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Molecular Characterization of an Egyptian Isolate of Penicillium Chrysogenum Virus

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ABSTRACT

The number and diversity of reported mycoviruses have significantly increased with the utilization of next generation sequencing technologies. In the present study, an Egyptian isolate of a known mycovirus was identified in *Penicillium chrysogenum* isolated from Damietta governorate, Egypt. The genome of the identified mycovirus is divided into four double-stranded RNA (dsRNA) segments, three of which were completely sequenced and analyzed (dsRNA1; 3562 nts, dsRNA2; 3198 nts and dsRNA4; 2902 nts). The nucleotide sequence of the fourth segment (dsRNA3) has been partially identified. DsRNAs 1 and 2 encode RNA-dependent RNA polymerase (RdRp) and capsid protein (CP), respectively. The mycovirus of the current study was identified as an isolate of Penicillium chrysogenum virus (PcV) based on the high RdRp identity (99.37%) in BLASTX searches. The identified dsRNAs have conserved untranslated regions (UTRs) that were identical to those observed in other chrysoviruses. According to the phylogenetic study, PcV belongs to family *Chrysoviridae* genus *Alphachrysovirus*.

Keywords: dsRNA; Mycovirus; PcV; *Chrysoviridae*; *Penicillium chrysogenum.*

INTRODUCTION

Penicillium is a highly diverse and ubiquitous ascomycetous fungus that is a source of several enzymes and antibiotics and able to affect food industry by infecting food products and producing fungal toxins (Visagie *et al.*, 2014). One of the most studied species of this genus is *P. chrysogenum*, which has been widely used as a source of β-lactam antibiotic (Guzmán-Chávez *et al.*, 2018). Several mycoviruses have been reported to infect *Penicillium* species such as *P. chrysogenum* (Banks *et al.*, 1969), *P. digitatum* (Niu *et al.*, 2018*), P. crustosum* (Wang *et al.,* 2019) and *P. janthinellum* (Sato *et al*., 2020).

Mycoviruses have attracted much attention in recent years as they are widespread in the major taxa of fungi (Pearson *et al.,* 2009), one of the largest and most diverse kingdoms of microorganisms (Choi and Kim 2017). Among known mycoviruses, penicillium chrysogenum virus (PcV) is one of the early reported fungal viruses classified in the *Chrysoviridae* family (Jiang and Ghabrial 2004). Family *Chrysoviridae* harbors members with segmented dsRNA genomes, individually encapsidated within small isometric capsids of ~40 nm in diameter. The family includes two genera namely *Alphachrysovirus* (represented by PcV) and *Betachrysovirus* (represented by Botryosphaeria dothidea chrysovirus 1 (BdCV1; Kotta-Loizou *et al*., 2020). Alphachrysoviruses have genomes ranging in length from 8.9 to 13.1 kbp divided between three or four segments. In addition to fungi, plants and probably insects were reported as hosts of Alphachrysoviruses (Jiang and Ghabrial 2004; Kotta-Loizou *et al*., 2020; Zhang *et al*., 2017). On the other hand, betachrysoviruses have 10.9-16 kbp long segmented genomes of four, five or seven genomic segments (Kotta-Loizou *et al.,* 2020; Zhai *et al.,* 018). Except for some cinquechrysoviruses, which negatively affect their hosts (Urayama *et al.,* 2010); chrysoviruses are normally associated with latent infections. The present study aimed to determine the identity and molecular characteristics of a multi-segmented dsRNA viral genome isolated from an Egyptian isolate of *P. chrysogenum*.

MATERIALS AND METHODS

Isolation and idetification of *Penicillium chrysogenum*

Standard isolation procedures were employed to isolate associated fungi from a soil sample collected from New Damietta city, Damietta governorate, Egypt. Among the purified isolates, isolate designated D18 was identified by sequencing the internal transcribed spacer (ITS) region of its ribosomal DNA (rDNA) using primer pair ITS4/ITS5 (White *et al.,* 1990). As explained below in the results section, isolate D18 was identified as *P. chrysogenum*. During this study, isolate D18 was cultured and maintained on potato dextrose agar (PDA) media at 4^{o}C. For liquid cultures, fungal mycelia were grown on potato dextrose broth (PDB) media for 5-7 days at 25^{o}C.

