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A novel victorivirus from the phytopathogenic fungus *Neofusicoccum parvum*

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Sequence data availability. The Neofusicoccum parvum victorivirus 3-NFN (NpVV3-NFN) complete nucleotide genomic sequence was deposited in the GenBank/ENA/DDBJ database under accession number MZ868719.

Abstract

Neofusicoccum parvum is an important plant pathogenic ascomycetous fungus that causes trunk diseases in a variety of plants. A limited number of reports on mycovirus from this fungus are available. In this study, we report the characterization of a novel victorivirus (Neofusicoccum parvum victorivirus 3, NpVV3). An agarose gel dsRNA profile of a Pakistani strain of *N. parvum*, NFN, showed a band of ~5 kbp, which was not detectable in Japanese strains of *N. parvum*. Taking a high-throughput and Sanger sequencing approach, the complete genome sequence of NpVV3 was determined to be 5226 bp in length with two open reading frames (ORF1 and ORF2) that encode capsid protein (CP) and the RNA-dependent RNA polymerase (RdRP). RdRP appears to be translated by the stop/restart mechanism facilitated by the junction sequence AUGucUGA as in some other virctoriviruses. Blastp searches showed that NpVV3 CP and RdRP share the highest amino acid sequence identities (80.5% and 72.4%) with the corresponding proteins of NpVV1 isolated from a French strain of *N. parvum.* However, NpVV3 was found to be different from NpVV1 in the terminal sequences and the stop/restart facilitator sequence. NpVV3 particles ~ 35 nm in diameter were semi-purified and transfected into an antiviral RNA silencing deficient strain (Δ*dcl2*) of an experimental ascomycetous fungal host, *Cryphonectria parasitica*. NpVV3 showed symptomatic but unstable infection in the new host strain.

Fungal viruses (mycoviruses) are found ubiquitously in all major groups of fungi [9] and now classified into a total of 23 families [32] by the International Committee on Taxonomy of Viruses (ICTV). It should be noted that there is a bias in fungal virus hunting studies. For example, ascomycetous fungi, particularly phytopathogenic fungi from the US, Europe and East Asia, have extensively been used as virus hosts [2, 24]. Other groups of fungi have also been explored as virus hosts to a lesser degree [20, 30, 31]. Many of such studies with technically unbiased high-throughput virus detection methods clearly indicate that the majority of fungal viruses have double-stranded (ds) and positive-sense (+) single-stranded (ss) RNA genomes.

Among fungal dsRNA RNA viruses are members of the family *Totiviridae* (order *Ghabrivirales*) includes five genera: *Totivirus*, *Victorivirus*, *Giradiavirus*, *Leishmaniavirus* and *Trichomonasvirus* (https://talk.ictvonline.org/taxonomy/). Of these five, two genera *Totivirus* and *Victorivirus* accommodate icosahedral viruses for fungi. The subject of the current study is a victorivirus. Victoriviruses are filamentous fungi and characterized by their approximately 5-kbp (4.6 to 7.0) undivided dsRNA genome, two-open reading frames (ORF) genome architecture, stop-restart translation mechanism for the downstream ORF, and icosahedral particle structure of 30–40 nm in diameter with a T=1 capsid [15, 19, 35]. The 5′-proximal ORF encodes the capsid protein, while the 3′-proximal one encodes RNA-dependent RNA polymerase (RdRP). Between the two ORFs is the junction sequence, typically *AUG*A or UA*AUG* in which the italicized AUG serves as the start codon while UGA or UAA (underlined) functions as the stop codon in co-translational re-initiation or stop/re-initiation [15]. Only a few members of the genus have been characterized biologically [4, 36].

We started screening of diverse fungi from Pakistan for viruses in 2011 [13]. Some peculiar and omnipresent fungal RNA viruses were characterized [11, 12, 25, 28]. The current study is an extension of the characterization study of Pakistani fungal viruses. In this study we report on the characterization of a novel victorivirus termed Neofusicoccum parvum victorivirus 3 (NpVV3) from a phytopathogenic ascomycete, *Neofusicoccum parvum.*

The fungal strain NFN used in this study (Fig. 1A) was isolated from the trunk of a diseased banana tree in Hangu District, Punjab Province, Pakistan in the year 2018. The trunk surface was sterilized by soaking in 1% sodium hypochlorite solution and washing with autoclaved distilled water three times. A small part from the dried sample was cut and inoculated on potato dextrose agar (PDA, Becton, Dickinson and Co.), then allowed to grow at room temperature. The Pakistani fungal strain NFN was identified as *N. parvum* (anamorph of *Botryosphaeria parva*) by sequencing the ribosomal internal transcribed spacer region (ITS) region. Two Japanese strains of *N. parvum* (MAFF numbers 237895 and 239155) were used as references.

