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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 (Sanger 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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- 181
- 182

(a)		(272)	(285) I					
RcMV1-RF1	264	GKLSVIEE W GGKARIVAQMDY W TQMALTP LHNTINHFLRALKEDGTFNQH						
RMV-like	276	GRLHSFEE W GGKTRVVAIVDY W TQILLTPLHNAIFHFLGNIPSDGTFDQD						
TcMV	336	SQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQE						
CpMV	219	GKLSVVYDQAGKARIVAITNS W IQTAFYSLHLHVFKLLKNIDQDGTDFDQE						
TeMV	280	SRLHLIFEKGIKSRIIAIGDYYSQCLLSPFMVTLRSCLECIPODYTYNQE						
HmMV1-18	255	ARLVCIPAPEGKSRIIGEMNF W AQCALKPLHDKEMKALRSIRQDLTFYQG						
	:::	* * : . .	: * : . .	* : . * * : *				
		(322)	II					
RcMV1-RF1	314	AIAERVRQ W TADPSMEVFS F DLTAATDRVPITFQESILSYLMTSKSFNGG						
RMV-like	326	AACARVAAFADANAEVYSY D DLTAATDRLPISIQREILEYLCP-KGFAGL						
TcMV	378	ASIARSQEKAVTAG-KAFSY D DLTAATDRLPVILTAFILSTIVGIRTFGGGL						
CpMV	269	RPFKLLIK W LNEPTQKFYGF D DLTAATDRLPIDLQVDILNIIFK-NSPGSS						
TeMV	330	AGFSKVLDFR-RLGKTCYSL D LKATDRLPLALQERVMGIILGDSELARL						
HmMV1-18	305	---IGPQVLKHLHPGSKY S FDLKSATDRFPVELQEKVIQAFYQ-EDFARS						
		::	** . **** . * : :	iii (395)	(404)			
RcMV1-RF1	364	WASILVDREF-LTPN-GDLIS-----YNTGQPM G ARSSFPMLALTHHI						
RMV-like	375	WASLLVDRDY-FYASSGTSYR-----YAVGQPM G SKSS W AMLALTHHV						
TcMV	435	WRSILVKRPFPGFNSNVAEKLKVS DGPYFYEVGQPM G ALSS W PGLALTHH W						
CpMV	318	WRSLLRIKYK----SPQGFLT-----YAVGQPM G AYSSFAMLALTHHV						
TeMV	379	WSSLLSSREF--VTQTGHKVR-----YEVGQ P Q G FKSSFHSLAMTHHV						
HmMV1-18	351	WRSLLITDQAF---AYGESEVR-----YGCQ P I G AYSS W ATFTLCHHM						
	* * : :	:	* * * * *	* * : :	::: **			
		(417)	IV					
RcMV1-RF1	405	IVQIAAARAGLTV--YRDYVVL G DDVTLTNAQVAAHYQTIMRCLGVPINL						
RMV-like	417	IVQASAASVSES--YMDYALL G DDITLTGSSI AKHYLQHMSTLGVSINM						
TcMV	485	IVQVAAFRVNTSKS W NTEYEIL G DDIVIFNELIAQEYLNIMAVIGCEINL						
CpMV	357	IVQVAALNSGFTTR-FTDYCIL G DDIVIAHDTVASEYLKLMETLGLSIS						
TeMV	420	IVRLAALKAGEMN--FTDYVIL G DDIVLTNEKVVKDYMIIMELLGVKISL						
HmMV1-18	391	IVQMLCHRYRAPR---SHYIIL G DDIVIAHDKVAEGYCEIMRALSVDISD						
	** :	.	* * * * * : . .	: . * * : . .	* .			
(b)		(272)	(285)	(322)	(364)	(395)	(404)	(417)
RcMV1-RF1		UGG	UGG	UGG	UGG	UUC	AUU	---
RMV-like		UGG	UGG	UUU	UGG	UGG	GUC	---
TcMV		GCU	AUA	AAG	UGG	UGG	UGG	UGG
CpMV		CAA	UGA	UGA	UGA	UUC	GUG	---
TeMV		AAG	UAC	UUU	UGA	UUU	GUC	---
HmMV1-18		CCU	UGA	---	UGA	UGG	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

