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Molecular and biological characterization of a novel botybirnavirus identified from a Pakistani isolate of *Alternaria alternata*

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ABSTRACT

Mycoviruses ubiquitously infect a wide range of fungal hosts in the world. The current study reports a novel double stranded RNA (dsRNA) virus, termed Alternaria alternata botybirnavirus 1 (AaBbV1), infecting a Pakistani strain, 4a, of a phytopathogenic ascomycetous fungus *Alternaria alternata*. A combined approach of next generation and conventional terminal end sequencing of the viral genome revealed that the virus is a distinct member of the genus *Botybirnavirus*. This virus comprised of two segments (dsRNA1 and dsRNA2) of sizes 6127 bp and 5860 bp respectively. The dsRNA1-encoded protein carrying the RNA-dependent RNA polymerase domain showed 61% identity to the counterpart of Botrytis porri botybirnavirus 1 and lower levels of amino acid similarity with those of other putative botybirnaviruses and the fungal dsRNA viruses such as members of the families *Totiviridae, Chrysoviridae* and *Megabirnaviridae*. The dsRNA2-encoded protein showed resemblance with corresponding proteins of botybirnaviruses. Electron microscopy showed AaBbV1 to form spherical particles of 40 nm in diameter. Biochemical analyses showed that two structural proteins encoded by dsRNA1 and dsRNA2 underwent processing to some extent during particle purification, resulting in the appearance of multiple smaller products. Phylogenetic analyses of structural proteins suggested that their coding region might have been duplicated once and maintained without recombination. Protoplast fusion technique allowed for the introduction of AaBbV1 into a virus free Japanese strain of *A. alternata* and demonstrated its symptomless infection by the virus. Interesting similarities and dissimilarities between AaBbV1 and other previously reported botybirnaviruses are also discussed.

1. Introduction

Fungal viruses (mycoviruses) are universally found in all major groups of fungi (Ghabrial et al., 2015). Presently 17 taxa, 16 families and one genus of mycoviruses are recognized by International Committee for the Taxonomy of Viruses (ICTV), including both linear double stranded (ds) RNA and single stranded (ss) RNA genomes, and circular ssDNA genomes. Mycoviruses with dsRNA genomes occur more frequently and are currently classified into six recognized families (*Totiviridae, Chrysoviridae, Partitiviridae, Reoviridae, Megabirnaviridae,* and *Quadriviridae*) and one recognized genus *Botybirnavirus*. Except for members of the family *Totiviridae* all have multi-segmented dsRNA genomes, including the two proposed families Alternaviridae and Polymycoviridae (Kotta-Loizou and Coutts, 2017; Sato et al., 2018). There are six families of mycoviruses or myco- and plant viruses with positive-sense ssRNA genomes, namely *Alphaflexiviridae, Gammaflexiviridae, Barnaviridae, Endornaviridae, Hypoviridae* and *Narnaviridae* (Ghabrial et al., 2015). The DNA mycovirus, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1, and the negative-sense ssRNA virus, Sclerotinia sclerotiorum negative-stranded RNA virus 1 are accommodated in the family *Genomoviridae* and *Mymonaviridae,* respectively (Liu et al., 2014; Yu et al., 2010).

Alternaria spp. are largely saprophytic, while some are endophytic or phytopathogenic (Thomma, 2003; Woudenberg et al., 2013). Pathogenic *Alternaria* spp. cause leaf spots, leaf rot and blight in many crop plants and their disease induction is often associated with non-

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host- and host-specific toxins (Akimitsu et al., 2014; Tsuge et al., 2013). *Alternaria* spp. are also well known as post-harvest pathogens (Tripathi and Dubey, 2004) and cause black spots in many fruits and vegetables worldwide during cold storage and marketing periods (Thomma, 2003). *A. alternata* has been reported to attack a large variety of agricultural products around the world including in Pakistan, China, and Australia (Alam et al., 2017; Bashar et al., 2012; Espinoza-Verduzco et al., 2012; Harteveld et al., 2013; Yan et al., 2015). In Pakistan, *Alternaria alternata* has been reported to cause postharvest losses of tomato by Akhtar et al. (1994 and 2004) and Fatima et al. (2009).

