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LETTER

A novel mitovirus from *Buergenerula spartinae* infecting the invasive species *Spartina alterniflora*

Dear Editor,

Spartina alterniflora, a coastal saltmarsh plant, has been classified as one of sixteen invasive alien species by the State Environmental Protection Administration of China. Nearly forty years of propagation and intrusion of this plant has resulted in some harmful effects on the economic development of coastal areas, due to its proliferation. Because *S. alterniflora* is an example of a prolific invasive plant species in coastal beach areas, its value in research is immense, and it is very beneficial to study the pathological characteristics of the plant. In our present work, we have isolated twenty-two fungal strains from the leaf and culm tissues of *S. alterniflora*, from the coastal beach areas of Jiangsu Province in China. According to analyses of rRNA-ITS sequences and of colony morphology, the strain YDC07 has been confirmed to be an isolate of *Buergenerula spartinae*. As reported some years ago (Gessner R V, 1977), *B. spartinae* exists on the leaves and culm of *S. alterniflora* (Supplementary information, Figure S1), consistent with the tropism of YDC07.

The majority of mycoviruses have single-stranded RNA (ssRNA) genomes, and others have genomes of double-stranded RNA (dsRNA) or DNA. But, in most cases, both ssRNA and dsRNA viruses form dsRNA intermediates during the infection process (Barnett O W, 1993). Mitoviruses belong to the genus *Mitovirus*, of the family *Narnaviridae* (Ghabrial S A, et al., 2009). To date, members of the genus *Mitovirus* are found only in fungi, and lack true virions. The genome is of approximately 2.5 kb, and contains only one ORF, with a low GC content, which encodes RdRp (Hong Y, et al., 1998. The potential stem-loop and panhandle secondary structures of the 5′- and 3′-UTR sequences of mitoviruses could be deduced from their genomes and mitoviruses are located and translated within the mitochondria, where they mostly exist as dsRNA replicative forms (Polashock J J, et al., 1994).

In this report, we have determined and analyzed the nucleotide sequences of one novel mycovirus, which encodes a distinct but related RdRp-like protein. Using phylogenetic analysis and northern blot analysis, evidence is presented for the existence of mitoviruses in fungal isolates obtained from *S. alterniflora*. In addition, the conserved motifs of its putative RdRp sequence have been analyzed, together with the secondary structures of the 5′- and 3′-UTR sequences.

Some fungi similar to *B. spartinae* were obtained from both culms and leaves of *S. alterniflora* plants that were exhibiting signs of diseases. All the plants were growing naturally across the Dafeng Port Coastal Beach, in the eastern part of Jiangsu Province, China.

Extraction of dsRNA from mycelia of strain YDC07 was conducted using the procedures described by Liu and coworkers (Liu W X, et al., 2009). A dsRNA was detected by electrophoresis on agarose gel (1%) and viewed on a UV trans-illuminator. The dsRNA was approximately 3.0 kbp in size, and was purified using an AxyPrep DNA Gel Extraction Kit (Axygen, Wujiang, China). The purified dsRNA was used as a template for the amplification of the complete genome, using a modified single-primer amplification technique (M-SPAT), as per our previous work (Chen L, et al., 2006). Primer A (5′-PO_4-TCTTCGGGTGTCCTTCCTCG-NH_2-3′) and primer B (5′-CGAGGAAGGACACCCGAAGA-3′) were used for cDNA synthesis. RT-PCR products were ligated to a pMD18-T vector (TaKaRa, Dalian, Japan) and the recombinants were transformed into competent *E. coli* DH5α cells. The identity of the cloned cDNAs was confirmed by restriction digestions with *Hin*d III and *Eco*l I. Three clones were sequenced to obtain a consensus sequence. The sequence of the ORF fragment was confirmed manually, and similar sequences were retrieved by NCBI BLAST programs. CLUSTALX was used for multiple alignments of sequences, and the results were displayed using GeneDoc 2.7. Phylogenetic analysis was performed using MEGA 4.0. Secondary structures of 5′- and 3′-UTR were predicted using the RNA structure 4.6 program.