Identification of Viral Infection

Purification and sequencing of dsRNAs associated with isolate D18 was carried out to confirm the viral infection of *P. chrysogenum*. DsRNAs were purified from 2 grams of D18 fungal mass based on the selective binding of dsRNAs to CF-11 cellulose in the presence of 16.5% ethanol, as previously described by Valverde *et al*., (1990). DsRNAs were run on 1% (w/v) agarose gel, in TAE buffer, pre-stained with RedSafe nucleic acid staining solution and visualized under UV light. Purified dsRNAs were collectively used as a template for random cDNA synthesis in a 2-step RT-PCR using primers Tag04-N (CTAACATAGGGAG-ATTGCNNNNNN) and Tag04 (CTAACATAGGG-AGATTGC) as described by Khalifa *et al*. (2016). Random dsDNA fragments of more than 100bp long were purified and sequenced using an Illumina HiSeq-

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2000 platform. Illumina reads were quality checked, trimmed and filtered as described by Khalifa *et al*. (2016). Filtered reads were initially de novo assembled using Geneious R11 software (https://www.geneious.com) and assembled contigs identified through BLASTX analysis against the non-redundant (nr) database of NCBI. Short sequence reads were mapped against the genome of the most closely related virus PcV (accession numbers AF296439, AF296440, AF296441 and AF296442).

Sequence and phylogenetic analysis

Open reading frames (ORFs) were detected using the ORFfinder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The terminal sequences of the viral genomic dsRNA segments were aligned and compared using MUSCLE sequence alignment software. To determine the conserved motifs and for phylogenetic analysis, amino acid (aa) sequences of RNA-dependent RNA-polymerase (RdRp) of chrysoviruses were aligned using MUSCLE sequence alignment software (Edgar 2004). Motifs were visualized and illustrated using Weblogo3 software (Crooks 2004). The neighbor joining phylogenetic tree was constructed using MEGA X software (Kumar *et al*., 2018).

RESULTS

Presence of dsRNA in isolate D18 of *P. chrysogenum*

The association of isolate D18 with dsRNA was tested by extracting dsRNA using cellulose chromategraphy and electrophoresis (Valverde *et al*., 1990). Following DNase treatment, multiple nucleic acid bands that range in size from ~2.3 to 3.2~ kb were observed (Figure 1). The nucleic acid bands were collectively gel-extracted and used as a template for random RT-PCR and sequencing. As shown below, the extracted dsRNA represents four segments of the same virus, the nucleotide (nt) sequences of three of which were complete, whereas the sequence of the fourth segment was only partially determined.

Sequence properties of D18-dsRNA

Initial *de novo* assembly of cDNA sequences (Illumina reads) amplified from dsRNA associated with isolate D18 revealed that the dsRNA consists of four segments. BLAST searches of the assembled sequences against the non-redundant protein sequences (nr) showed that D18-dsRNAs share high nucleotide sequence identities with *Penicillium* PcV. As a result, the four dsRNA segments presented in this paper represent the segmented genome of a PcV isolate (D18). Sequence reads were compared to previously sequenced PcV dsRNAs in GenBank, and sequences of PcV-D18 segments were deposited in GenBank with accession numbers OK032547, OK032548, OK032549, and OK032550, respectively.

The full-length nucleotide sequence of dsRNA1 is 3562 nts in length and encodes a single ORF that is 3354 nts long, starts at an AUG codon (nt positions 145-147) and terminates at a UAA codon (nt positions 3496-3498). The 5' and 3' untranslated regions (UTRs) of dsRNA1 are 144 and 64 nts, respectively. The nucleotide sequence of dsRNA1 shares 97.19 and 83.22% identities with PcV (accession number AF29-6440) and penicillium italicum chrysovirus (PiCV; accession number MK214381), respectively. DsRNA1-ORF has the potential to encode a 1117 aa long protein with an estimated molecular mass of 128.58 kDa. BLASTP search of the aa sequence of dsRNA1-ORF revealed that it shares identities with RdRp sequences of chrysoviruses. The RdRp aa sequence of PcV-D18 shared the highest identity with that of PcV (99.37%; accession number AAM95601) (Table 1).