Several RNA mycoviruses have been identified and characterized in *Alternaria* spp. Unlike many other mycoviruses, some viruses of *A. alternata* have been reported to alter host phenotype. A tetra-segmented dsRNA virus, Alternaria alternata virus 1 appears to induce impaired growth of host colonies with enlarged vesicles in mycelial cells (Aoki et al., 2009). Another example is Alternaria alternata chrysovirus 1 that impairs the growth of its host fungus; on the other hand, it enhances the pathogenicity of the fungus against the plant (Okada et al., 2018). Other mycoviruses reported in *A. alternata* include unidentified dsRNA viruses (Hayashi et al., 1988; Shepherd, 1988), and Alternaria alternata partitivirus 1 (Xavier et al., 2018). Some other ds or ssRNA viruses have also been reported in *Alternaria* spp., such as Alternaria longipes dsRNA virus 1 (Lin et al., 2015), Alternaria botybirnavirus 1 (ABRV1) (Xiang et al., 2017), Alternaria brassicicola mitovirus 1 (Chen et al., 2017), Alternaria arborescens mitovirus 1 (Komatsu et al., 2016), Alternaria brassicicola fusarivirus 1 (Zhong et al., 2016), and Alternaria brassicicola endornavirus 1 (Shang et al., 2015).

In the present study, we identified a novel bi-segmented dsRNA virus, which is termed Altenaria alternata botybirnavirus 1 (AaBbV1), that belongs to a new species of the genus *Botybirnavirus* from a Pakistani isolate of *A. alternata* infecting tomato at a postharvest stage. Previously, one assigned and five potential members of the genus *Botybirnavirus* (Wu et al., 2012) have been reported. These include Botrytis porri botybirnavirus 1 (BpBRV1, formerly Botrytis porri RNA virus 1, the type member of the genus), Sclerotinia sclerotiorum botybirnavirus 1 (SsBRV1), soybean leaf-associated botybirnavirus 1 (SlaBRV1), Sclerotinia sclerotiorum botybirnavirus 2 (SsBRV2), ABRV1, and Bipolaris maydis botybirnavirus 1 (BmBRV1) (Liu et al., 2015; Marzano and Domier, 2016; Ran et al., 2016; Wu et al., 2012; Xiang et al., 2017). Here we describe the molecular and biological characterization of AalBbV1 that is more closely related to BpBRV1 than a previously reported *Alternaria* botybirnavirus, ABRV1, and shows asymptomatic infection in *A. alternata.*

2. Materials and methods

2.1. Fungal strain and growth conditions

A virus infecting *A. alternata* strain called 4a was collected from a diseased tomato fruit at the postharvest stage from Islamabad, Pakistan, which was identified by its morphology and Internal Transcribed Spacer (ITS) region sequence. A virus free Japanese strain of *A. alternata* f. sp *lycopersici* (Ally-12) (Taga and Murata, 1994) was kindly provided by Dr. Masatoki Taga. The fungal strains were grown on Difco™ potato dextrose agar (PDA; BD Difco Laboratories, Detroit, MI, USA) on a benchtop at 22–25 °C for 5 to 7 days for phenotypic observation or in Difco™ potato dextrose broth (PDB; BD Difco Laboratories) for purification of viral particles and spheroplast preparation.

2.2. DsRNA extraction and sequencing

DsRNA was extracted from fungal mycelia cultured on PDA overlaid with cellophane membrane (for aiding mycelial growth) by a method using cellulose (Advantech, Tokyo, Japan) as described by Eusebio-Cope and Suzuki (Eusebio-Cope and Suzuki, 2015). The dsRNAs were analyzed by electrophoretic mobility on 1% agarose gel. For removing traces of genomic DNA and ssRNA, the dsRNA preparation was treated with DNase I (Qiagen, Hilden, Germany) and SI nuclease (Takara, Shiga, Japan), respectively. A mixture of dsRNA (1.7 µg) including three independent dsRNA samples derived from *A. alternata* strain 4a and two other phytopathogenic fungi was used for next-generation sequencing (NGS). The cDNA library construction using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and subsequent deep cDNA sequencing on the Illumina platform (HiSeq 2500, 100 bp single-end reads) were performed by Macrogen Inc (Tokyo, Japan). After deep sequencing, the 30,684,321 reads were assembled *de novo* into 7836 contigs (~1000–17,010 nt in length) using CLC Genomics Workbench (version 11, CLC Bio-Qiagen). Assembled fragments were then subjected to local BLAST searches against the viral reference sequence dataset obtained from National Center for Biotechnology Information (NCBI). Virus-like sequences detected from the other two fungal strains will be reported elsewhere. To determine the 5′ and 3′ terminal sequences, 3′-RLM-RACE was performed as described previously (Suzuki et al., 2004). Briefly the 3′ termini of the two dsRNAs were ligated to a 3RACE adaptor (5′ phosphorylated oligodeoxynucleotide, 5′-PO_4-CAATACCTTCTGACCATGCAGTGACAGTCAGC ATG-3′) at 16 °C for 16–18 h using T4 RNA ligase (Takara). For cDNA synthesis, oligonucleotide 3RACE -1st (complementary to 3′ half of 3RACE adaptor, 5′-CATGCTGACTGTCACTGCAT-3′) was used with the ligated DNA-RNA strands denatured in 90% DMSO. A gene specific and 3RACE-2nd primer (complementary to 5′ half of 3RACE adaptor, 5′-TGCATGGTCAGAAGGTATTG-3′) was used for PCR amplification of cDNA. Amplified PCR products were ligated into pGEMT-Easy (Promega, Madison, WI, USA) for sequencing.