The full genome of this dsRNA was determined to be 2735 nucleotides (nt) in length (Figure 1A), with a low G+C content of 39.49%, and the nucleotide composition was found to be 28.04% A, 19.23% C, 20.26% G, and 32.47% U. Analysis of the sequence revealed that one ORF, on one strand, contained nine UGA codons (Supplementary information, Figure S2). Most of mitoviruses in fungi have been reported to use UGA to encode

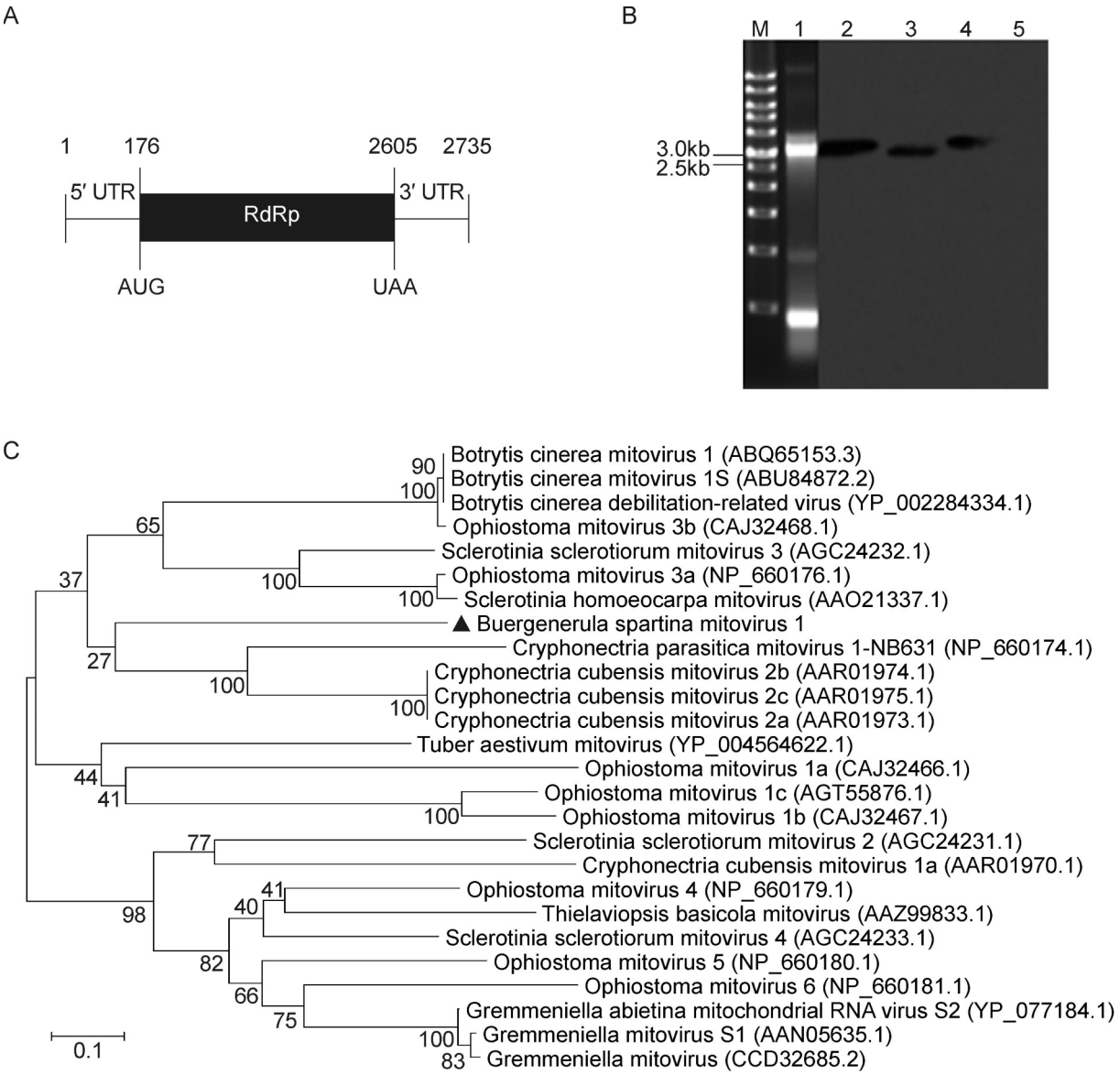
A
1
176
2605
2735
5′ UTR
RdRp
3′ UTR
AUG
UAA
B
M
1
2
3
4
5
3.0kb
2.5kb
C
Botrytis cinerea mitovirus 1 (ABQ65153.3)
Botrytis cinerea mitovirus 1S (ABU84872.2)
Botrytis cinerea debilitation-related virus (YP_002284334.1)
Ophiostoma mitovirus 3b (CAJ32468.1)
Sclerotinia sclerotiorum mitovirus 3 (AGC24232.1)
Ophiostoma mitovirus 3a (NP_660176.1)
Sclerotinia homoeocarpa mitovirus (AAO21337.1)
▲ Buergenerula spartina mitovirus 1
Cryphonectria parasitica mitovirus 1-NB631 (NP_660174.1)
Cryphonectria cubensis mitovirus 2b (AAR01974.1)
Cryphonectria cubensis mitovirus 2c (AAR01975.1)
Cryphonectria cubensis mitovirus 2a (AAR01973.1)
Tuber aestivum mitovirus (YP_004564622.1)
Ophiostoma mitovirus 1a (CAJ32466.1)
Ophiostoma mitovirus 1c (AGT55876.1)
Ophiostoma mitovirus 1b (CAJ32467.1)
Sclerotinia sclerotiorum mitovirus 2 (AGC24231.1)
Cryphonectria cubensis mitovirus 1a (AAR01970.1)
Ophiostoma mitovirus 4 (NP_660179.1)
Thielaviopsis basicola mitovirus (AAZ99833.1)
Sclerotinia sclerotiorum mitovirus 4 (AGC24233.1)
Ophiostoma mitovirus 5 (NP_660180.1)
Ophiostoma mitovirus 6 (NP_660181.1)
Gremmeniella abietina mitochondrial RNA virus S2 (YP_077184.1)
Gremmeniella mitovirus S1 (AAN05635.1)
Gremmeniella mitovirus (CCD32685.2)
0.1
D
5′-terminus
ΔG=−66.40(kal/mol)
3′-terminus
ΔG=−37.30(kal/mol)