Three-day old mycelia were used for dsRNA extraction by cellulose affinity chromatography [8]. DNase I (Thermo Fisher Scientific) and S1 nuclease (Thermo Fisher Scientific) treatments were carried out for eliminating the DNA and ssRNA from the sample. The dsRNA-enriched samples were resolved by electrophoresis on 1% agarose gel. As shown in Fig. 1B, a dsRNA band of ~5 kbp was detected from the Pakistani strain NFN, but not from the Japanese strains (MAFF 237895 and 239155).

Total RNA and dsRNA samples from three fungal species including *N. parvum* strain NFN were pooled together and sent for next-generation sequencing (NGS) analysis to Macrogen Inc (Tokyo, Japan), as described by Khan et al. [12]. The three fungal isolates included *N. parvum* strain NFN, *Geotrichum candidum* (formerly species was not identified) strain E, and *Fusarium nygami* strain 1NL. After *de novo* assembly of raw NGS data using CLC Genomics work bench 11 (CLC Bio-QIAGEN, Aarhus, Denmark), local BLAST analyses were conducted with viral reference sequences from the NCBI database. As previously described, several virus-like contigs, including segments of a strain of hadakavirus 1 in *F. nygami* [12], were detected. Among those obtained sequences, contig numbers 630 (5,199 nt, 27,397 assembled reads) and 1683 (2055 nt, 7,093 assembled reads) were confirmed by to be derived from the *N. parvum* strain NFN, which showed sequence similarity to victoriviruses and splipalmiviruses (novel segmented RNA viruses related with narnaviruses) [5, 31], respectively (data not shown). Contig 1683 and other contigs from *Geotrichum candidum* are not the subject of this study and will be published elsewhere after a thorough characterization of biological and molecular properties.

The contig 630 sequence was likely derived from a victorivirus (tentatively termed Neofusicoccum parvum victorivirus 3, NpVV3) infecting the strain NFN; thus, the virctorivirus sequence was re-confirmed by Sanger sequencing of overlapping RT-PCR clones. The terminal sequences of this virus were determined using RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) in which five independent RACE clones from each of the 5′ and 3′ ends were sequenced, as described by Khan et al. [12]. The complete genome sequence of NpVV3 was 5226 bp in length (accession no: MZ868719) and possessed two large ORFs (ORF1 and ORF2), namely ORF1 and ORF2, respectively, which encode capsid protein (CP, 83.7 kDa) and RNA-dependent RNA polymerase (RdRP, 92.7 kDa) (Fig. 1C). The dsRNA was also detectable from a semi-purified preparation of NpVV3 particles approximately ~35 nm in diameter (Fig. 1D, data not shown). The ORF2-encoded RdRP domain is considered to be expressed by a stop/restart translation mechanism, which is possibly mediated by the $_{2634}$*AUG*ucUGA (Fig. 1C) and a pseudoknot structure predicted to form between positions 2,588 and 2,646 (Fig. 1E). The *AUG*ucUGA sequence is rarely observed in victoriviruses [11]. The tetra-nucleotide *A*UGA and penta-nucleotide UA*AUG* are commonly observed in the junction between ORF1 and ORF2 in victoriviruses [11], while the facilitator has been substantiated only in a few cases [10, 15, 16]. The italicized and underlined triplets are expected to serve