2.3. Bioinformatics analyses

Sequence analysis was performed using GENETYX DNA-processing software (SDC, Tokyo, Japan). To predict the Open Reading Frames (ORFs) in each of the cDNA sequences, the NCBI ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) with standard codon usage was utilized. Database searches of full length cDNA sequences were performed using the NCBI BLAST (BLASTn and BLASTp) program. For detection of any conserved domains, the NCBI conserved domain database (CDD) (Marchler-Bauer et al., 2017) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was utilized.

In order to perform phylogenetic analysis, the deduced protein sequence of virus and virus-like sequences were aligned using the online MAFFT server (version 7) (Katoh and Toh, 2008). Neighbor joining (NJ) trees were constructed based on the gap-free and/or moderately conserved amino acid alignments using MAAFT (Saitou and Nei, 1987) with 1000 bootstrap replications. The tree was visualized using FigTree (version 1.4.3) (http://tree.bio.ed.ac.uk/software/). In order to predict the possible secondary structure of the 3′ and 5′ terminal sequences of the positive-sense strands of two viral dsRNAs, RNA structure software (version 6.0.1) was employed (Mathews et al., 2004) (https://rna.urmc.rochester.edu/RNAstructure.html). The amino acid sequences of all botybirnaviruses were aligned to identify motifs in the deduced polypeptide using the online CLUSTAL Omega tool. For calculating proline frequency on the deduced polypeptides, Microsoft Office Excel was utilized as reported by Spear et al. (Spear et al., 2010). The proline frequency was determined, spanning a nine amino acid interval with a threshold value of 0.33 as the minimum frequency necessary to generate a left-handed polyproline II (PPII)-like helix (PxxPxxPxx).

2.4. Purification of virus particles

Virus particles were purified by a method described by Chiba et al. (2009). Fungal strains were cultured for 10 days in PDB or cellophane PDA. Approximately 6–10 *g* of fresh mycelia was ground to fine powder with the help of liquid nitrogen, followed by addition of 0.1 M phosphate buffer (pH 7.0), 0.1% (w/v) 2-merceptoethanol and two rounds