The nucleotide sequence of dsRNA2 shares similarities with two chrysoviruses; PcV (97.19%; accession number AF296440) and PiCV (83.22%; accession number MK214381). The full-length dsRNA2 is 3198 nts long and encodes a 982 aa long protein (calculated molecular mass: 108.72 kDa) that shares high similarity with coat protein (CP) sequences of chrysoviruses including PcV (99.39 %; accession number YP_392483). The 2949 nts long CP-ORF starts and terminates with AUG and UAA codons at nt positions 157-159 and 3103-3105, respectively. UTRs of dsRNA2 have lengths of 156 and 93 nts at the 5' and 3' termini, respectively.

The nt sequence of PcV-D18 dsRNA3 was only partially determined. The 130 nts long sequenced portion shared 91.15% nt sequence identity, using BLASTN search, with the corresponding dsRNA stretch of segment 3 of PcV (accession number: AF296441) which encodes a functionally unknown protein. The nt sequence of the 5'-UTR of dsRNA3 was determined whereas that of the 3'-UTR was not.

DsRNA4 of PcV-D18 is 2902 nts long and also consist of a single ORF (2544 nts) that starts at nt positions 163-165 with an AUG codon, terminates at nt position 2704-2706 with a UAG codon and encodes a 847 aa long protein with calculated molecular mass of 94.8 kDa. DsRNA4 shared 97.35 and 84.16% nt sequence identities with PcV (accession number AF296439) and PiCV (accession number MK214383), respectively. The protein encoded by dsRNA4 is highly similar to that encoded by corresponding dsRNA segments of several chrysoviruses including that of dsRNA4 of PcV (99.17%; accession number YP_392485) which has an unknown function.

Terminal sequences of PcV-D18 dsRNAs

As shown in Figure (2), the 5' terminal sequences of the four dsRNA segments of PcV-D18 are highly conserved. The first 10 nts of the 5' UTRs of the four dsRNA segments are identical (GATAAAAAAA) and followed by several stretches of highly conserved nucleotides along the UTR sequences. Upstream of the initiation codon of the three fully-sequenced dsRNAs as well as the corresponding sequence of partially sequenced dsRNA3, stretches of CAA repeats $(CAA)^n$ were found. As the nucleotide sequence of the 3' UTR of dsRNA3 is missing, the 3' terminal sequences of the three remaining dsRNAs were compared and found to be highly conserved (Figure 2).

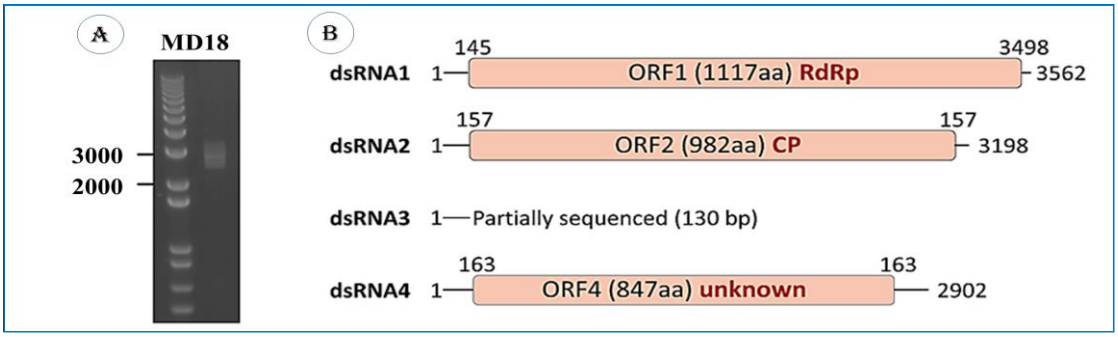

Figure (1): (A), Image of agarose gel electrophoresis shows the dsRNAs purified from *Penicillium chrysogenum* isolate D18; M: 1 kb plus marker (Invitrogen). (B), Schematic representation of the multisegmented genome of Penicillium chrysogenum virus (PcV-D18).