as the ORF2 start and ORF1 stop codons. The terminal sequences of the NpVV3 plus-strand RNA are partially shared with those of other victoriviruses such as Neofusicoccum parvum victorivirus 1 (NpVV1), Btryosphaeria dothidea victorivirus 2 (BdVV2), and Rosellinia necatrix victorivirus 1 (RnVV1). The extreme 5′ terminal sequence, 5′-G/U/CGAAA---, is strictly conserved (Fig. 1F). Blastp searches showed that the NpVV3 proteins exhibited a high level of sequence identity to NpVV1 (RdRp, 72.4%; CP, 80.5%) [18], Sphaeropsis sapinea RNA virus 1 (RdRP, 62.9%; CP, 67.4%) [22] and BdVV2 (RdRp, 61.0%; CP, 69.8%) [37]. The close relationship to the aforementioned victoriviruses was also confirmed by multiple RdRP sequence alignment (Fig. S1) and phylogenetic analyses (Fig. 2). NpVV3 of Pakistani origin was placed phylogenetically in the same clade as NpVV1 from a French *N. parvum* strain. However, it should be noted that NpVV1, the closest relative of NpVV3 of Pakistani origin, appears to have the stop/restart facilitator sequence *AUG*uc<u>UAG</u> at map positions 2625-2632 (GenBank accession number: MW175879.1) and a pseudoknot structure different from those of NpVV3.

Despite a large number of victoriviruses reported from diverse filamentous fungi, only a limited number of them have been biologically investigated [4, 36]. In order to examine the possible effects of NpVV3 infection on host fungi, we took a few approaches. First, virus curing was attempted by mycelial fragmentation [14], protoplasting [3], and hypha tipping [17], given the fact that *N. parvum* strain NFN did not produce asexual spores on PDA plates. At least 30 sub-isolates obtained by each aforementioned method were tested by one-step colony RT-PCR for NpVV1 infection by using PrimeScript one-step RT-PCR kit ver.2 (Dye Plus) (TaKaRa Bio, Inc.) [1, 34]. Consequently, no virus-free sub-isolate was obtained by the methods (data not shown), as in the case for other victoriviruses [23].

We next attempted to transfect an experimental model ascomycetous fungus, *C. parasitica*, with semi-purified NpVV3, as described earlier by Chiba et al. [3]. The semi-purified preparation containing approximately 300 ng of the NpVV3 genomic dsRNA was introduced into 3 x 10^6 protoplasts of an antiviral RNA silencing deficient *C. parasitica* mutant, Δ*dcl2* (*dcl2*-knockout with EP155 background) [26]. This mutant strain has been utilized as a host for diverse fungal viruses not only from *C. parasitica* but also from fungi of orders or suborders different from the accommodating *C. parasitica* [11, 27, 33, 36]. As observed for other victoriviruses, Δ*dcl2* was found to be susceptible to NpVV3 in which its genomic dsRNA accumulated to a detectable level by agarose gel electrophoresis (Fig. 3A). However, unlike the biologically well-characterized victoriviruses (RnVV1 and Helminthosporium victoriae virus 190S) [4, 36], NpVV3 caused no phenotypic alteration in Δ*dcl2* (Fig. 3B). It should be noted that NpVV3 was lost from Δ*dcl2* after repeated subculturing and storage (data not shown). Asymptomatic infection of Δ*dcl2* has been observed for a few viruses such as Cryphonectoria hypovirus 4 (CHV4) strain C18 [1] and Alternaria alternata victorivirus 1 (AalVV1) [11].

This study described the molecular and biological attributes of NpVV3 from a Pakistani strain of *N. parvum*, which are common to and different from previously reported victoriviruses. The fact that there are only a few papers featuring viruses from *N. parvum* [18, 21] shows the fungus to be poorly studied as a virus host. Based on the species demarcation criteria (<60% RdRP amino acid sequence identity) reported by the International Committee on Taxonomy of Viruses in 2011 [35], NpVV3 and its closest relative, NpVV1, may belong to the same new species. However, the two viruses are different in their terminal sequences (Fig. 1C) and the ORF1 and ORF2 junction sequence serving as the putative stop/restart translation facilitator (data not shown). The symptomless and unstable nature of the virus in Δ*dcl2* is of great interest.

Supplementary Information

The online version contains supplementary material available at ====.

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Compliance with ethical standards

Conflicts of interest

The authors declare that there are no conflicts of interest.

Human and animal rights

This article does not contain any studies with human participants or animals performed by any of the authors.

