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MOLECULAR CHARACTERISATION OF AN EGYPTIAN ISOLATE OF NIGROSPORA ORYZAE NONSEGMENTED RNA VIRUS 1

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ABSTRACT

Mycoviruses are widely distributed among fungi, including plant-associated species. In this study, a *Nigrospora oryzae* isolate (NDC1) was isolated from Egypt and characterised based on morphology and molecular analysis, confirming its identity through ITS sequencing and phylogenetic analysis. Screening for mycoviruses revealed the presence of a ~3 kb dsRNA genome, which was sequenced using next-generation sequencing (NGS) and identified as an isolate of Nigrospora oryzae nonsegmented RNA virus 1 (NoNRV1-NDC1). The viral genome consists of 2858 nucleotides and contains two open reading frames (ORFs), with ORF2 encoding an RNA-dependent RNA polymerase (RdRp) that shares 98% identity with NoNRV1 isolates previously reported in China. Phylogenetic analysis clustered NoNRV1-NDC1 with closely related unclassified viruses, suggesting a common evolutionary origin. A conserved slippery sequence near the ORF1 stop codon indicates a potential -1 ribosomal frameshifting mechanism for RdRp expression. This study contributes to the expanding knowledge of fungal viromes and provides insights into the diversity of mycoviruses associated with *N. oryzae*. It also expands our understanding of NoNRV1 diversity, offers new insights into the genomic organisation of unclassified mycoviruses, and highlights mycoviruses' occurrence in *N. oryzae* from Egypt.

Keywords: *Nigrospora oryzae*, Mycoviruses, DsRNA genome, Nigrospora oryzae nonsegmented RNA virus 1

INTRODUCTION

The kingdom of Fungi is among the most diverse organisms on Earth, occupying various environments such as soil, plant parts, water, and food sources. As heterotrophic organisms, fungi can exist as saprophytes, parasites, or symbionts associated with other microorganisms **(Jobard et al., 2010; Tedersoo et al., 2017)**. Fungal pathogens pose significant economic and environmental threats to agriculture and food production worldwide. The standard approach to managing these pathogens involves the use of fungicides. However, the extensive application of fungicides has several drawbacks, including adverse effects on human health and the environment, high costs, and development of resistance. Consequently, interest in biological control agents as an alternative to chemical fungicides has grown in recent years, including fungal and bacterial antagonists **(Bressan, 2003; Köhl et al., 2011; Thambugala et al., 2020; Azeem et al., 2022; Bonaterra et al., 2022)**. Recently, mycoviruses, or fungal viruses, have emerged as promising eco-friendly biocontrol agents capable of mitigating the negative impacts of chemical fungicides and traditional biological control methods. These viruses offer a targeted approach against specific fungal pathogens and can be adapted to counteract microbial resistance, which may evolve **(Abid et al., 2018; Bocos-Asenjo et al., 2022; Tonka et al., 2022)**. Mycoviruses are widespread across all major fungal groups, and while many cause little to no apparent harm to their fungal hosts, some can significantly impair fungal growth and virulence **(Pearson et al., 2009)**.

Several virus families, encompassing a wide range of genomic types, including those with dsRNA, ssRNA, and DNA genomes, have been related to hypovirulence in fungi. These include well-known families like *Hypoviridae*, *Alphaflexiviridae*, *Botybirnaviridae*, *Chrysoviridae*, *Endornaviridae*, *Megabirnaviridae*, *Narnaviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae* **(Ghabrial et al., 2015)** and recently described groups like *Mymonaviridae* **(Liu et al., 2014)** and *Genomoviridae* **(Yu et al., 2010; Khalifa & MacDiarmid, 2021)**. The RNA silencing machinery of fungal hosts can reduce viral multiplication and protein synthesis, but it does not always stop mycovirus survival. Some mycoviruses have evolved RNA silencing suppressors to resist this host response, enabling survival and modifying the host's phenotypic **(Rodriguez Coy et al., 2022)**. The capacity of several mycoviruses to attenuate their fungal host virulence, coupled with the ability of some to evade host RNA silencing mechanisms, makes them promising candidates for the biological control of fungal diseases.