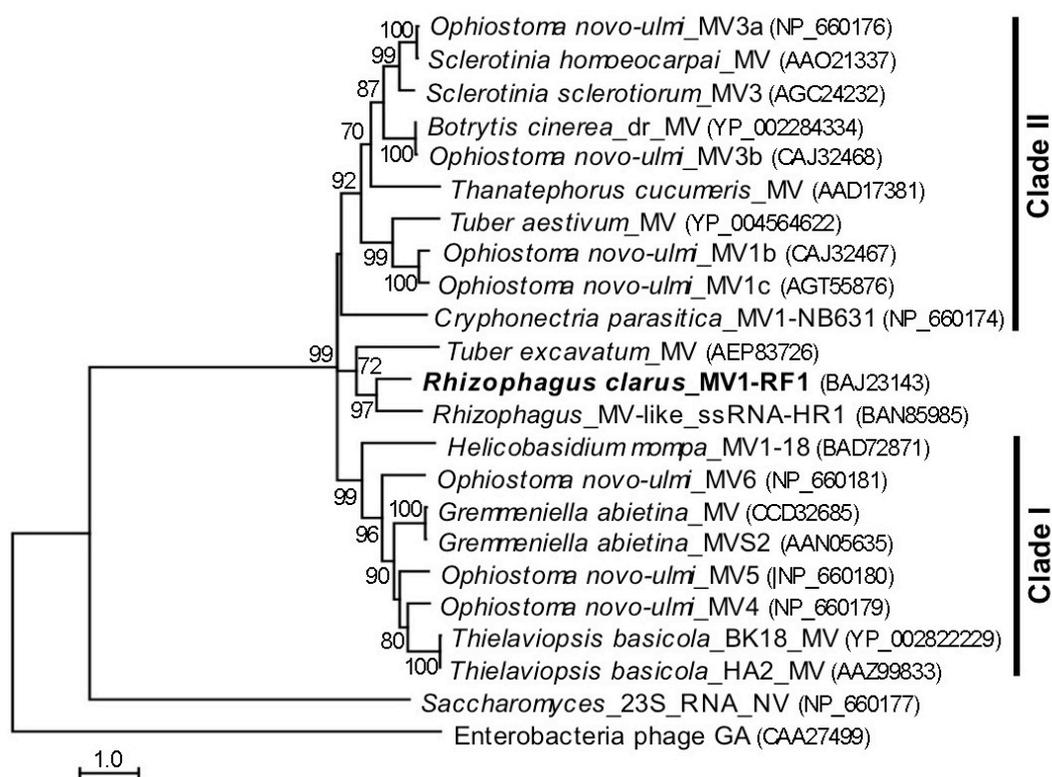
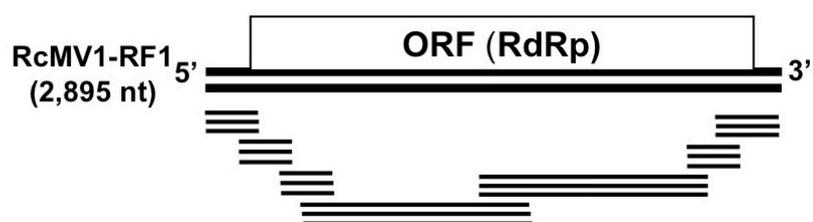


Fig. 2 Phylogenetic position of RcMV1-RF1. Maximum-likelihood tree was constructed based on the amino acid sequences of mitovirus RdRP according to the JTT matrix-based model. Percentage bootstrap values (1000 replication) are indicated at the nodes. Two major clades (I and II) in *Mitovirus* are labeled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

