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**Title:** A unique mitovirus from Glomeromycota, the phylum of arbuscular mycorrhizal fungi

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1 **Abstract**

2

3 Arbuscular mycorrhizal (AM) fungi that belong to the phylum Glomeromycota associate  
4 with most land plants and supply mineral nutrients to the host plants. One of the four viral  
5 segments found by deep-sequencing of dsRNA in the AM fungus *Rhizophagus clarus* strain  
6 RF1 showed similarity to mitoviruses and is characterized in this report. The genome  
7 segment is 2,895 nucleotides in length, and the largest ORF was predicted by applying either  
8 the mold mitochondrial or the universal genetic code. The ORF encodes a polypeptide of 820  
9 amino acids with a molecular mass of 91.2 kDa and conserves the domain of the mitovirus  
10 RdRp superfamily. Accordingly, the dsRNA was designated as *R. clarus* mitovirus 1 strain  
11 RF1 (RcMV1-RF1). Mitoviruses are localized exclusively in mitochondria and thus  
12 generally employ the mold mitochondrial genetic code. The distinct codon usage of  
13 RcMV1-RF1, however, suggests that the virus is potentially able to replicate not only in  
14 mitochondria but also in the cytoplasm. RcMV1-RF1 RdRp showed the highest similarity to  
15 the putative RdRp of a mitovirus-like ssRNA found in another AM fungus, followed by RdRp  
16 of a mitovirus in an ascomycotan ectomycorrhizal fungus. The three mitoviruses found in the  
17 three mycorrhizal fungi formed a deeply branching clade that is distinct from the two major  
18 clades in the genus *Mitovirus*.

## 19 Introduction

20

21 Arbuscular mycorrhizal (AM) fungi that belong to the phylum Glomeromycota associate  
22 with most land plants and supply mineral nutrients, in particular phosphorus, to the host  
23 plants through extensive hyphal networks constructed in the soil [15]. The plant-AM fungal  
24 symbiosis occurred more than 400 million years ago, and the coincidence of the appearances  
25 of early land plants and AM associations suggests that the associations were instrumental in  
26 the colonization of land by plants [14]. Although AM fungi have been playing a significant  
27 role in terrestrial ecosystems via enhancing P-cycling in the soil, biological characteristics of  
28 the fungi have been poorly understood due to their obligate biotrophic nature.

29         Members of the genus *Mitovirus* in the family Narnaviridae composed of a single  
30 genome segment of positive-sense RNA that encodes only RNA-dependent RNA  
31 polymerase (RdRp) [3]. Mitoviruses are localized exclusively in mitochondria of the host  
32 fungi, except for *Thanatephorus cucumeris* mitovirus that is potentially able to replicate  
33 both in the cytosol and mitochondria [6]. The infection of mitoviruses often causes  
34 malformation of mitochondria, which leads, in the case of plant pathogenic fungi, to  
35 debilitation in virulence [18] due to attenuation of mitochondrial function [12]. Accordingly,  
36 their possibility as a biological control agent has been studied extensively [1]. The impact of  
37 mitoviruses on AM symbiosis is also of interest, but no mitovirus has been described in the  
38 Glomeromycota so far.

39           One technical limitation for virological study in AM fungi was the difficulty in  
40 obtaining a sufficient amount of fungal material for characterization of viral genomes. We  
41 have established an open culture system for mass production of AM fungal mycelia and  
42 initiated virological studies of the fungi recently, in which four distinct dsRNA viruses,  
43 including a new class of virus, were described for the first time in the phylum [5]. In the  
44 present study, one dsRNA that was found to be similar to mitoviruses in the previous study  
45 is characterized with reference to the members of the genus *Mitovirus*.