Numerous fungal species spend part of their life cycle closely associated with soil environments. Examples include *Aspergillus niger*, *A. flavus*, *A. terreus*, *Penicillium notatum*, *P. funiculosum*, *Nigrospora* sphaerica, and *N. oryzae* **(Moubasher & Moustafa, 1970; Bridge & Spooner, 2001; Iram et al., 2003; Muhsin & Hachim, 2014; Rathod et al., 2014)**. *Nigrospora* sp. is a filamentous fungus found in soil, air and as a plant pathogen **(Wu et al., 2009; Thalavaipandian et al., 2011; Uzor et al., 2015; Wang et al., 2017; Hao et al., 2020)**. This widely distributed fungus can act as an endophyte colonising stems and leaves or as a saprobe decomposing organic matter, including dead larvae and leaf litter **(Chen et al., 2016; Wang et al., 2017; Hao et al., 2020)**. Several mycoviruses with RNA genomes have been identified in *N. oryzae*, including Nigrospora oryzae nonsegmented RNA virus 1 (NoNRV1) **(Zhou et al., 2016; Wang et al., 2022)**, Nigrospora oryzae victorivirus 1 (NoVV1) **(Zhong et al., 2016a)**, Nigrospora oryzae fusarivirus 1 (NoFV1) **(Zhong et al., 2016b)**, Nigrospora oryzae partitivirus 1 (NoPV1) **(Yu et al., 2018)**, and Nigrospora oryzae mitovirus 1 (NoMV1) and mitovirus 2 (NoMV2) **(Liu et al., 2019)**. NoNRV1, an unclassified mycovirus distantly related to members of the *Partitiviridae* family, possesses a nonsegmented double-stranded RNA (dsRNA) genome of 2857 base pair (bp) containing two open reading frames (ORFs). ORF1 encodes a protein of unknown function, while ORF2 is predicted to encode an RNA-dependent RNA polymerase (RdRp) **(Zhou et al., 2016; Wang et al., 2022)**. Although NoNRV1 does not impact the fungal host's growth rate on PDA plates, it enhances pigmentation and reduces both the pathogenicity of the fungus on cotton leaves and its thermotolerance **(Wang et al., 2022)**. The current study reports the characterisation of an Egyptian isolate of NoNRV1.

MATERIALS AND METHODS

Isolation, maintenance, and identification of the host fungal isolate

Soil fungi were isolated on potato-dextrose agar (PDA) media from a soil sample using the pour-plate technique described by **Warcup (1955)**. Pure cultures were transferred onto PDA plates and maintained on PDA slants at 4°C. The fungal isolates were sub-cultured on PDA plates and incubated at 25°C for 5 days to obtain fresh cultures. To prepare liquid cultures, flasks containing potato dextrose broth (PDB) medium were inoculated with fungal discs from the actively growing margins and incubated with shaking (180 rpm) for 5 days at 25°C. Mycelia were harvested by filtration, washed with autoclaved water, and used for further experiments. DNA was extracted from fungal isolates of interest using the phenol-chloroform purification method. Purified DNA was used to identify the fungal isolate used in this study by amplifying and sequencing the non-coding internal transcribed spacer (ITS) region of its ribosomal DNA (rDNA) using the primer pair ITS4/ITS5 **(White et al., 1990)**.

Extraction and purification of dsRNAs

DsRNAs were extracted based on their selective binding to CF11 cellulose in the presence of ethanol, as **Valverde et al. (1990)** described with some modifications. 2-3g (fresh weight) of fungal mycelium was ground to a fine powder with a mortar and pestle in liquid nitrogen. The fine powder was suspended in 8 ml of Sodium-Tris-EDTA (1× STE) buffer, 1 ml of 10% sodium dodecyl sulfate (SDS), and 0.5 ml of 2% bentonite, and 9ml ml of STE-saturated phenol was added to each sample. Each sample was shaken for 30 minutes and centrifuged at 4000 rpm for 20 minutes. After centrifugation, ethanol (final concentration of 16% (v/v)) was added to 10 ml aliquots of the aqueous phase of each sample and applied to CF11 columns. The columns were washed with 40 ml of 16% ethanol-STE buffer, followed by a final washing step using 1.25 ml of 1× STE buffer. dsRNA was eluted with 5.4 ml of STE buffer and precipitated with 5.4 ml of isopropanol and 1.2 ml of 3M sodium acetate at -20°C overnight. The dsRNA was pelleted by centrifugation for 20 minutes at 8000 rpm, air-dried, and re-suspended in a 100µl aliquot of UltraPure Gibco water (Invitrogen, USA). The RNA nature of each sample was confirmed by DNase treatment. The DNase-treated RNA was then analysed using agarose gel electrophoresis.

Reverse transcription PCR (RT-PCR) and sequencing of dsRNA

Complementary DNA (cDNA) was synthesised based on the protocol of Roossinck et al. (2010), with some modifications. An 8 µl aliquot of purified dsRNA from the selected fungal isolate was mixed with 1.2 µl of a primer with the sequence 5′-CATTGAGTTGTCCCNNNNNN-3′. The dsRNA was denatured by boiling for 2 minutes and quenched on ice for 15 minutes. A 10.8 µl volume of an RT mixture containing 0.8 µl of Superscript III and 10 µl of 2× Reaction mix was added (Superscript™ III one-step RT-PCR system, Invitrogen, USA). This mixture was incubated at 50°C for 60 minutes, treated with 1 µl of RNase A (Geneaid, Taiwan) at room temperature for 15 minutes, then incubated at 85°C for 2 minutes. The remaining primers from the previous step were removed using a PCR clean-up & gel extraction kit (GeneDireX, Taiwan) as recommended by the manufacturer. For cDNA amplification, a 5 µl aliquot of the pure cDNA was amplified in a reaction volume of 50 µl, including 2 µl of a primer with the sequence 5′-CTATGCCATTGAGTTGTCCC-3′, 25µl of 2× TOP simple PCR DyeMIX-nTaq (Enzynomics, Korea), and 18µL of UltraPure Gibco water. The amplification program began at 95°C for 1 minute, 65°C for 30 seconds, 72°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, a single cycle for 5 minutes at 72°C and a final extension step for 5 minutes at 37°C. Random dsDNA fragments over 100 bp long were purified using a PCR clean-up & gel extraction kit as recommended by the manufacturer. The purified PCR product was sequenced using an Illumina NovaSeq6000 with paired-end libraries and a read length of 150 bp at Macrogen, South Korea.