Figure legends

Figure 1 Properties of the new victorivirus NpVV3 from *Neofusicoccum parvum*. (A) Colony morphology of NpVV3-carrying Pakistani strain NFN and NpVV3-free Japanese strains MAFF237895 and MAFF239155. Fungal colonies were grown on PDA for three days on the bench and photographed. The scale bar represents 3 cm. (B) Double-stranded RNA profile of the fungal strains. DsRNA fractions were isolated from the fungal strains shown on the top of the gel, electrophoresed on an agarose gel using the 1x TBE buffer system, and stained with ethidium bromide. The dsRNA markers 1 and 2 are purified genomic dsRNA of RnVV1 [4] and mycoreovirus 1/S10ss [29], respectively. The dsDNA marker is GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Inc.). (C) Genome organization of NpVV3. The plus-strand of the NpVV3 genome has two ORFs (ORF1 ad ORF2), encoding CP and RdRP, which are separated by the sequence *AUG*ucUGA. (D) NpVV3 virus particle morphology. NpVV3 particles were purified from NFN fungal culture and examined by electron microscopy using a Hitachi electron microscope H-7650 after samples were negatively stained using an EM stainer (Nissin EM Co., Tokyo, Japan). NpVV3 particles are denoted by white arrows. (E) Predicted H-type pseudoknot structure upstream of the junction octanucleotide *AUG*ucUGA. The structure was predicted using the Dot Knot online program (https://dotknot.csse.uwa.edu.au/) and drawn with PseudoViewer3 (http://pseudoviewer.inha.ac.kr/). The AUG start codon is highlighted with the red open rectangle. (F) Comparison of the 5′- and 3′-terminal sequences of victoriviruses. The positive-strand RNA of several victoriviruses were compared and conserved residues are shown in white letters with black background. The compared victoriviruses are: The compared victoriviruses are: Neofusicoccum parvum victorivirus 3 (NpVV3, MZ868719), Neofusicoccum parvum victorivirus 1 (NpVV1, MW175879), Botryosphaeria dothidea victorivirus 2 (BdVV2, MH301088.1), Beauveria bassiana victorivirus 1 (BbVV1, NC_024151.1), and Rosellinia necatrix victorivirus 1 (RnVV1, NC_021565.1). The multiple sequence alignment was performed by MUSCLE [7] in GENETYX-MAC ver. 20.1.0 with default settings.

Figure 2. Phylogenetic analysis of NpVV3 RdRP. Amino acid sequences of totiviruses RdRPs (A) and CP (B) were aligned using MAFFT and the alignment was trimmed by BMGE [6]. The maximum likelihood tree (LG+G+I+F model for RdRP and LG+G +F model for CP) was generated using the online tree generation software NGPhylogeny.fr (https://ngphylogeny.fr/). Bootstrap values in 1000 replications are shown next to the nodes. Accession numbers are shown in the tree. iToll (online webserver) was used for tree visualization (https://itol.embl.de/).

Figure 3. Transfection of *Cryphonectria parasitica* strain Δ*dcl2* with NpVV3 particles. (A) DsRNA purified from Δ*dcl2* candidate transfectants with NpVV3. Semi-purified virus particles (Fig. 1D) were transfected into protoplasts of an RNA silencing-deficient Δ*dcl2*. The line #1 was successfully transfected with NpVV3, while the line #2 was not. The dsDNA marker is GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Inc.). (B) Colony morphology of virus-free Δ*dcl2* and NpVV3-transfected Δ*dcl2.* An obtained fungal transfectant was cultured on PDA for one week along with a non-transfectant. The scale bar represents 1 cm.

Figure S1

Multiple alignments of the amino acid sequences of the conserved motifs in RdRps of NpVV3 and other victoriviruses. Virus names and abbreviations are as follows: NpVV3, Neofusicoccum parvum victorivirus 3 (accession MZ868719); NpVV1, Neofusicoccum parvum victorivirus 1 (accession QTE76048.1); RnVV1, Rosellinia necatrix victorivirus 1 (accession YP_008130308.1); UvRV1, Ustilaginoidea virens RNA virus 1 (accession YP_007761589.1); MpVV1, Macrophomina phaseolina victorivirus 1 (accession QKI37143.1); PlTV1, Phomopsis longicolla totivirus 1 (accession ALD89108.1); AaVV1, Alternaria arborescens victorivirus 1 (accession YP_009553478.1); CmRV, Coniothyrium minitans RNA virus (accession YP_392467.1); PdV1, Penicillium digitatum virus 1 (accession AMY26886.1); MoV2, Magnaporthe oryzae virus 2 (accession BBG92298.1); SsRV2, Sphaeropsis sapinea RNA virus 2 (accession NP_047560.1); UvRV1, Ustilaginoidea virens RNA virus 1 (accession YP_007761589.1).

