root lesion nematode from the family *Pratylenchidae*, *Pratylenchus penetrans*.

P. penetrans is an endoparasitic species that attacks a broad range of crops [6–11]. Breeding for resistance to root lesion nematodes is difficult and disease management practices are limited to sanitation and the use of nematicides [12].

While analysing transcriptomic datasets previously generated from two independent pools of *P. penetrans* [9], we have identified by BLAST searches transcripts associated with an unknown positive-sense single-stranded RNA virus. Virus transcripts were found in both nematode libraries. The total number of raw reads in both libraries was 149 688 264, of which 367 641 mapped to the virus genome. The full-length viral genome was obtained by *de novo* assembly using CLC Genomics Workbench (v. 8). The assembly from high-throughput sequence data was supplemented by sequencing of the 5′ RACE-amplified cDNA ends of the virus. 5′RACE reactions were performed with the virus-specific primers GSP1, GSP2 and LN715 (Table S1, available in the online version of this article) in three independent assays using 5′RACE system from Thermo Fisher Scientific (Waltham, MA, USA) and the SMARTerRACE 5′/3′ system from Takara Bio USA, Inc. (Mountain View, CA, USA).

The 3′ terminal sequence of the virus contained a poly(A) tail and thus appeared to be complete. To additionally confirm this, we performed 3′RACE utilizing the SMARTerRACE 5′/3′ system (Takara Bio USA, Inc.). For PCRs with the 3′RACE-derived cDNA, RLNV1-specific forward primer LN716 (Table S1) was used together with the universal primer mixture supplied by the company. The cloned PCR products were sequenced and found to be identical with the predicted sequence of the RLNV1's 3′ terminal end.

The virus identity was further confirmed by reverse transcription (RT)-PCR with a set of primers LN721 and LN722, designed from the *in silico* assembled genome sequence (Table S1). The total RNA was extracted from the following sources using the RNeasy Plant Mini kit (Qiagen

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Abbreviations: 5′ UTR, 5′ untranslated region; CP, capsid protein; CRPV, cricket paralysis virus; IRES, internal ribosome entry site; ISH, in situhybridization; PCN, potato cyst nematode; PCNPLV, potato cyst nematode picorna-like virus; RACE, rapid amplification of cDNA ends; Rhv, rhinovirus; RdRp, RNA dependent RNA polymerase; RLNV1, root lesion nematode virus 1; SBCNV1, sugar beet cyst nematode virus 1; SCN, soybean cyst nematode.

GenBank accession number MK138531.

Five supplementary figures and three supplementary tables are available with the online version of this article.

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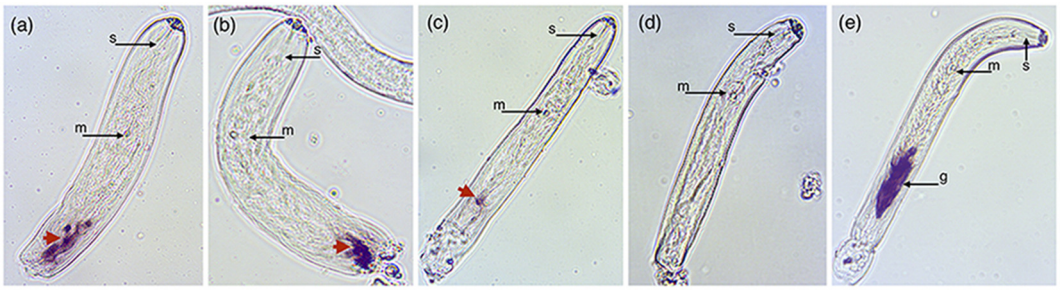

Fig. 1. Detection of root lesion nematode virus (RLNV1) within the body of *Pratylenchus penetrans* by *in situ* hybridization. (a–c) A positive detection (brown colour) of the virus by antisense digoxigenin (DIG)-labelled DNA probe in the esophageal gland region of different specimens (red arrow). (d) Negative control hybridization performed with the sense DIG-labelled probe. (e) Positive control *in situ* hybridization performed with the antisense probe complementary to the transcripts of *Pp-eng-1*, a gene encoding cell wall-degrading enzyme endoglucanase, which specifically localizes within the esophageal glands of the nematode. g, glands; m, metacorpus; s, stylet.