Processing of Illumina short reads

PCR Primers used to generate cDNA from dsRNA and sequencing library preparation adapters were removed from the dataset using Geneious Prime software. Short read quality was assessed based on the FastQC report. Nucleotide sequences with quality scores less than Q20 were discarded. Unreliable stretches at the ends of each read were also trimmed using the same software. Quality-filtered reads were assembled into long contigs using the *de novo* assembly tool of Geneious Prime set to medium sensitivity and default parameters. Contigs shorter than 500bp were discarded, whereas the identity of those longer than 500bp was determined using BLASTx analysis against the non-redundant (nr) database of NCBI.

Terminal sequence determination of the virus genome

The terminal sequences were determined as previously described by Khalifa and Pearson (2013). T4L adapter (5′-PO_4-CCCGTCGTTTGCTGGCTCTTT-NH_2-3′) was ligated to the 3′ ends of the dsRNA using T4 RNA ligase. T4L-ligated dsRNA was used as a template for RT-PCR amplification genome termini using primer T4LC together with NoRV1-5R primer (5′-ATGCCTGCGTCTTTCCAC-3′) and NoRV1-3F primer (5′-GTTCCTGGGTGGGGAAGG-3′) to amplify the 5′ and 3′ terminal regions, respectively. The RT-PCR reactions were run at the following conditions: 55°C for 40 minutes, 94°C for 2.5 minutes, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 68°C for 20 seconds, a final extension of 68°C for 3 minutes, and holding at 10°C. The RT-PCR products were visualised by gel electrophoresis and Sanger sequenced at Macrogen, South Korea.

Viral sequence confirmation and detection

The generated consensus sequence of viral origin, based on BLASTx analysis, contained a short nucleotide stretch with low-quality base calling. Therefore, it was necessary to design sequence-specific primers to amplify and sequence the viral genome around this region. A pair of primers (NoRV1-S2R: 5′-AGAGCGTGTTATGCGGGG-3′ and NoRV1-S2F: 5′-CTGGGATACAAATCGTTCGG-3′) was designed based on the available sequence. Total RNA was extracted from 150 mg (fresh weight) of fungal mycelium. The extracted RNA was used as a template for the one-step RT-PCR amplification of fragment S2 of the viral genome. RT-PCR reaction mixture contained 1µL of the template, 1µL of NoRV1-S2F primer, 1µL of NoRV1-S2R primer, 25µL of 2x Reaction Mix, 2µL of the SuperScript III RT/Platinum Taq mix, and 20µL of autoclaved distilled water. The reaction was run at the following conditions: 55°C for 40 minutes, 94°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 53°C for 30 seconds, 68°C for 1 minute and 30 seconds, a final extension at 68°C for 3 minutes and holding at 10°C. The amplified PCR product was checked using gel electrophoresis, and Sanger sequenced at Macrogen, South Korea.

Sequence and phylogenetic analyses

Sequences obtained by Sanger sequencing for the viral fragment S2 and terminal sequences were assembled with the contiguous sequences obtained by Illumina sequencing to generate a full-length viral genome. ORFs were determined using the ORF Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). Potential secondary structures at the terminal regions of the viral genome were detected using MFOLD software **(Zuker et al., 1999)**. The possible functions of proteins encoded by detected ORFs were determined using BLASTp analysis. For conserved motifs detection and phylogenetic analysis, multiple sequence alignments were performed using MUSCLE alignment **(Edgar, 2004)**. Neighbour-joining phylogenetic tree based on the multiple alignments of the RdRp deduced amino acid (aa) sequences of the virus of this study, together with those of other related viruses, was constructed using MEGA-X software **(Kumar et al., 2018)**.

RESULTS AND DISCUSSION

N. oryzae is a widely distributed fungus that is either an endophyte or a weak pathogen and is capable of infecting a wide range of crops such as maize **(Standen, 1945)**, rice **(Liu et al., 2021)**, sorghum (Fakhrunnisa & Ghaffar, 2006), cotton **(Zhang et al., 2012)**, watermelon **(Chen et al., 2019)**, Kentucky bluegrass **(Zheng et al., 2012)**, kiwifruit **(Li et al., 2018)**, and many more. Various mycoviruses have been identified in *N. oryzae*, e.g., NoVV1 **(Zhong et al., 2016a)**, NoVV2 **(Liu et al., 2019)**, NoMV1, NoMV2 **(Liu et al., 2019)**, NoFV1 **(Zhong et al., 2016b)**, NoPV1 **(Yu et al., 2018)**, and NoNRV1 **(Zhou et al., 2016)**. In this study, an Egyptian isolate, NDC1, of a *Nigrospora* sp. was isolated, characterised, and screened for RNA viruses.