Phylogenetic identity of PcV-D18

Based on multiple aa sequence alignments between the RdRp of PcV-18 and those of other reported chrysoviruses, PcV-18 RdRp contained the aa conserved motifs I-VIII of dsRNAs (Figure 2). RdRp sequence identities between PcV-18 and other chrysoviruses are presented in Table (1). PcV-18 RdRp shared identities ranging from 29.58 to 99.37% with members of the *Alphachrysovirus* genus. The phylogenetic tree in Figure (3) shows that PcV-18 clusters with other *Penicillium* alphachrysoviruses in one clade.

DISCUSSION

Chrysoviridae is a family of viruses with non-enveloped isometric particles of ~40 nm that separately harbor multi-segmented genomic dsRNAs of 8.9-16 kbp. The multi-segmented genomes may consist of three (trichrysoviruses; (Li *et al.*, 2013), four (tetrachrysoviruses; Covelli *et al.*, 2004; Jiang and Ghabrial 2004), five (cinquechrysoviruses; Darissa *et al.*, 2011; Urayama *et al.*, 2012) or seven linear dsRNA segments (settechrysoviruses; Zhai *et al.*, 2018), with tetrachrysoviruses being the most commonly reported. PcV-18 reported in this study belongs to tetrarchrysoviruses which is the case for its most closely related viruses; PcV and PiCV (Jiang and Ghabrial 2004; Zhang *et al.*, 2019). Most members of *Chrysoviridae* have been reported from fungi, however plants and probably insects have been recently found to host chrysoviruses (Li *et al.*, 2013; Shi *et al.*, 2016; Zhang *et al.*, 2017).

Genomic dsRNA segments are assigned numbers (dsRNA1 to 7) based on their decreasing size and the proteins they encode are assigned the number of their corresponding dsRNA (Kotta-Loizou *et al.*, 2020; Zhai *et al.*, 2018). RdRp and CP of chrysoviruses, including those of PcV, are coded for by dsRNA1 and 2, respectively, whereas the rest of dsRNAs encode proteins with unknown functions (Kotta-Loizou *et al.*, 2020).

The 5′- and 3′-UTRs of the dsRNA segments are highly conserved and have the potential to fold into-