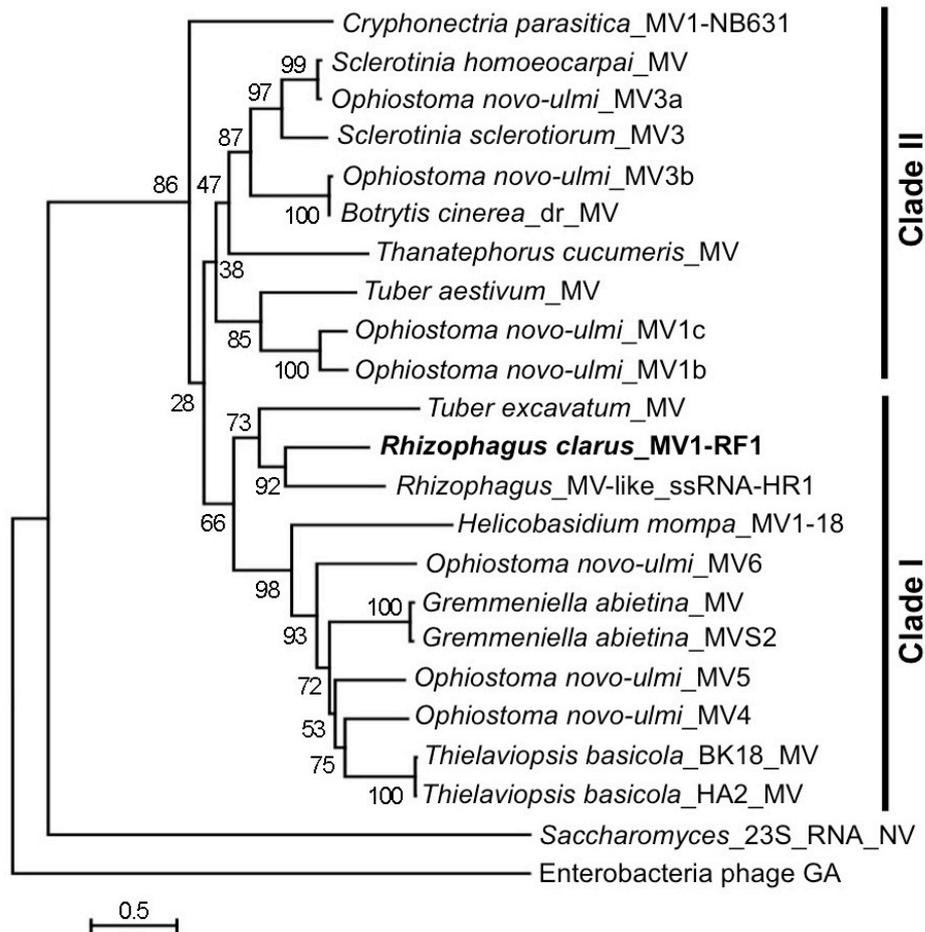
186

187

acgatgctacaagcggagaggttggacgcttgggtgataaaaagccaatcattctagtagg 60
 ttagtgcccgaacggatctcacaacactagtggggttgagcagacacggctcggtat 120
 tgcacgttaagcagcggcctggtagcttgggtgacgactgacgctgagcggagccca 180
 ttatattcaaaagtagctttatagtgaccactcttacattatcctttcccaaaattccc 240
 cggagcggcagtgactctcactcaactcaatacattctacttgggtgctatg 300
 M
 tcaacatttaattccaactctcctaatgaggtgagacgtgacacgctcctaacaatagct 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
 tatacggaccacggcagacgttatttactcttttactatagaccgtatcctcgtt 720
 Y T D H A A Q L I F T L L S I D R I I V
 gtactcgcagttcctaactattctactatagaggatggcctgtcattgaacctgtggc 780
 V P A V P N Y S T I E D G P V I E P V G
 tatctactaacgagagcttggtaacgctctctctctctctcatattgaccacggcc 840
 Y P T N E E L V N A L S S L H I D P A A
 ttcaagcctctataaccaacaggtacacgactctgattacagggttctttcaactagg 900
 F K A F Y N Q Q V H D F D Y E V L S T R
 ggccccaatgggatgctacgtggactgccatttagacggagagctgggctttggac 960
 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
 cgtgacctttcgggtgtattcgttcagcagcggctgaggctatccctaaccttagtccc 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
 atgactactggacgcaaatggctctcacacctctcacaataactatcaaccatttctt 1200
 M D Y W T Q M A L T P L H N T I N H F L
 cgcgctcttaagagagtgaccactttcaatcaacatgccatcgcagagcaggttcgtcag 1260
 R A L K E D G T F N Q H A I A E R V R Q
 tggacagcggatccgctcgtatggaggtctctctcttctcgtatttaactgtgactatcga 1320
 W T A D P S M E V F S F D L T A A T D R
 gtacctattactttccagagagtcacactcttcttcttcttcttcttcttcttcttctt 1380
 V P I T F Q E S I L S Y L M T S K S F G
 aacgggtgggaccttattctcgttagatagaggttcttcttcttcttcttcttcttctt 1440
 N G W A S I L V D R E F L T P N G D L I
 tcttataactggccaaccaatggggcagcggctcattccctatggttggcactacg 1500
 S Y N T G Q P M G A R S S F P M L A L T
 catcatattatcgtcagattgctcagcggcggcaggtcttaccgtatcagggattat 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
 ataatgggtgcttgggtgctcctatcaacctttctaagtctatagtcacggtggatgga 1680
 I M R C L G V P I N L S K S I V H V D G
 ggcgtatcaatggcgaatctgtaaacgtgttttcatggatggagtgagattagccgc 1740
 G V S M A E I C K R V F M D G V E I S R