Figure 1. A: Schematic representation of the genomic organization of the novel mitovirus infecting *B. spartinae*. B: M is a 500 bp Marker (Fermentas, SM0311); Lane 1 shows the total dsRNAs extracted from *B. spartinae* (YDC07); Lane 2 shows gel-purified viral genomic dsRNA; Lane 3 shows the PCR products with cloned plasmid DNA; Lane 4 shows the RT-PCR products of primer 1F and 1R; Lane 5 is the negative water control. C: Phylogenetic analysis based on the NJ method with 1000 bootstrap replicates, and performed with putative RdRps of members of the *Mitovirus* genus, including BsMV1. Numbers at the nodes of branches represent bootstrap values. Clade I is representative of viruses with a larger 5′-UTR, and clade II of viruses with a larger 3′-UTR. Accession numbers are given in parentheses. D: The predicted secondary structures of the 5′-terminus (nucleotides 1-175) and the 3′-terminus (nucleotides 2606–2735) of the novel virus.

tryptophan rather than as a translation terminator, and all the reported sequences of mitoviruses exhibit the same phenomenon (Cole C E, et al., 2000). Thus, we assume that the mitovirus contains a single ORF of 2429 nt, in which a mitochondrial codon is used (UGA=Trp), and that this ORF encodes the RdRp. Translation of the ORF may be initiated at the AUG codon at nucleotide position 176 and terminated at the UAA stop codon at nucleotide position 2605. A 175-nt-long 5′-UTR and 130-nt-long 3′-UTR were found to be located at each end, respectively, as depicted in Figure 1A. The deduced 810 aa sequence has a calculated molecular mass of 89.1 kDa; the highest degree of identity (43%) is shared with Cryphonectria cubensis mitovirus 2b(CcMV2b), and at least 35% aa sequence identity is shared with C. cubensis mitovirus isolates 2a, 2c (CcMV2a 2c) and with C. parasitica mitovirus 1 (CpMV1). Therefore, it appears that the 3.0kb RNA genome may be a new mycovirus that infects the fungus present on *S. alterniflora*. Based on the above, we tentatively designate the new virus as Buergenerula spartinae mitovirus 1 (BsMV1).