46

#### 47 Provenance of the virus material

48

49 *Rhizophagus clarus* (Nicolson & Schenck) Walker & Schüßler strain RF1 (= *Glomus* sp.  
50 strain RF1) MAFF520086 was isolated by plant trap culture of *Petasites japonicus* subsp.  
51 *giganteus* grown in acidic soil in Hokkaido, Japan in 2005 [5] and has been maintained with  
52 sorghum and groundnut grown in a greenhouse. To obtain fungal material, the strain was  
53 grown with seedlings of *Lotus japonicus* cv. Miyakojima in the mesh bag-separated open  
54 culture system [2], and dsRNA was extracted from extraradical mycelia, purified, and  
55 electrophoresed [5]. Four dsRNA segments observed in the gel were excised from the gel,  
56 purified, and randomly amplified using the anchored-N6 primer according to Márquez et al  
57 [7]. The amplicons were directly sequenced by Roche 454 FLX GS Titanium using a  
58 1/8-scale gasket, and assembled. Among contigs obtained in the sequencing, an ORF of a

59 2.5-kbp contig showed similarity to RNA-dependent RNA polymerase (RdRp) of mitoviruses.  
60 The nucleotide sequence of the coding region of 2.5-kbp dsRNA was reconfirmed by  
61 sequencing three clones for each of two >1-kbp cDNAs obtained by nested RT-PCR, and the  
62 extreme ends were determined by sequencing three clones for each of three and two RACE  
63 products of the 5' and 3' ends, respectively (Supplementary Table S1 and Fig. S1). The  
64 sequences were analyzed and annotated with Artemis (Sangar Institute) and has been  
65 deposited in the DDBJ under accession no. AB558120. The amino acid (aa) sequence of  
66 predicted ORF was subjected to BLASTp searches and aligned with those of other  
67 mitoviruses using MUSCLE implemented in MEGA 5 [17]. Neighbor-joining (NJ) and  
68 maximum-likelihood (ML) trees were constructed with MEGA 5 for phylogenetic analysis.  
69 Four well-characterized mitoviruses and an uncharacterized mitovirus-like ssRNA were  
70 selected for comparative sequence analysis of the dsRNA of *R. clarus* RF1: TeMV found in  
71 the ectomycorrhizal fungus *Tuber excavatum* in Germany [16], CpMV found in a  
72 hypovirulent strain of the chestnut blight fungus *Cryphonectria parasitica* in USA [10],  
73 TcMV found in a hypovirulent strain of *Th. cucumeris* in USA [6], HmMV1-18 found in the  
74 violet root rot fungus *Helicobasidium mompa* in Japan [8], and an uncharacterized  
75 mitovirus-like ssRNA found in the AM fungus *Rhizophagus* sp. strain HR1 (= *Glomus* sp.  
76 strain HR1 [2]) (RMV-like ssRNA-HR1) in Japan.

77

## 78 Sequence properties

79

80 The sequencing of the RACE products revealed that complete genome of the dsRNA was  
81 2,895 nucleotides (nt) in length, which was approx. 400-nt longer than that predicted by the  
82 454 sequencing. Between a 297-nt 5' UTR and a 135-nt 3' UTR, the largest ORF (2,463 nt)  
83 was predicted by applying either the mold mitochondrial or the universal genetic code  
84 (Supplementary Fig. S2). The predicted ORF encodes a polypeptide of 820 amino acids (aa)  
85 with a molecular mass of 91.2 kDa and conserves the domain of mitovirus RdRp superfamily  
86 (Pfam PF05919), including the GDD motif (Fig. 1a). Accordingly, the dsRNA was  
87 designated as *R. clarus* mitovirus 1 strain RF1 (RcMV1-RF1). Generally functional RdRp in  
88 mitoviruses can be translated only if the mold mitochondrial genetic code is invoked [13].  
89 This is because tryptophan residues in mitovirus RdRps are usually encoded either by a  
90 UGA or a UGG codon, but the former codon encodes a translation terminator in the  
91 universal genetic code (in the cytosol). In fact, 55, 52, and 84% of tryptophan residues are  
92 encoded by the UGA codon in the RdRps of TeMV, CpMV, and HmMV1-18, respectively.  
93 On the other hand, all tryptophan residues in RcMV1-RF1 RdRp are encoded by the UGG  
94 codon (Supplementary Fig. S2, TGG in cDNA) as well as those in TcMV RdRp [6] and  
95 putative RdRp of RMV-like ssRNA-HR1 (data not shown), suggesting that functional RdRp  
96 could be translated both in the cytosol and in mitochondria. The codons for all tryptophan  
97 residues within the conserved domain of the selected mitoviruses are shown in Fig. 1b. The