Cultural characteristics and molecular identification of isolate NDC1

Hyphae of isolate NDC1 are branched, septate, smooth and hyaline, becoming brown closer to the conidiogenous region. Conidia appeared globose, black, shiny, smooth and aseptate. The fungal colony on PDA was woolly in appearance with circular margins, grey to black with age, and reached 9 cm in 5 days at 25°C (Figure 1A). The fungal isolate was characterised as N. oryzae according to the morphological characteristics. To confirm the identity of the fungal isolate, molecular characterisation was performed by sequencing the ITS-5.8s rRNA gene region, which produced a 555 bp sequence. BLASTn and multiple alignments revealed that isolate NDC1 shared identities with multiple *N. oryzae* and *N. sphaerica* isolates. Since it was previously reported that *N. oryzae* and *N. sphaerica* are sometimes misidentified (Starratt & Loschiavo, 1974), several confirmed isolates belonging to both species were used, together with isolate NDC1, to construct a neighbour-joining phylogenetic tree. Results shown in Figure 1B showed that isolate NDC1 clustered with *N. oryzae* isolates in a distinct, well-supported clade. Hence, isolate NDC1 was confirmed to be part of the species *N. oryzae*.

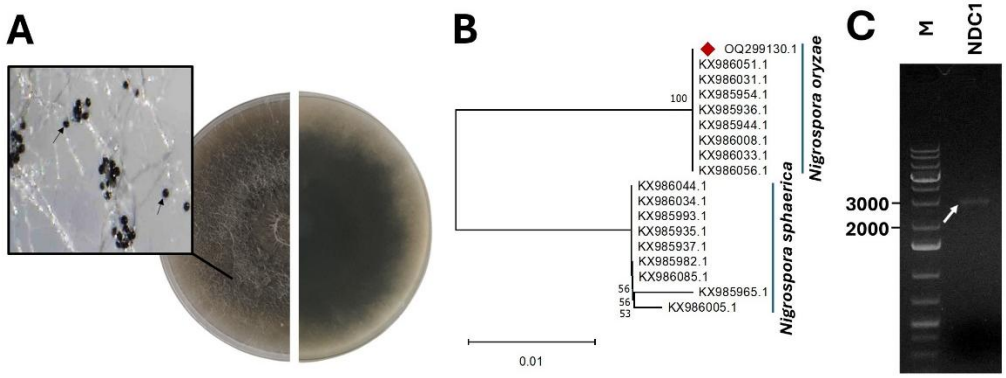

Figure 1 A. Colony morphology of *Nigrospora* isolate NDC1 after 5 days of culture on PDA at 25°C in the dark. The upper surface (left), reverse overview (right), and conidia (arrows) of the fungal culture are shown. **B.** Neighbour-joining phylogenetic tree constructed based on the ITS-5.8s rRNA gene sequence alignment of isolate NDC1 and confirmed isolates of *N. oryzae* and *N. sphaerica*. Similar DNA sequences were obtained from NCBI GenBank and aligned using CLUSTAL Omega. phylogenetic tree analysis was performed using MEGA version X and the maximum composite likelihood methods. The values 30 or above were only considered and represented next to the phylogenetic tree branches with confidence levels estimated by 1000 bootstrap replicates. **C.** Agarose gel (1% (w/v) in 1x TAE buffer) electrophoresis of dsRNA extracted from isolate NDC1 (~3000 bp). M: 1kb Plus DNA ladder.

RT-PCR, sequencing and pre-assembly processing of dsRNA associated with isolate NDC1

Most mycoviruses contain dsRNA or plus-sense single-stranded RNA (+ssRNA) genomes (Ghabrial et al., 2015), and therefore, they are easily detectable with dsRNA purification and subsequent electrophoresis (Valverde et al., 1990). Isolate NDC1 contained a single dsRNA band with an estimated molecular weight of ~3 kilobase pairs (kbp) (Figure 1C). RT-PCR generated cDNAs of random lengths from dsRNA isolated from this dsRNA-containing sample. The generated cDNAs ranged in size from 300 to 1250 bp. High molecular weight cDNAs were fragmented to produce cDNAs with a maximum size of 550 bp, which is the size compatible with the library construction kit and sequencing platform. The advent of next-generation sequencing (NGS) has made the discovery of mycoviruses from various sources possible (Ho & Tzanetakis, 2014). NGS platforms can generate near full-length, high-quality sequences equivalent to the sequences generated by traditional methods (Khalifa et al., 2016). In the present study, NGS was employed for sequencing and identification of the dsRNA component of the *N. oryzae* isolate NDC1. Following library construction, the Illumina NovaSeq6000 generated 416,180 reads of 151 nucleotides (nts). PCR primers and sequencing barcodes were trimmed, sequence reads were filtered by quality, and the number of nts to be trimmed was determined according to the FastQC report. Following quality trimming and filtering, 388,889 reads with a maximum length of 131 bp (with >Q20) remained and were ready for de novo assembly.

De novo assembly and preliminary identification

93% of quality-filtered reads were assembled to produce seven contigs. Contigs with lengths of 500 bp or more were kept for further analysis. After length filtering, only three contigs were kept. The three contigs with lengths of 500 bp or greater were subjected to BLASTx analysis against the nr database of NCBI. A single contig (Contig 1) was identified to be of viral origin as it had similarities to sequences of previously identified mycoviruses. Contig 1 was 2720 nts long and produced from the assembly of 260,555 overlapped sequence reads.