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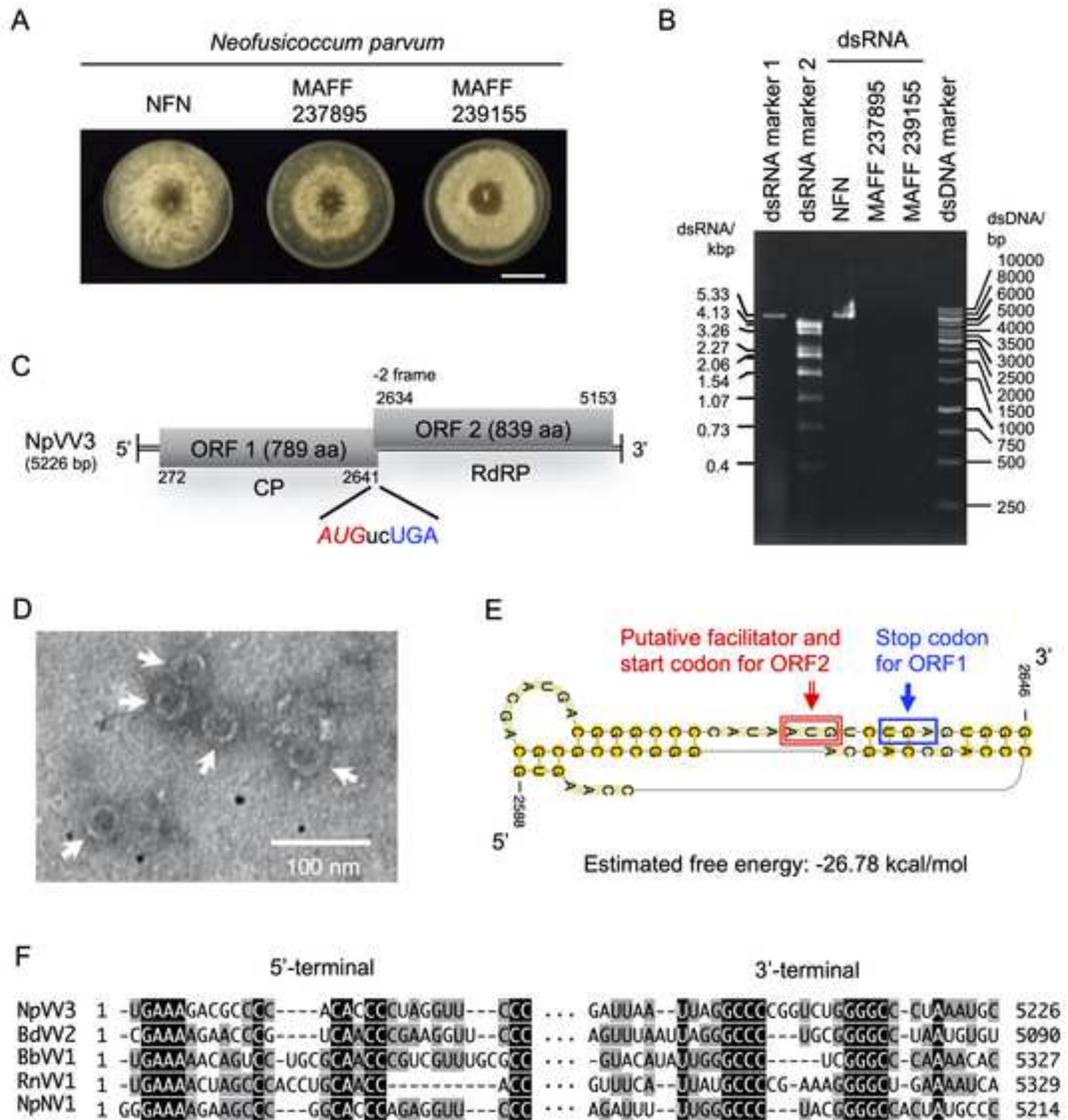