98 RdRp aa sequence of RcMV1-RF1 shows high levels of similarity to those found in the two  
99 mycorrhizal fungi throughout the ORF: 34% identity to that of RMV-like ssRNA-HR1 at  
100 98% coverage and 28% identity to TeMV RdRp at 96% coverage. Significant similarity to  
101 TcMV RdRp in which all tryptophan residues are encoded by the UGG codon, however,  
102 was observed only within the conserved domain (43% identity at 23% coverage). The three  
103 RdRps of RcMV1-RF1, RMV-like ssRNA-HR1, and TeMV found in the mycorrhizal fungi  
104 form a subclade within the *Mitovirus* clade I [3] in the NJ-tree (Supplementary Fig. S3),  
105 although the node separating the clades I and II is poorly supported by a low-bootstrap value  
106 (28%). Whereas in the ML-tree the three viral sequences form a deeply branching clade with a  
107 bootstrap value of 99%, which is distinct from the two major clades (Fig. 2). A similar tree  
108 topology was also reported recently [4]. These observations suggest that the mitoviruses from  
109 the mycorrhizal fungi is likely to create the third distinct group in the genus.

110           The first member of *Mitovirus* in the Glomeromycota has been characterized in the  
111 present study. It seems likely that the distinct codon usage found in RcMV1-RF1 is a  
112 common feature of mitoviruses in AM fungi. The virus is potentially capable of replicating  
113 in the cytoplasm as well as in mitochondria. This might be an advantageous trait for  
114 horizontal transmission among the fungi, because those that belong to the same anastomosis  
115 group can exchange not only nuclei but also cytosol. Given the 400-million-year history of  
116 the close association of the fungi with plants, we also consider another possibility that  
117 ancestors of RcMV1-RF1 might be able to shuttle between the fungi and the host plant



118 during a certain stage of their evolution. This idea is supported by the evidence that RdRps  
119 of the members in the genus *Ourmiavirus*, plant ssRNA viruses, are phylogenetically related  
120 to those of the members in the Narnaviridae [11], suggesting that mitoviruses and  
121 ourmiaviruses diverged from a common ancestor. It is thus expected that more mitoviruses  
122 employing the universal genetic code will be found in AM fungi when their sequences  
123 become available.

124

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126

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129 Science (TE).