To obtain a full-length sequence of the viral dsRNA represented by Contig 1, sequences of the 5′ and 3′ terminal regions were obtained by adapter ligation to both ends, RT-PCR amplification, and sequencing of the terminal regions. Using RT-PCR and primer pair T4LC and NoNRV1-3F, a 190 bp DNA fragment was amplified from the 3′ end of viral dsRNA (Figure 2A). Similarly, RT-PCR amplification using primer pair T4LC and NoNRV1-5R produced a 138 bp DNA fragment representing the 5′-terminal region of the viral dsRNA under investigation (Figure 2B). The short sequence stretch of approximately 100bp in Contig 1, which was produced from the assembly of a small number of sequence reads, required sequence confirmation using sequence-specific primers. This resulted in the amplification of a DNA fragment of 1417 bp (Figure 2C). The amplification of this DNA fragment can also be used as a detection method for the presence of the viral RNA. The 1417 bp long fragment was Sanger sequenced, and then the sequence of that fragment and those of the 3′ and 5′ termini were assembled with the sequence of Contig 1 to produce a full-length genome of viral RNA.

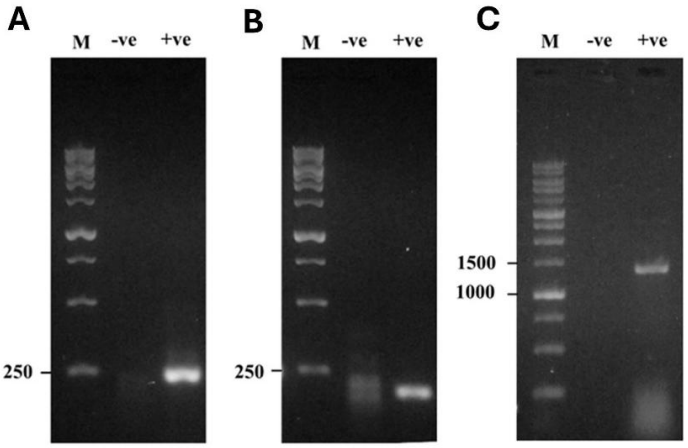

Figure 2 Agarose gel (1% (w/v)) shows RT-PCR products of 3′ **(A)**, 5′ **(B)** termini and segment S2 **(C)** of the viral dsRNA. Lane M: 1 Kb DNA ladder (GeneDireX), -ve: negative RT-PCR control, and +ve: RT-PCR positive reaction.

Sequence properties of the dsRNA segment associated with isolate NDC1

The entire sequence of the dsRNA is 2858 nts long, codes for two ORFs, and has a G+C content of 57.7%. The N-terminal ORF (ORF1), in frame 2, consists of 525 nts (nt positions 35-559), initiates at an AUG codon, terminates at a UAG codon, and codes for a 174aa long protein with an estimated molecular mass of 19.24 kDa. In frame 3, the second ORF (ORF2) at the C-terminus of the (+) strand is 2184 nts long (nt positions 618-2801), starts with an AUG codon, terminates at a UAA codon, and codes for a 727aa long protein with an estimated molecular mass of 82.84 kDa.

The coding region is surrounded by 5′ and 3′ untranslated regions (UTRs), which are 34 and 57 nts long, respectively. The BLASTP searches showed that ORF1 shared 30.7% - 97.7% identities with hypothetical proteins of several unassigned mycoviruses (Table 1). ORF1 shared the highest identity of 97.7%, with the corresponding ORF of NoNRV1 (accession number UJH94048). The protein encoded by ORF2 shared identities with RdRp proteins of numerous unassigned mycoviruses (Table 2), with the highest identity of 98% shared with the RdRp of NoNRV1 (accession number ALR87111). Based on the previous results, this study's virus was considered an isolate of NoNRV1 and was named NoNRV1-NDC1. A schematic representation of the genome organisation of NoNRV1-NDC1 is shown in Figure 3A. The sequence has been deposited in GenBank under accession number OQ589928. NoNRV1 was initially identified and molecularly characterised in 2016 (isolate HN-21). Further research in 2022 (isolate ZJ) explored its biological properties (Zhou et al., 2016; Wang et al., 2022).

The NoNRV1-NDC1 genome has two non-overlapping ORFs. A well-characterized slippery sequence, which can facilitate ribosomal -1 frameshifting and induce gag-pol protein fusion, has been described in totiviruses (Dinman et al., 1991; Khalifa & MacDiarmid, 2023; Xu et al., 2022). Surprisingly, NoNRV1-NDC1 contains an identical slippery sequence (CCCUUUC) 5 nts ahead of the ORF1 stop codon, which is shared by other unclassified mycoviruses such as NoNRV1, Purpureocillium lilacinum nonsegmented virus 1 (PlNV-1), and Ustilaginoidea virens nonsegmented virus 2 (UvNV-2). From this observation, we suggest that -1 frameshifting is employed by these unclassified, non-segmented mycoviruses, including NoNRV1, to produce their RdRP. Moreover, It has been proposed that NoNRV1, UvNV1, UvNV2, and PlNV1 express their RdRp (ORF2) and a hypothetical protein (ORF1) as a fusion protein through a +1 ribosomal

frameshifting mechanism (Herrero, 2016; Wang et al., 2022). However, signals for +1 ribosomal frameshifting were not identified in Phytophthora cactorum usti-like virus 1 (PcUV1) and Conidiobolus non-segmented RNA virus 1 (CNRV1), suggesting that these viruses may use alternative mechanisms to express their RdRp proteins (Wang et al., 2022).