Figure 2A

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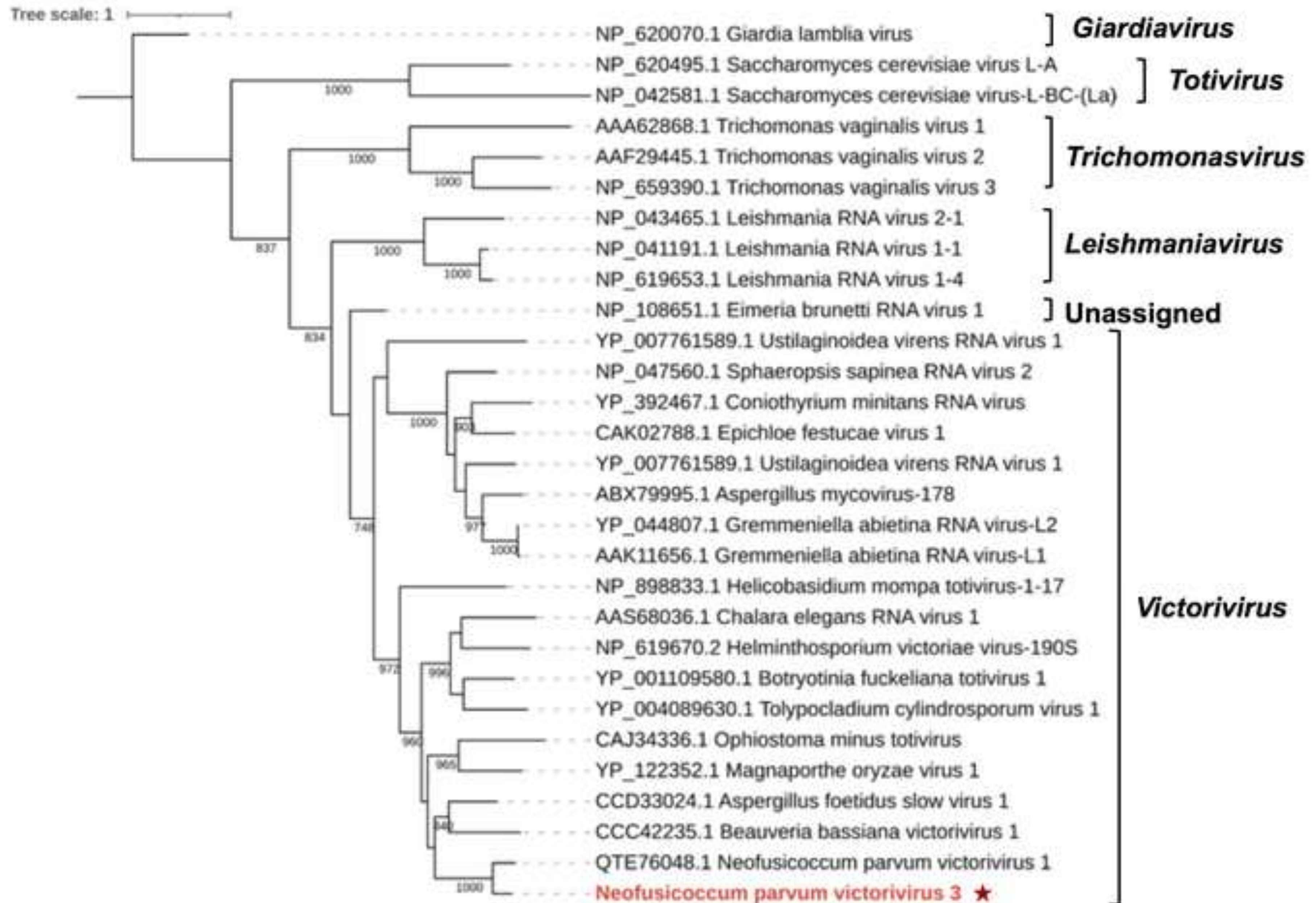