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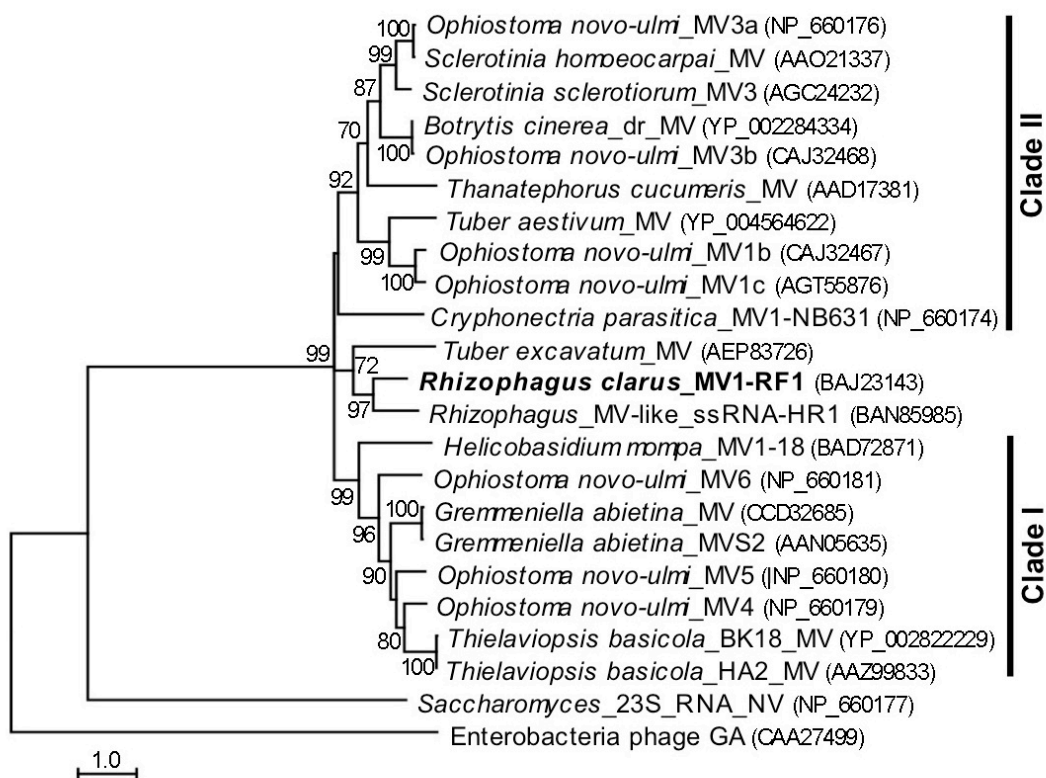
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- 182