To confirm the virus derived from genome of mitovirus extracted from the strain YDC07 of *S. alterniflora*, northern blotting hybridization was conducted as previously described (Liu W X, et al., 2008), using biotin-labeled cDNA of BsMV1 as a probe. The specific primers for the probe template were 1F (AGCTGGTCAAGACAACACTCG) and 1R (ACTGTATCGGTAGTCTATCTGTCGC). The hybridization results for the probe with the viral genome, the plasmid PCR products, the purified dsRNA from YDC07 and the negative water control are shown in Supplementary Material 2-B. The results of the northern blotting hybridization indicated that the dsRNA was derived from the genome of mitoviruses.

From the results of the NCBI BLAST search, the putative protein was shown to be homologous to RdRp proteins from the genus *Mitovirus*, including those from CcMV2a, CcMV2b, CcMV2c and *Botrytis cinerea* debilitation-related virus (BcDRV), B. cinerea mitovirus 1 (BcMV1), Ophiostoma mitovirus 3b (OMV3b), O. mitovirus 3a (OMV3a), Sclerotinia homoeocarpa mitovirus (ShMV), and S. sclerotiorum mitovirus 3 (SsMV3).

As shown in Supplementary Figure S3, the putative RdRp of BsMV1 contains six conserved amino acid sequence motifs (I to VI) identical with those of the thirty-three members of the genus *Mitovirus* recognized by Poch et al (Poch O, et al., 1989). According to Hong et al (Hong Y, et al., 1999), motif I has been considered to be a characteristic of mitoviruses. Motifs II and VI are similar to RdRp motifs described in other viruses; the active-site, conserved GDD motif in motif IV is also present, which is found in other RNA virus genomes.

Phylogenetic analysis on the basis of RdRp sequences indicated that BsMV1 was clustered to a group containing eleven members in the genus *Mitovirus*, and was particularly closely related to CcMV2b, CcMV2c, CcMV2a and C. parasitica mitovirus 1-NB631(CpMV1-NB631), but far removed from two members, including Glomus sp. RF1 small virus (GsRF1SV) and Tuber excavatum mitovirus (TeMV), as shown in Supplementary Figure S3. The previous finding (Stielow B, et al., 2010) that some mitoviruses with either a larger 5′- or 3′-UTR are phylogenetically separated into two large clades is further supported by the present results and by the phylogenetic position of BsMV1 (in Figure 1C, clades I and II). The previous finding (Stielow B, et al., 2006) that closely related mitoviruses with either a larger 5′- or 3′-UTR are generally in the same clades, confirmed by the sequence properties of BsMV1 (Supplementary information, Table S1), is in agreement with the hypothesis (Mathews DH, et al., 2004) that mitoviruses might have evolved from two different ancestral viral precursors during fungal evolution.

The 5′- and 3′-UTR sequences of BsMV1 were examined for potential secondary structures using RNA structure software (version 4.6). The results showed that the 5′-UTR (nucleotides 1–175) could be folded into a stem-loop structure with a ΔG value of –66.40 kcal/mol. The 3′-UTR (nucleotides 2606–2735) could also be folded into a potentially stable stem-loop structure with a ΔG value of –37.30 kcal/mol. Stem-loop structures of the 5′- and 3′-UTR sequences are found in many RNA viruses (Hong Y, et al., 1998). These structures are considered to be a characteristic feature of fungal mitochondrial viruses, including the genus *Mitovirus*. Both the 3′- and 5′-UTR sequences of BsMV1 could form hairpin-like structures which might support RdRp recognition in the

process of virus replication (Kim J W, et al., 2003) (Figure 1D).

In conclusion, our work is the first to report a novel mitovirus from *B. spartinae*, a parasitic fungus from *S. alterniflora*. To date, the classification of mitoviruses is usually based on their genome sequences and on phylogenetic analyses. Research on BsMV1 offers potential for the improved control of *S. alterniflora*, and possibly provides a basis for effective utilization of resources.

FOOTNOTES

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Supplementary materials are available on the website of Virologica Sinica: http://www.virosin.org.

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