Inc., Germantown, MD, USA): (1) three different batches of nematodes, each containing a mixture of adults, juveniles and eggs, and (2) individual nematode developmental stages, i.e. males, females, juveniles (J2–J4) and eggs. The nematodes (all stages) were obtained from *in vitro* cultures as described in Vieira *et al.* [9]. Different nematode stages (female, male, juveniles and eggs) were hand-picked; a total of 200–250 nematodes/eggs were processed for RNA extraction for each corresponding cDNA library.

The presence of the virus was confirmed in all batches containing a mixture of the nematode stages, and in males and females, but not in the juveniles or eggs (Figs. S1a and S1b). All amplified products were cloned into pCRII TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA), sequenced and found to be 100 % identical with the corresponding fragments of the assembled genome. The new virus has been provisionally named root lesion nematode virus 1 (RLNV1).

To demonstrate the actual presence of the virus within the nematode bodies, we conducted *in situ* hybridization (ISH) assays with sense and antisense digoxigenin-labelled DNA probes generated from the virus-specific PCR products amplified using primers ISH-F and ISH-R (Table S1). ISH was performed following the protocol of de Boer *et al.* [13]. Using the antisense probe, the virus was detected in the infected nematodes near the esophageal glands (Fig. 1). Esophageal glands are the main producers of nematode effector proteins and have a high level of transcriptional activity, which could also be an optimal environment for virus replication.

To experimentally confirm viral replication in RLN, we performed a negative-strand-specific reverse transcription PCR assay. The primer designed for the cDNA synthesis (LN712, Table S1) was complementary to the 3′ region of the predicted negative RNA strand. First-strand cDNA was synthesized using the SuperScript III RT system (Thermo Fisher Scientific, MA, USA) and PCR was performed with primers LN712 and LN715 (Table S1), employing AmpliTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Negative-strand RT-PCR led to amplification of the correct size products only from the negative-strand viral cDNA, thus confirming the presence of the double-stranded RNA intermediates that occur during virus replication (Fig. S2).

The full-length genome of the virus consisted of 8614 nucleotides (excluding the poly(A) tail), and was deposited in NCBI GenBank (accession number MK138531). BLAST [14] and PASC [15] analyses using the RLNV1 nucleotide sequence as a query indicated that RLNV1 belongs to the order *Picornavirales* and has a monocistronic genome that incorporates one open reading frame (ORF), encoding a single large polyprotein. When NCBI PSI-BLAST was performed with the largest predicted ORF of the RLNV1 (2833 aa), the top hits corresponded to the potato cyst nematode picorna-like virus (PCNPLV; 39 % identity) and sugar beet cyst nematode virus 1 (SBCNV1; 40 % identity) (Table S2). The remaining hits produced alignments with significantly lower coverage and percentage identity. A BLAST search of the complete viral nucleotide sequence resulted in an equal outcome, producing low-coverage (~5 %) alignments only with SBCNV1 and PCNPLV. This indicated that RLNV1 is a new species that is most closely related to SBCNV1 and PCNPLV, both of which do not group with other viruses in the order *Picornavirales* and may require a new taxonomic rank [4, 5].

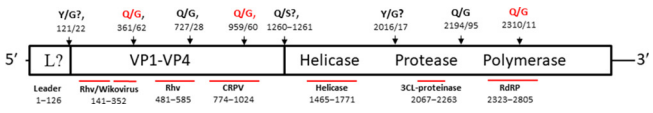

Fig. 2. Tentative genome organization of the root lesion nematode virus (RLNV1). The positions of the predicted structural (VP1–VP4) and non-structural proteins (helicase; protease and RNA-dependent RNA polymerase) are indicated inside the rectangles. Conserved capsid protein domains (rhinovirus, Rhv; waikivirus; and cricket paralysis virus, CRPV) and conserved domains of the helicase, protease and polymerase are indicated by red lines and amino acid positions in the polyprotein. Predicted conserved cleavage sites are shown in red.