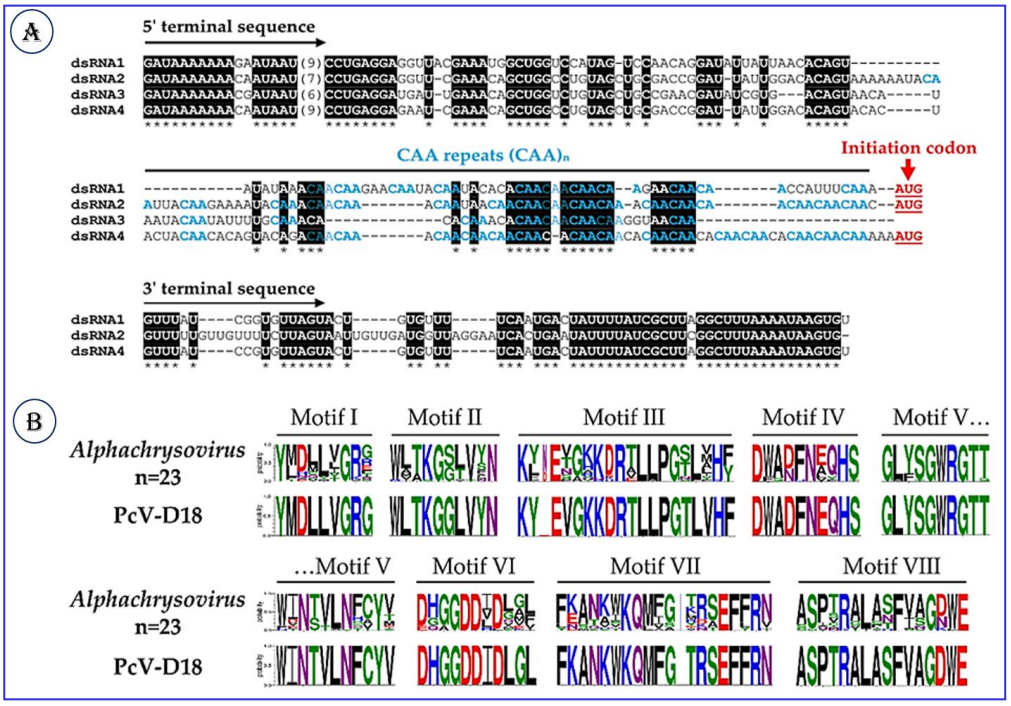

Figure (2): (A) Multiple nucleotide sequence alignment of the terminal sequences of PcV-D18 dsRNAs. Identical nucleotides are indicated by asterisks "*". (B) Amino acid (aa) sequence alignments of RNA-dependent RNA-polymerase (RdRp) sequences of Penicillium chrysogenum virus (PcV-D18) and other members of the same genus; *Alphachrysovirus*. Conserved motifs (I-VIII) of RdRps are indicated.

Table (1): Relation between Penicillium chrysogenum virus (PcV-D18), isolated in this study, and other members of the family *Chrysoviridae* based on the complete deduced percentage of amino acid sequences identity of the RdRP.

Genus	Virus	Abbreviation	Identity (%)	GenBank Accession No.
Alphachrysovirus	Amasya cherry disease associated chrysovirus	ACDACV	36.86	CAH03664
	Anthurium mosaic-associated virus	AMAV	29.58	ACU11563
	Aspergillus fumigatus chrysovirus	AfCV	70.15	BCH36618
	Beauveria bassiana chrysovirus 1	BbCV1	59.08	AZT88571
	Bipolaris maydis chrysovirus 1	BmCV1	37.79	ARM36035
	Brassica campestris chrysovirus 1	BcCV1	34.78	AKU48197
	Chrysothrix chrysovirus 1	CCV1	58.73	QGR26538
	Colletotrichum gloeosporioides chrysovirus 1	CgCV1	37.33	QCY49458
	Cryphonectria nitschkei chrysovirus 1	CnCV1	49.62	BBJ21307
	Fusarium oxysporum chrysovirus 1	FoCV1	40.33	ABQ53134
	Grapevine associated chrysovirus 1	GACV1	61.51	ADO60926
	Helminthosporium victoriae 145S virus	HvV145S	38.23	AAM68953
	Isaria javanica chrysovirus 1	IjCV1	59.78	APR73428
	Macrophomina phaseolina chrysovirus 1	MpCV1	54.01	ALD89090
	Penicillium chrysogenum virus	PcV	99.37	AAM95601
	Penicillium italicum chrysovirus	PiCV	98.75	QCZ35876
	Penicillium raistrickii chrysovirus 1	PrCV1	88.35	AZT88567
	Penicillium roseopurpureum chrysovirus 1	ProCV1	83.8	AYP71812
	Persea americana chrysovirus	PaCV	30.35	AJA37498
	Raphanus sativas chrysovirus 1	RsCV1	35.26	AFE83590
	Shuangao chryso-like virus 1	SCLV1	26.43	ASA47445
	Verticillium dahliae chrysovirus 1	VdCV1	46.36	ADG21213
Betachrysovirus	Alternaria alternata chrysovirus 1	AaCV1	18.5	BBC27878
	Aspergillus mycovirus 1816	AMV1816	20.26	ABX79996
	Aspergillus thermomutatus chrysovirus 1	AtCV1	19.54	AWC67507
	Botryosphaeria dothidea chrysovirus 1	BdCV1	18.59	AGZ84312
	Colletotrichum fructicola chrysovirus 1	CfCV1	19.58	AXP19674
	Coniothyrium diplodiella chrysovirus 1	CdCV1	19.6	QDB74971
	Fusarium graminearum dsRNA mycovirus 2	FgV2	17.26	ADW08802
	Magnaporthe oryzae chrysovirus 1	MoCV1	19.31	BAJ15133
	Penicillium janczewskii chrysovirus 1	PjCV1	19.62	ALO50142
	Penicillium janczewskii chrysovirus 2	PjCV2	19.52	ALO50149
	Tolypocladium cylindrosporum virus 2	TcV2	16.94	CBY84993
	Wuhan insect virus 29	WuIV29	17.74	APG76052