 ttcaaccccaacttattgttaacgtcacaacgtcagcggctcgtttaggtcctgacctcag 1800
 F N P K L I V N V I R D G R L G P D L Q
 aacgatcttatttccgtgggtgggatccctcacaacgaggtattcgggaagtccatggct 1860
 N D L I I R G W D P S N E V F W K F M A
 ggtctcttctatcagataaacttactcttcttatacgccttaactgtgcaacctatctct 1920
 G L L S I D N L T L L I R L N C A P I S
 attactggctcttctcggcaatttgcctccaactcgaattggcacaactctcagcttgg 1980
 I T G L R Q F A S N S K L A Q L S A W
 atccggcctaccagatctcaaacggcaacatctagttgagctcttacttactcagctacc 2040
 I P A Y Q D L K P E H L V E L F T Y V T
 gcttctgaggtctgaaagcgttggacgtatcctacgtcggcgtgctactatataacgat 2100
 A S E A L K R L D G I L R A A V T I N D
 tctcttctatcagctgctcgaacgctcaccctgaccgtattccacagtagctacgg 2160
 S L S I I A A A N A H P D R I P Q Y V R
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 D T W L G E G L T K E E R A R L E G L I
 gctctacagcccccataactcctaaccatcctttagtatcagcttctcgtgcagaagct 2280
 A S T T G P I T P N H P L V S A S R A E A
 aaccgtattcggagctctcctcagctcaattcacatgatactgctattatcaccagg 2340
 N R I S E L L H Q L N S H D T A I I T R
 gccagacttggcttctggatggttccgtacttctatctcttcttatttggctcagcagat 2400
 A R L G L L D V F R T S I S S I W L D D
 ggtaacatcagagcgggtgagctcgttctatctcaccgcgtatgtaactactcttcta 2460
 G N I R A G E S R S I F T R M L T T L V
 tccctgttctactagtgaagcgggtatcgaatcaggacgtaactatcactgtcatac 2520
 S L F T S E K R V S K S G R N L S L S Y
 tcagttgttcaactcttctcgtctctggactgtagccttagactcggcggctcag 2580
 S V V L T S L S R L W T V A L D F G G Q
 gtaactgttaacgcacttctcgtcgaacgttactcgtgatccacaacgctgtggataac 2640
 V T V N A L R A N V T R D I H N A V D N
 ctgaaagccggagggctgctgttcttctctcttcttctcagtcctactacatct 2700
 L K A A E E A A V L I S S S V P T T S
 cctacgctcagctcctaaggcctcgcagacggcgtgctcagcaggttctcctaa 2760
 P T P A T P K G I R R R A F A R I S
 aaatccccccgggattctgagtaggggacttgaattttatcatagggggttctct 2820
 cttgcaacttagttagtagtaagcacagtgtaatgaaatccacacaagggcggagcataa 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

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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