<b>(a)</b>		(272)	(285) <b>I</b>				
RcMV1-RF1	264	GKLSVIEE <b>W</b> GGKARIVAQMDY <b>W</b> TQMALTP LHNTINHFLRALKEDGTFNQH					
RMV-like	276	GRLHSFEE <b>W</b> GGKTRVVAIVDY <b>W</b> TQILLTPLHNAIFHFLGNIPSDGTFDQD					
TcMV	336	SQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQE					
CpMV	219	GKLSVVYDQAGKARIVAITNS <b>W</b> IQTAFYSLHLHVFKLLKNIDQDGTDFDQE					
TeMV	280	SRLHLIFEKGIKSRIIAIGDYYSQCLLSPFMVTLRSCLECIPODYTYNQE					
HmMV1-18	255	ARLVCIPAPEGKSRIIGEMNF <b>W</b> AQCALKPLHDKEMKALRSIRQDLTFYQG					
	:::	* * : . .	: * : . .	* : . * * : *			
		(322)	<b>II</b>				
RcMV1-RF1	314	AIAERVRQ <b>W</b> TADPSMEVFSF <b>DL</b> TAATDRVPITFQESILSYLMTSKSFGNG					
RMV-like	326	AACARVAAF <b>TADANA</b> EVYSY <b>DL</b> TAATDRLPISIQREILEYLCP-KGFAGL					
TcMV	378	ASIARSQEKAVTAG-KAFSY <b>DL</b> TAATDRLPVILTAFILSTIVGIRTFGGGL					
CpMV	269	RPFKLLIK <b>W</b> LNEPTQKFYGF <b>DL</b> TAATDRLPIDLQVDILNIIFK-NSPGSS					
TeMV	330	AGFSKVLDF <b>T</b> -RLGKTCYSL <b>DL</b> SKATDRLPLALQERVMGII <b>LG</b> DSE <b>LARL</b>					
HmMV1-18	305	---IGPQVLKLHPGSKY <b>SF</b> DLKSATDRFPVELQEKVIQAFY <b>G</b> -EDFARS					
		::	** . **** . * : :	iii (395)	(404)		
RcMV1-RF1	364	<b>W</b> ASILVDREF-LTPN-GDLIS-----YNTGQ <b>PMGAR</b> SSF <b>PMLAL</b> THHI					
RMV-like	375	<b>W</b> ASLLVDRDY-FYASSG <b>TSYR</b> -----YAVGQ <b>PMGSK</b> SS <b>W</b> AMLAL <b>THHV</b>					
TcMV	435	<b>W</b> RSILVKRPF <b>GFNS</b> NVAEKLK <b>VSD</b> GPYFYEVG <b>QPMGAL</b> SS <b>W</b> PGLAL <b>THHW</b>					
CpMV	318	<b>W</b> RSLLRIKYK----SPQG <b>FLT</b> -----YAVGQ <b>PMGAY</b> SS <b>FAM</b> LAL <b>THHV</b>					
TeMV	379	<b>W</b> SSLSSREF--VTQ <b>TGH</b> KVR-----YEVGQ <b>PQGF</b> SS <b>FH</b> SLAM <b>THHV</b>					
HmMV1-18	351	<b>W</b> RS <b>LITDQAF</b> ---AYGESEVR-----YGC <b>GQPIGAY</b> SS <b>W</b> AT <b>FTL</b> CH <b>HM</b>					
	* * : :	:	* * * * *	* * : :	::: **		
		(417)	<b>IV</b>				
RcMV1-RF1	405	IVQIAAARAGLTV--YRDYV <b>VVLGDD</b> VTLTNAQVA <b>AHYQT</b> IMRCLGVPINL					
RMV-like	417	IVQASAASV <b>SSES</b> --YMDYALL <b>GDD</b> ITLTGSSIAKH <b>YLQHM</b> STLGVSINM					
TcMV	485	IVQVA <b>AFRV</b> TNSK <b>SW</b> NTEYE <b>ILGDD</b> IVIFNELIAQ <b>EYLN</b> IMAVIGCEINL					
CpMV	357	IVQVAALNSG <b>FTTR</b> -FTDY <b>CILGDD</b> IVIAHDTVASE <b>YLKLM</b> ETLGLS <b>ISS</b>					
TeMV	420	IVRLAAL <b>KAGEMN</b> --FTDY <b>VILGDD</b> IVLTNEK <b>VVKDYMI</b> IMELLGVKISL					
HmMV1-18	391	IVQ <b>MLCHRYR</b> APR---SHY <b>IILGDD</b> IVIAHDKVAEGY <b>CEIMRAL</b> SVDISD					
	** :	.	* * * * * : :	.. * * * : .			
<b>(b)</b>	(272)	(285)	(322)	(364)	(395)	(404)	(417)
RcMV1-RF1	<b>UGG</b>	<b>UGG</b>	<b>UGG</b>	<b>UGG</b>	UUC	AUU	---
RMV-like	<b>UGG</b>	<b>UGG</b>	UUU	<b>UGG</b>	<b>UGG</b>	GUC	---
TcMV	GCU	AUA	AAG	<b>UGG</b>	<b>UGG</b>	<b>UGG</b>	<b>UGG</b>
CpMV	CAA	<b>UGA</b>	<b>UGA</b>	<b>UGA</b>	UUC	GUG	---
TeMV	AAG	UAC	UUU	<b>UGA</b>	UUU	GUC	---
HmMV1-18	CCU	<b>UGA</b>	---	<b>UGA</b>	<b>UGG</b>	AUG	---

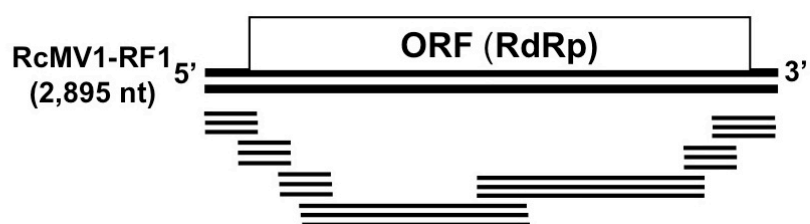
**Fig. 1 a)** Alignment of the conserved domain of *Mitovirus* RdRp in RcMV1-RF1. Motifs are labeled according to Poch et al. [9], and the consensus amino acids are written in bold letters. Numbers in parentheses represent the number of amino acid residue in RcMV1-RF1 RdRp. **b)** Codons for the tryptophan residues (W) corresponding to those indicated in the alignment