The formation of stem-loop structures is frequent in viral RNA, arising when complementary nucleotide sequences pair together. These structures are commonly found within viral genomes' 5′- and 3′-UTRs. Their primary role is to regulate key processes such as virus replication and translation (Alvarez et al., 2005; Lin et al., 2013). The 5′ and 3′ UTRs of NoNRV1-NDC1 (+) RNA have the potential for folding and forming stem-loop secondary structures with ΔG values of -4.8 and -18.6 kcal/mole, respectively. The 5′ UTR secondary structure is formed by two stem-loops with perfect base-pairing at their stem regions, whereas the 3' UTR consists of a single perfectly base-paired stem-loop secondary structure (Figure 3B).

Table 1 Percent amino acid (aa) sequence identity between the hypothetical protein encoded by open reading frame 1 (ORF1) of Nigrospora oryzae nonsegmented RNA virus 1 (NoNRV1-NDC1) and that of other closely related unassigned mycoviruses.

Virus name	Identity (%)	Accession number
Nigrospora oryzae nonsegmented RNA virus 1	97.70	UJH94048.1
Nigrospora oryzae nonsegmented RNA virus 1	97.13	ALR87110.1
Purpureocillium lilacinum nonsegmented virus 1	51.85	AOO52901.1
Ustilaginoidea virens nonsegmented virus 2	51.85	YP_009553681.1
Purpureocillium lilacinum nonsegmented virus 1	51.22	AOO52900.1
Ustilaginoidea virens nonsegmented virus 2	51.22	YP_009553680.1
Ustilaginoidea virens nonsegmented virus 1	34.73	AIE77247.1
Ustilaginoidea virens nonsegmented virus 1	34.73	AIE77246.1
Phytophthora cactorum usti-like virus 1	38.24	QUA12646.1
Conidiobolus non-segmented RNA virus 1	30.91	QKL20126.1

Table 2 Percent amino acid (aa) sequence identity between the RNA-dependent RNA polymerase (RdRp) encoded by open reading frame 2 (ORF2) of Nigrospora oryzae nonsegmented RNA virus 1 (NoNRV1-NDC1) and that of other closely related unassigned mycoviruses.

Virus name	Identity (%)	Accession number
Nigrospora oryzae nonsegmented RNA virus 1	98.07	ALR87111.1
Nigrospora oryzae nonsegmented RNA virus 1	97.80	UJH94049.1
Ustilaginoidea virens nonsegmented virus 2	45.15	YP_009553680.1
Ustilaginoidea virens nonsegmented virus 2	45.09	YP_009553682.1
Purpureocillium lilacinum nonsegmented virus 1	45.09	AOO52900.1
Purpureocillium lilacinum nonsegmented virus 1	45.19	AOO52902.1
Conidiobolus non-segmented RNA virus 1	32.53	QKL20127.1
Phytophthora cactorum usti-like virus 1	30.51	QUA12647.1
Ustilaginoidea virens nonsegmented virus 1	31.18	AIE77248.1
Ustilaginoidea virens partitivirus 11	31.18	UVX28927.1
Ustilaginoidea virens nonsegmented virus 1	31.18	AIE77246.1

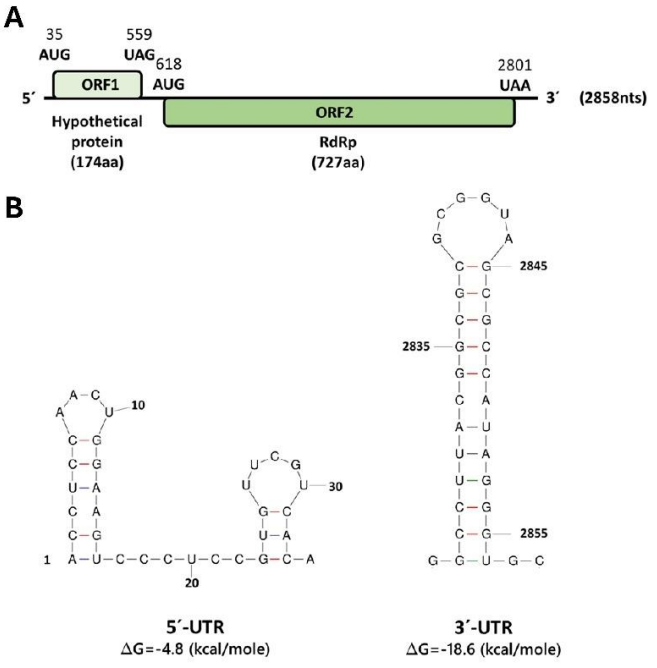

Figure 3 A. Schematic representation shows the genome organisation of the (+) strand of NoNRV1-NDC1. **B.** Potential predicted secondary structures of the 5′- and 3′-UTRs of the positive (+) strand of NoNRV1-NDC1. The RNAs were folded, and the free energy (ΔG) was estimated using MFOLD software (Mathews et al., 1999).