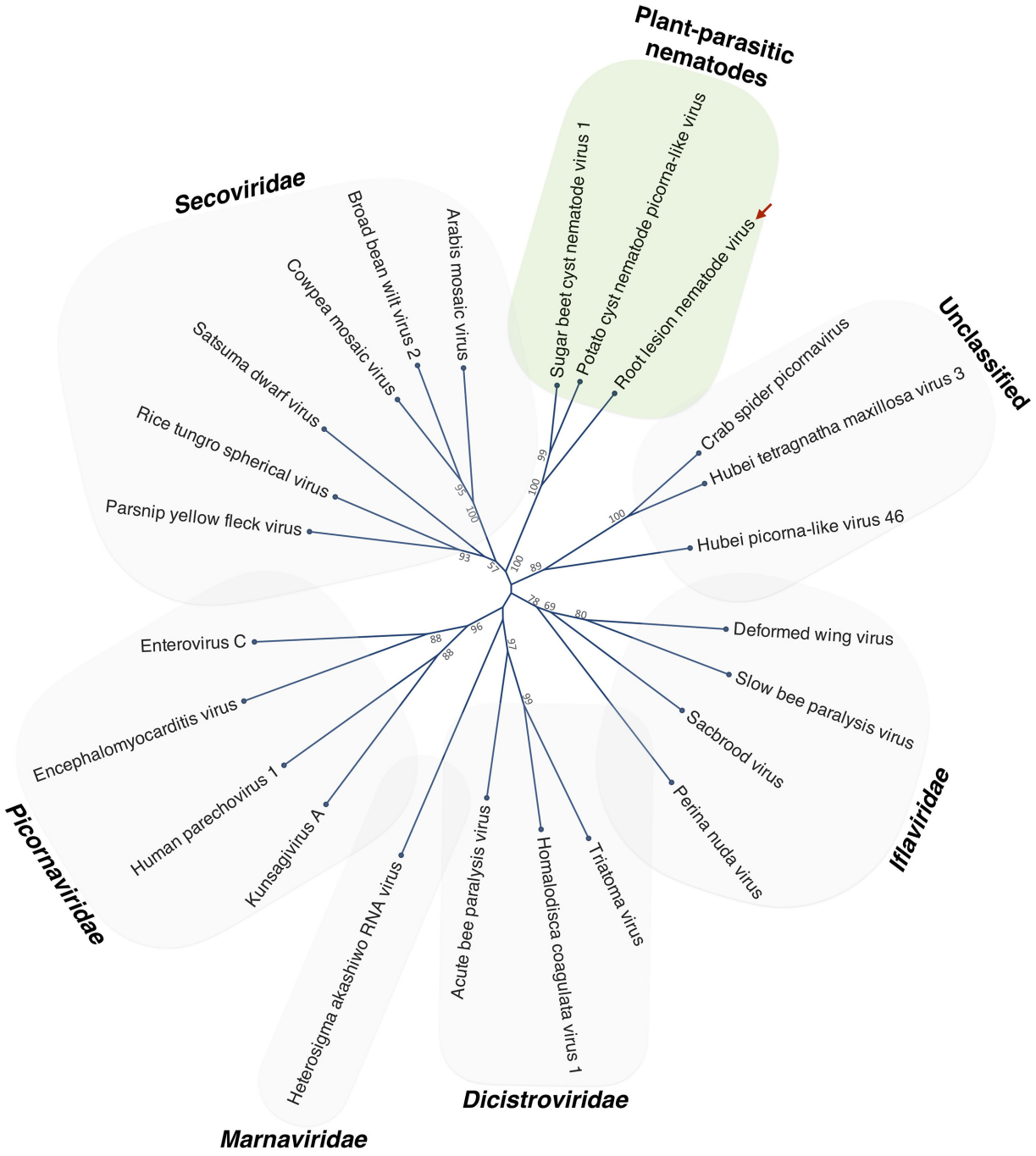

Fig. 3. Phylogenetic analysis of the predicted RdRp (RNA-dependent RNA polymerase) conserved domain of the root lesion nematode virus (RLNV1) and selected members of the order *Picornavirales*. All sequences were obtained from GenBank (NCBI). The protein sequences were aligned using MUSCLE, and maximum-likelihood trees were constructed using CLC Main Workbench v. 8, with 1000 bootstrap replications. Only bootstrap values above 50 % are shown. Red arrow indicates the position of the root lesion nematode virus. Accession numbers are shown in Table S2.

Several putative domains were predicted in the RLNV1 genome using the Pfam tool [16] incorporated into CLC Genomics Workbench software (v. 8) and publicly available InterPro software [17]: two rhinovirus (Rhv) picornavirus capsid protein domains, a waikavirus capsid protein domain, a cricket paralysis virus (CRPV) capsid protein-like domain, an RNA helicase domain and an RNA-dependent RNA polymerase (RdRp) domain (Fig. 2). Alignment of the predicted RNA helicase and the RdRp domains of RLNV1 and selected members of the order *Picornavirales* showed

the presence of the previously identified conserved motifs within both proteins (Fig. S3) [18]. Based on the Pfam predictions, RLNV1 has a similar genomic structure to the families *Picornaviridae*, *Iflaviridae* and *Secoviridae* (genera *Sequivirus* and *Waikavirus*) [19, 20] (Fig. 2).

Four predicted N-terminal protein domains of the RLNV1 correspond to the four capsid proteins, VP1–VP4 [21, 22]. As predicted by the Pfam tool, the first capsid protein (CP) domain starts at amino acid (aa) position 141 (447 nt) of the largest ORF (2833 aa), which suggests that its initiation codon is likely to be AUG at position 405–407 nt in the context aagaAUGcaa. There are three other AUG codons upstream of the AUG 405 nt that have a less favourable context for the initiation codon: AUG 27 nt, AUG 30 nt and AUG 57 nt. If translation is initiated at one of those three codons, the RLNV1 genome will encode a small