183

184



**Fig. 2** Phylogenetic position of RcMV1-RF1. Maximum-likelihood tree was constructed based on the amino acid sequences of mitovirus RdRP according to the JTT matrix-based model. Percentage bootstrap values (1000 replication) are indicated at the nodes. Two major clades (I and II) in *Mitovirus* are labeled according to Hillman and Cai [3]. Accession numbers are given in parentheses



**Supplementary Fig. S1** Genome structure of *R. clarus* mitovirus 1 strain RF1 (RcMV1-RF1). An ORF encoding RNA-dependent RNA polymerase (RdRp) was predicted. Relative positions and size of cDNA clones sequenced for confirmation are drawn below the genome

186

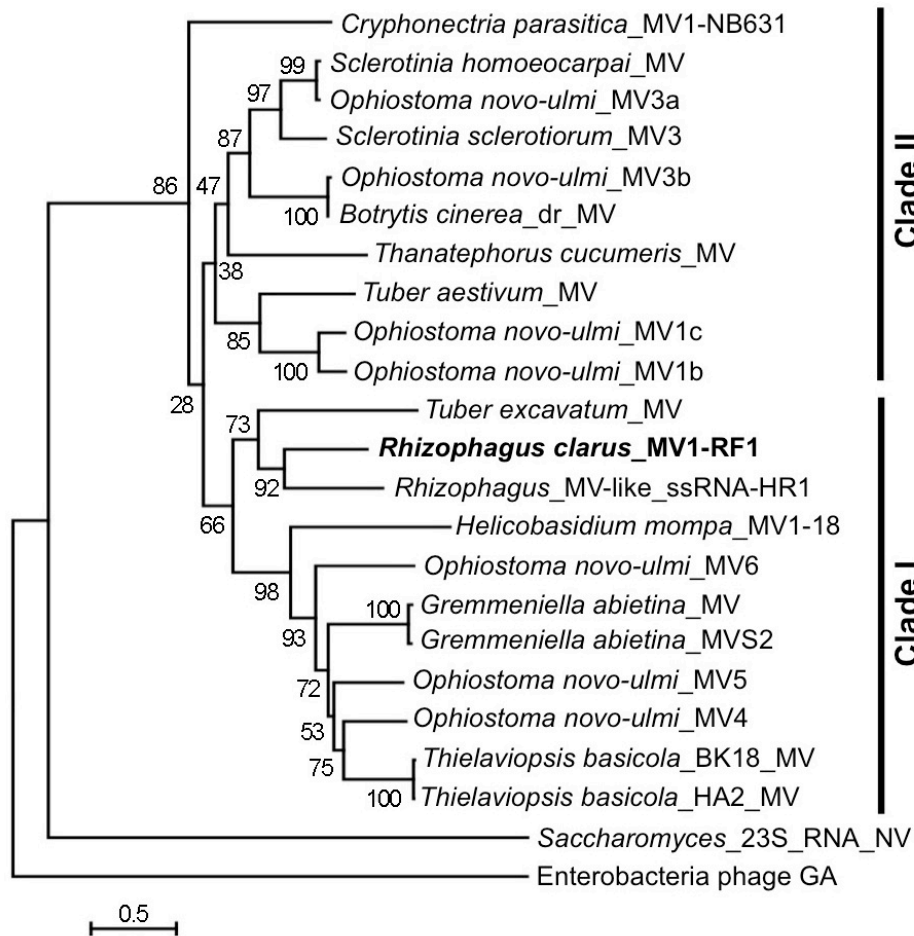
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 tgcacgttaagcagcggcctggtagcttggtagcttggtagctgacgctgacgctggagcc 180  
 ttatattcaaaagtagctttatagtgaccactcttacattatcctttcccaaaattccc 240  
 cggagcgcgagtgactctcactcactcaactcaatacattctacttgggtgctatg 300  
 M  
 tcaacatttaatttcaactctcctaatgaggtgagacgtgacacgctcctaacaatagct 360  
 S T F N F N S P N E V R R V T R S N I A  
 agcttggttgctgcctcctgcatctcaattctcttgaactgtaacttattctcaacgg 420  
 S L F A A L M H L N S L V T V T Y S Q R  
 ctcttggctttctgggagcagtgataatcgtattctctgctctttcgaatgccaacgca 480  
 L L A F L G A V Y N R I L V L F D A N A  
 cgttctcttatttccgagcttaagctcgttccggcgtgggtttcttgaattcctcgtaac 540  
 R S L I S E L K L V R R W F L E F I R N  
 gggaaactgataaccgggtctcgaatgggacaggtgggatgactcctaacaactgtccg 600  
 G N T D N P G L E W D R W D D S N N C P  
 gtactcttagaggtctcgaacgctcttgggatgagatagaatctccatcggactctcac 660  
 V L L E G L D A L W D E I E S P S D S H  
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 Y T D H A A Q L I F T L L S I D R I I V  
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 Y P T N E E L V N A L S S L H I D P A A  
 ttcaaggcctctataaccaacaggtacacgactctgattacagggctcttcaactagg 900  
 F K A F Y N Q Q V H D F D Y E V L S T R  
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 G P N G D A T W T A H L D A R A W A L D  
 actgagctttcagacgttttagtggcggctcagagagtcacgctgactcgtatctta 1020  
 T E L F R R F S A W L E E S R L T R I L  
 cgtgacctttcgggtgtattcgtcagcagcggctgaggctatccctaaccttagtccc 1080  
 R D L F G C I R S A A A E A I P N L S P  