NoNRV1-NDC1 encoded proteins and their relatedness to other mycoviruses

NoNRV1-NDC1 genomic structure is analogous to that of *Amalgaviridae* and *Totiviridae* family viruses, with its RdRp being distantly related to those of *Partitiviridae* family viruses. The same structure is observed in other viruses of the same family, such as NoNRV1-HN-21 (Zhou et al., 2016), UvNV-2 (Zhang et al., 2014), and PlNV1 (Herrero, 2016). To explore the evolutionary relationship of NoNRV1-NDC1, a phylogenetic analysis was performed on the full-length amino acid sequences of RdRps of selected unclassified viruses and *Partitiviridae*, *Amalgaviridae*, *Chrysoviridae*, and *Totiviridae* families. Moreover, neighbour-joining analysis was also performed using the hypothetical protein encoded by ORF1 of NoNRV1-NDC1 and other related viruses. In the phylogenetic tree constructed based on the hypothetical protein of ORF1 in all related viruses, NoNRV1-NDC1 formed a cluster with different isolates of the same species (Zhou et al., 2016), PlNV1 (Herrero, 2016), and UvNV2 (Zhang et al., 2014) (Figure 4A). Unlike the viral RdRp, the hypothetical protein of NoNRV1-NDC1 was found only in a few unassigned viruses included in the phylogenetic tree. The phylogenetic analysis based on the multiple alignment of the RdRp of NoNRV1-NDC1 and those of other unassigned viruses and members of closely related viral families (Figure 4B) showed similar results. The phylogenetic analyses showed that NoNRV1-NDC1 forms a distinct clade, along with NoNRV1, PINV-1, UvNV-2, and other nonsegmented, unclassified viruses. The NoNRV1-NDC1 isolate reported in this study displayed molecular features similar to the previously characterised isolates of the same species. However, the RNA genome of isolate NDC1 was one nucleotide longer (2858 nts) compared to the genomes of isolates HN-21 and ZJ (2857 nts). The RdRps of isolates HN-21 and ZJ were closely related, sharing 99.4% identity, whereas their identity to isolate NDC1 was slightly lower (97.8% and 98.1%, respectively).

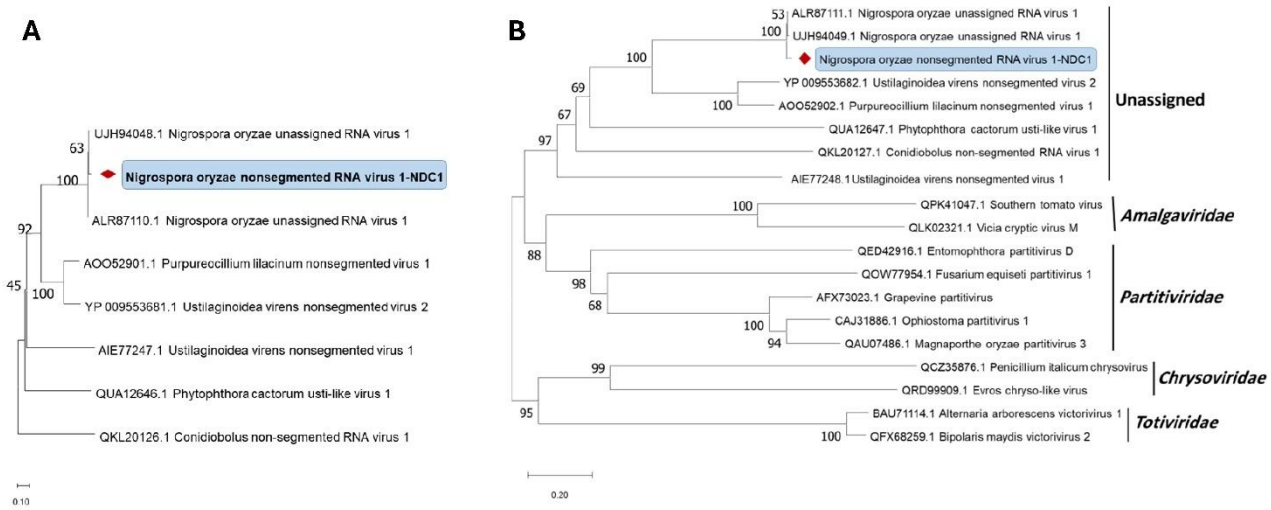

Figure 4 Phylogenetic relationship between the hypothetical protein encoded by ORF1 **(A)** and the RdRp encoded by ORF2 **(B)** of NoNRV1-NDC1 and other related viruses. The neighbour-joining trees were constructed using MEGAX software using the Poisson model and bootstrapping analysis of 1,000 replicates, as indicated by numbers on the branches.

NoNRV1-NDC1 RdRp comprises the aa conserved motifs of RdRp proteins, including the GDD region, which is highly conserved in motif C (Jablonski et al., 1991), according to multiple aa sequence alignments between NoNRV1 RdRp and similar sequences of closely related unassigned viruses (Figure 5). NoNRV1-NDC1 isolate and its closest relative, isolate HN-21, share similarities in their nonsegmented nature and totivirus-like genome organisation. They also have comparable genome lengths and 5' and 3' untranslated regions (UTRs) (Zhou et al., 2016).