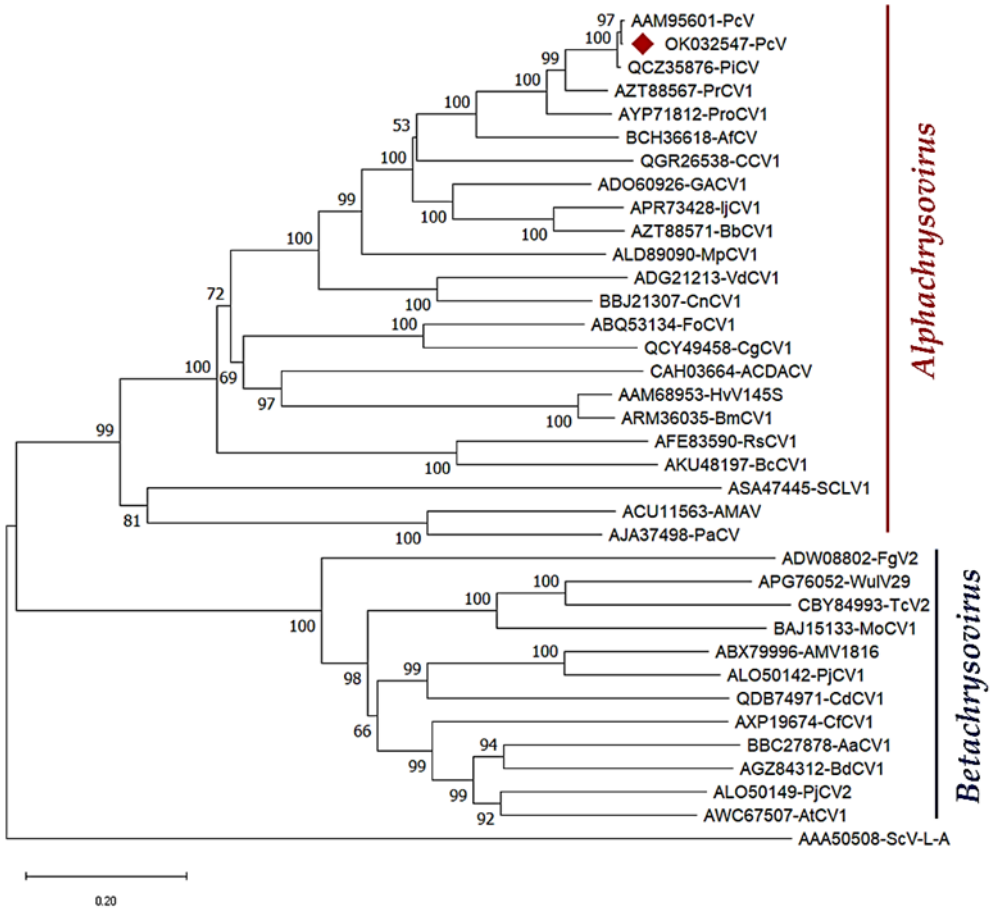

Figure (3): Neighbor-joining phylogenetic tree based on multiple alignments of RNA-dependent RNA-polymerase (RdRp) aa sequences of Penicillium chrysogenum virus (PcV-D18) and other chrysoviruses. RdRp sequence of Saccharomyces cerevisiae virus L-A (ScV-L-A) was used as outgroup to root the tree. The tree was displayed using MEGAX software using Poisson model. Values on the branches are the percentage of 1000 bootstrap replicates. Virus notations are as shown in Table (1).