leader protein (L) with molecular mass of ~14 kDa. In iflaviruses, the CP coding regions are often preceded by a leader protein of unknown function [21]. Alternatively, the 5′ terminal nucleotides of the RLNV1 genome may incorporate the internal ribosome entry site (IRES), which is commonly located in the 5′UTR of *Picornaviridae* [22] and some members of the family *Iflaviridae* [21].

Based on the amino acid alignment of RLNV1 and the most closely related species, PCNPLV and SBCNV1, we predicted some of the conserved polyprotein cleavage sites for the viral proteinase (Figs 2 and S4). We hypothesized that cleavage of the RLNV1 precursor polyprotein may occur at the conserved Q/G sites, complemented with processing at other motifs, possibly Y/G and Q/S (Figs 2 and S4). Tyrosine–glycine (Y/G) was suggested to be a preferential cleavage site for the 2A proteinase of *Picornaviridae* [23], while glutamine–serine (Q/S) was predicted to be one of the tentative cleavage sites for the 3C-like proteases of iflaviruses [24].

Phylogenetic analysis based on the conserved RdRp domains of RLNV1 and other related viruses (Table S3) [19, 25] placed RLNV1, together with PCNPLV and SBCNV1, within a distinct clade, which could potentially represent a new taxonomic rank within this order (Fig. 3). This arrangement was also obvious when phylogenetic analysis was performed with all of the polyproteins of the RLNV1 and the top NCBI PSI-BLAST hits (Fig. S5). Characteristically, within this well-defined group, RLNV1 clearly branched out from the other two viruses found in nematodes. We speculate that RLNV1, PCNPLV and SBCNV1 form a new family within the order *Picornavirales*. Within the proposed family, RLNV1 would represent a new genus.

To the best of our knowledge, RLNV1 is the first virus to have been identified in *Pratylenchus* spp. The effect ofthe virus infection on the viability and development of the nematodes has yet to be clarified.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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