In terms of diversity and geographical distribution, some mycoviruses display significant variation. For example, at least nine mitoviruses have been identified in *O. novo-ulmi* across Europe and North America (Doherty et al., 2006; Hintz et al., 2013). Similarly, *S. sclerotiorum* mitovirus isolates have been reported in strains from America, China, and Egypt (Xie & Ghabrial, 2012; Xu et al., 2015; Khalifa et al., 2022). Isolates of NoNRV1, including the one characterised in this study, have been reported in China (Zhou et al., 2016; Wang et al., 2022) and Egypt (this study).

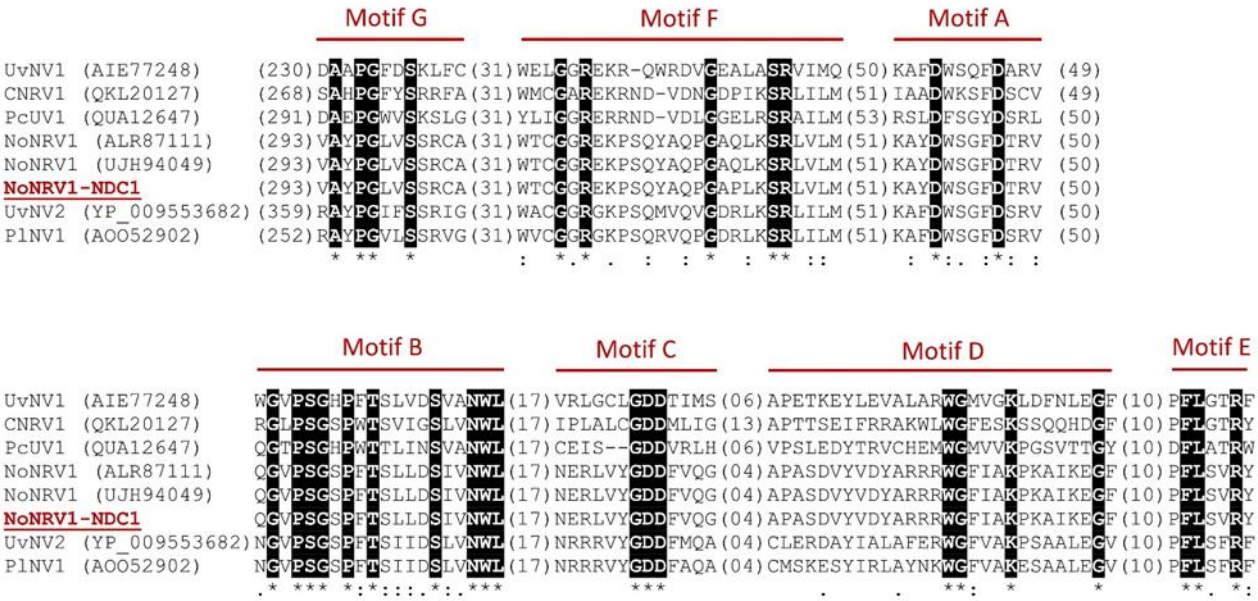

Figure 5 Conserved aa sequence motifs (A-G) of RdRps of NoNRV1-NDC1 and other viruses. Identical residues are highlighted in black and indicated by asterisks "*". Colons signify higher and lower chemically similar residues ":" and dots ".", respectively. Virus notations are UvNV1: Ustilaginoidea virens nonsegmented virus 1, CNRV1: Conidiobolus non-segmented RNA virus 1, PcUV1: Phytophthora cactorum usti-like virus 1, NoNRV1: Nigrospora oryzae nonsegmented RNA virus 1, UvNV2: Ustilaginoidea virens nonsegmented virus 2 and PlNV1: Purpureocillium lilacinum nonsegmented virus 1.

CONCLUSIONS

Growing data support the extensive distribution of mycoviruses among saprophytic and pathogenic fungi, including many plant-associated species. This work identified an isolate of Nigrospora oryzae nonsegmented RNA virus 1 (NoNRV1-NDC1) from an Egyptian isolate of *Nigrospora oryzae* (NDC1) collected from Damietta Governorate. Comprehensive genomic and phylogenetic analysis of the genome of NoNRV1-NDC1 revealed that it is similar to the Chinese isolate, supporting a similar evolutionary ancestry. As seen in other RNA viruses, a conserved slippery location near the ORF1 stop codon shows that the virus may translate its RdRp using -1 ribosomal frameshifting. This is the first report of NoNRV1 in *N. oryzae* from Egypt, broadening its host and geographical distribution. In previous investigations, NoNRV1 has been linked to changed host phenotypes. Future studies should examine the effects of NoNRV1-NDC1 on its fungal host, including growth, stress tolerance, and pathogenicity. The discovery of mycoviruses like NoNRV1 supports virus-based biocontrol strategies as ecologically friendly alternatives to chemical fungicides. Mycoviruses must be monitored and characterized in distinct fungal species from different regions to understand their ecological significance and possible usage in agriculture and biotechnology.

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