Figure 2B

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CP

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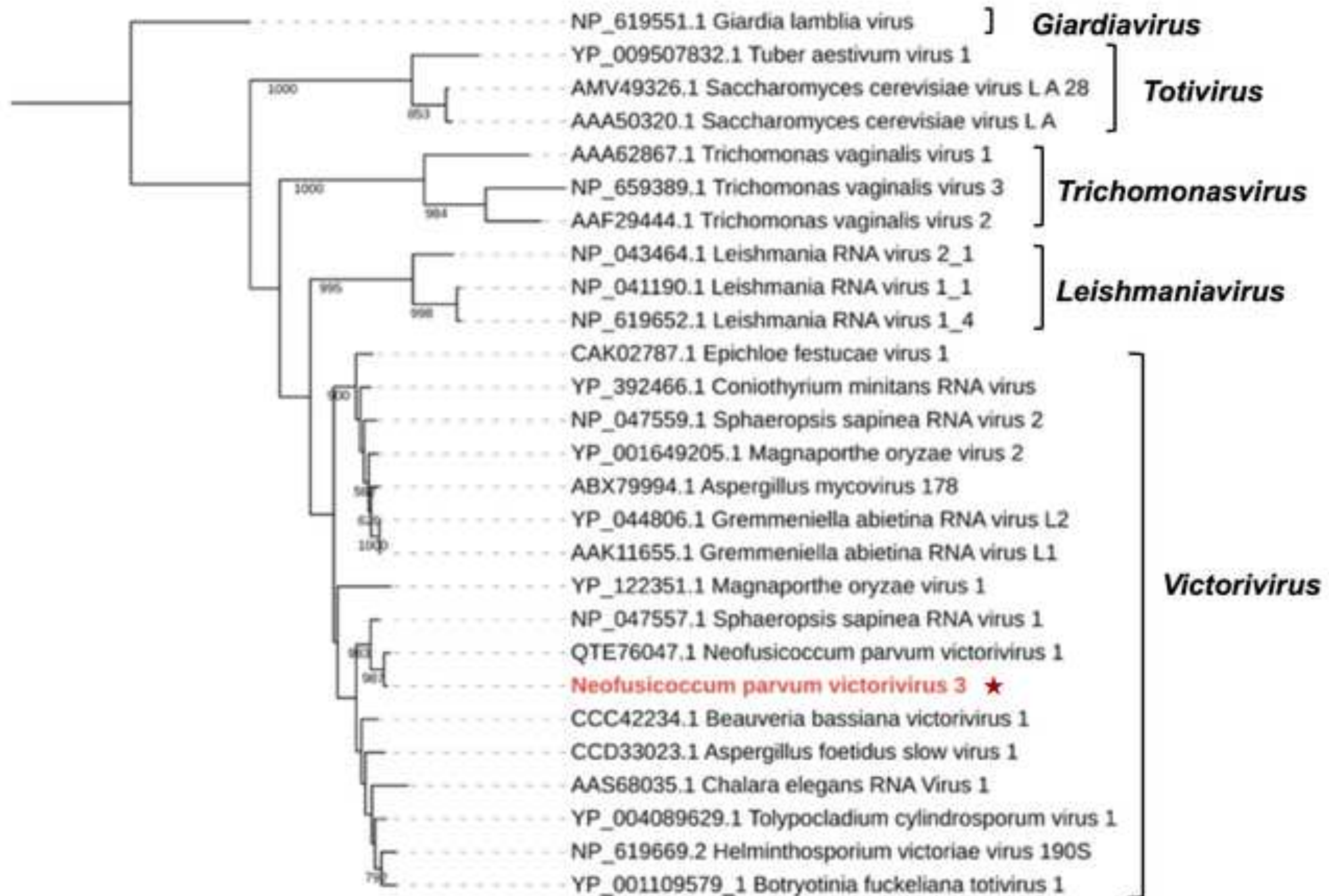