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 I L G K L S V I E E W G G K A R I V A Q  
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 V P I T F Q E S I L S Y L M T S K S F G  
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 N G W A S I L V D R E F L T P N G D L I  
 tcttataactggccaaccaatggggcagcggctcattccctatggttggcactcag 1500  
 S Y N T G Q P M G A R S S F P M L A L T  
 catcatattatctgacagattgctcagcggcggcaggtcttaccgtatcagggattat 1560  
 H H I I V Q I A A A R A G L T V Y R D Y  
 gtcgtacttggtagcagatggtacattaactaacgctcaggtagcagccactaccagacg 1620  
 V V L G D D V T L T N A Q V A A H Y Q T  
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 I M R C L G V P I N L S K S I V H V D G  
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 G V S M A E I C K R V F M D G V E I S R  
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 F N P K L I V N V I R D G R L G P D L Q  
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 N D L I I R G W D P S N E V F W K F M A  
 ggtctcttctatcagataaacttactcttcttatacgccttaactgtgcaacctatctct 1920  
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 A S E A L K R L D G I L R A A V T I N D  
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 S L S I I A A A N A H P D R I P Q Y V R  
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 A R L G L L D V F R T S I S S I W L D D  
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 G N I R A G E S R S I F T R M L T T L V  
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 S L F T S E K R V S K S G R N L S L S Y  
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 cttgcaacttagttagtagtaagcacagtgtaataagccacacaagggcggagcataa 2880  
 ccggggttgaccct 2895

**Supplementary Fig. S2**  
 cDNA sequence of RcMV1-  
 RF1 and predicted ORF  
 according to either the  
 mitochondrial or the universal  
 genetic code. All tryptophan  
 residues are written in a red  
 letter, and their codons are  
 highlighted

188  
189





**Supplementary Fig. S3** Phylogenetic position of RCMV1-RF1. The Neighbor-joining tree was constructed based on the JTT matrix-based model. Percentage bootstrap values (1000 replication) are indicated at the nodes. Two major clades (I and II) in *Mitovirus* are labeled according to Hillman and Cai [3]. Accession numbers are indicated in Fig. 2