stable stem-loop secondary structures. This conserve-ation is involved in packaging genomic RNA into the particles, virus replication and transcription (Anzola *et al*., 1987; Wei *et al*., 2003) in multipartite viruses including chrysoviruses. The conserved sequences of the 5′-UTRs are divided into three regions; a strictly conserved stretch of nucleotides at the terminal-most end, followed by a 40-75 bp conserved region known as Box1, downstream of which the third region of repeated CAA sequences (CAA repeat region) is present. The $(CAA)^n$ repeats present in the 5′-UTRs are a characteristic feature of most chrysoviruses and similar to those representing the enhancer elements found at the 5′-UTRs of tobamoviruses (Gallie and Walbot 1992). Those three regions were clearly identified in PcV-18 dsRNAs of the current study and are similar to those of other chrysoviruses.

Phylogenetic analysis of the RdRp aa sequences of dsRNA mycoviruses revealed that chrysoviruses are phylogenetically related to members of *Quadriviridae*, *Botybirnaviridae*, *Megabirnaviridae* and *Totiviridae* families. However, these families show differences in their virion and genome structures (Kotta-Loizou *et al*., 2020). Within *Chrysoviridae*, members are currently classified into two genera, namely *Alphachrysovirus* which accommodates 17 species with three or four genome segments and *Betachrysovirus* which accommodates eight species with four, five or seven genome segments (Kotta-Loizou *et al*., 2020). Phylogenetic analysis confirmed the identity of PcV-18 as an isolate of PcV (Jiang and Ghabrial 2004).

CONCLUSION

This study reported the molecular characteristics of an Egyptian isolate of PcV. The reported characteristics are in harmony with those reported for previously described chrysoviruses. To my knowledge, this study extends the geographical presence of PcV and chrysoviruses as it is the first report of a chrysovirus from Egypt. This report extends the distribution of mycoviruses (a chrysovirus in this study) to Egypt.

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التوصيف الجزيئي لعزلة مصرية من penicillium chrysogenum virus

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زاد عدد وتنوع فيروسات الفطريات بشكل كبير مع استخدام تقنيات الجيل التالي لتقنية تحديد التسلسل النيوكليوتيدي (next generation sequencing). في هذه الدراسة تم تعريف عزلة مصرية لفيروس فطري معروف من فطر *Penicillium chrysogenum* معزول من محافظة دمياط، مصر. ينقسم جينوم الفيروس الفطري الذي تم تعريفه إلى أربعة قطع RNA مزدوجة الشريط (dsRNA)، ثلاثة منها تم تحديد تسلسلها النيوكليوتيدي وتحليلها بالكامل (dsRNA1؛ 3562 نيوكليوتيدة، dsRNA2؛ 3198 نيوكليوتيدة و dsRNA4؛ 2902 نيوكليوتيدة). كما تم تحديد تسلسل النوكليوتيدات للجزء الرابع (dsRNA3) جزئيًا فقط. تقوم DsRNA 1و 2 بتشفير بوليميريز الحمض النووي الريبوزي (RdRp) والغلاف البروتيني (CP)، على التوالي. تم تعريف فيروس الفطريات في الدراسة الحالية كعزلة من فيروس Penicillium chrysogenum (PcV) بناءً على درجة تماثل RdRp العالية (99.37 ٪) في عمليات بحث BLASTX. وجد ان المناطق غير المترجمة (UTRs) من dsRNAs محافظ عليها (conserved) كما هو الحال في فيروسات مجموعه كريسو(chrysoviruses) الأخرى. بناءً على تحليل التطور الوراثي، ينتمي PcV إلى جنس *Alphachrysovirus* من عائلة *Chrysoviridae*.