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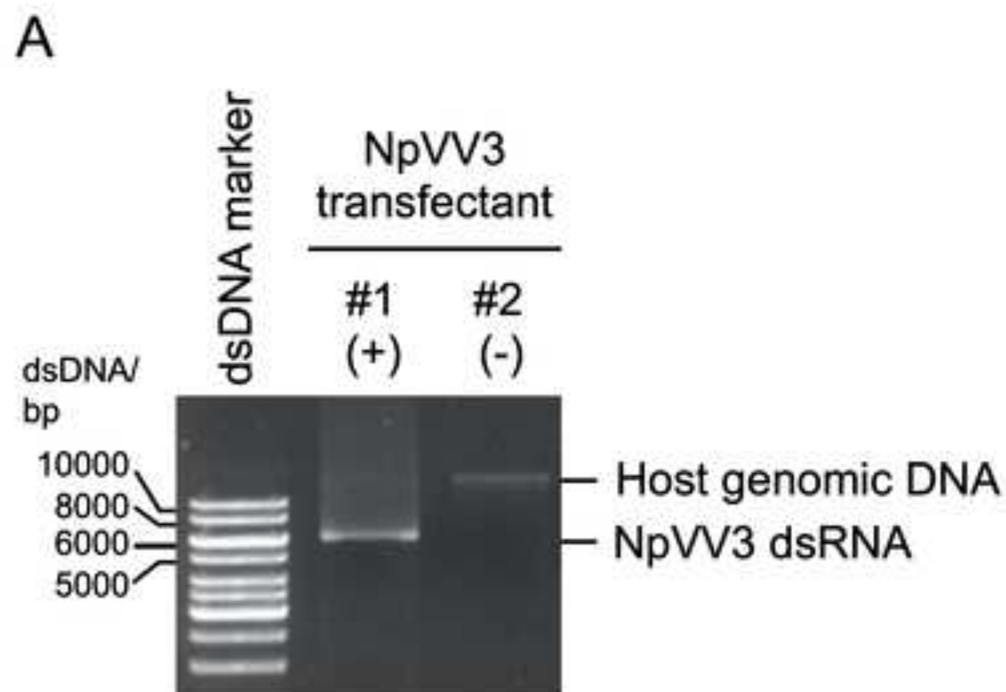
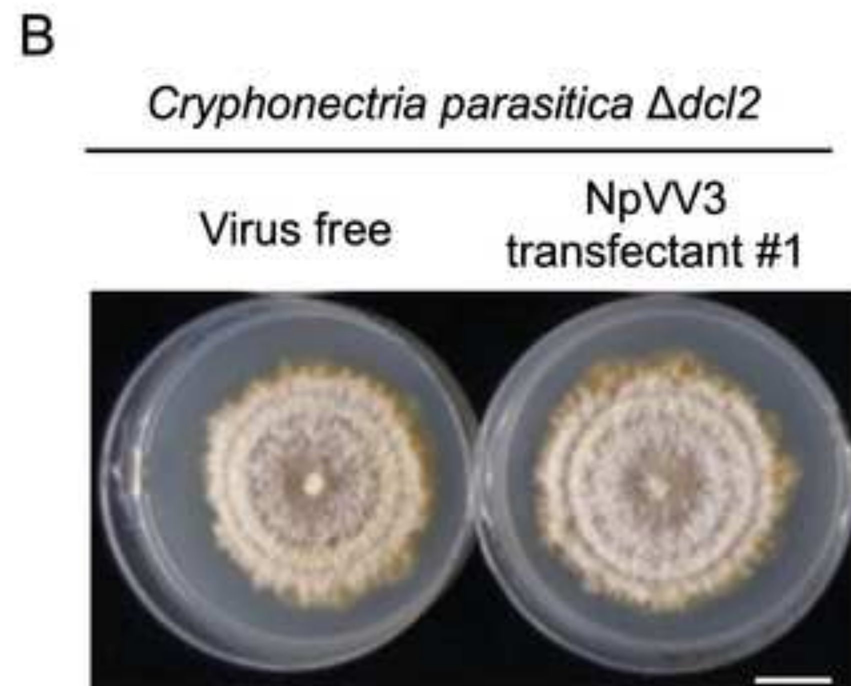
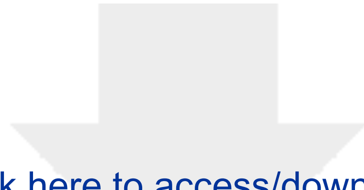




supplementary data



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Electronic Supplementary Material

Supplementary Data 2.pdf