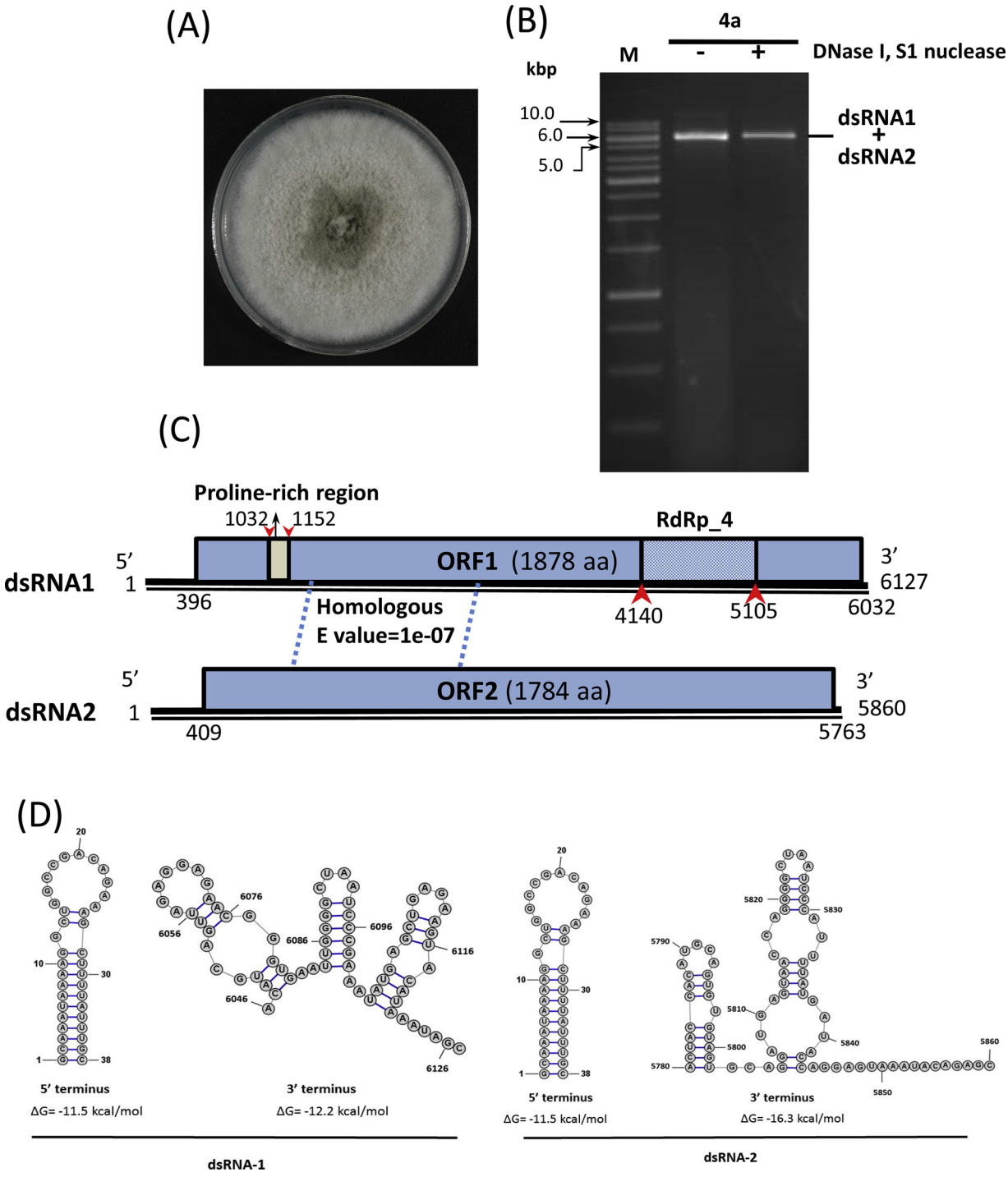

Fig. 1. Colony morphology of *Alternaria alternata* Pakistani strain 4a infected by Alternaria alternata botybirnavirus 1 (AaBbV1) and its genomic features. (A) Colony morphology of *A. alternate* 4a strain. The fungal isolate was grown on PDA for one week and photographed. (B) Agarose gel electrophoresis of dsRNA from strain 4a. A dsRNA fraction obtained from 4a with and without treatment by DNase I and SI nuclease was electrophoresed in 1% agarose gel. (C) Genomic organization of AaBbV1 with a bi-segmented genome. The positive strand of AaBbV1 dsRNA1 includes a 395-nt 5′ UTR, an ORF1 of 5637 nt (1878 aa residues) encoding a protein and a 95-nt 3′ UTR. An RdRp-4 superfamily domain was present at nt positions 4140–5105. A proline rich region was present at nt positions 1032–1152. The positive strand of AaBbV1 dsRNA-2 includes a 408-nt 5′ UTR, an ORF2 of 5355 nt (1784 aa residues) and a 97-nt 3′ UTR. A potential duplicated region of the structural proteins showing a moderate amino acid identity is denoted by dotted lines. (D) Predicted secondary structures for the terminal regions of the positive strands of dsRNA1 and dsRNA2 of AaBbV1.

of clarification with 20% carbon tetrachloride. The resulting supernatant was mixed with 1% (w/v) NaCl and 8% (w/v) PEG 6000 on ice for 2–3 h. Following centrifugation (10,000 × *g*) the pellet was suspended in 0.1 M phosphate buffer and insoluble matters were discarded by further centrifugation (6000 × *g*). The supernatant was layered onto a 20% (w/v) sucrose cushion, subjected to ultracentrifugation (100,000 × *g*) and subsequently fractioned with 20–50% (w/w) cesium chloride by ultracentrifugation (~150,000 × *g*). Virus containing fractions were precipitated in 0.05 M phosphate buffer, followed by ultracentrifugation (100,000 × *g*). The resulting pellet was re-suspended in 0.01 M

phosphate buffer and was further used for transmission electron microscopy (TEM), transfection, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mass fingerprinting (PMF).

After staining with an EM stain (EM stainer, an alternative for uranyl acetate, Nissin EM Co., Tokyo, Japan) (Nakakoshi et al., 2011), the virus particles were analyzed using a Hitachi H-7650 TEM (Tokyo, Japan). The proteins subjected to SDS-PAGE were denatured with modified Laemmli sample buffer containing 6% (v/v) of 2-mercaptoethanol at final concentration. The SDS-PAGE gels were stained with a Rapid Stain CBB Kit (Nacalai Tesque Inc., Kyoto, Japan) and Silver-Quest™ Silver Staining Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each protein band of interest was excised from the stained gels and subjected to in-gel tryptic digestion according to the protocol provided by the manufacturer (Bruker Daltonics Inc., Billerica, MA, USA). The digested peptides were subjected to LC−MS/MS analysis with the HPLC-Chip/QTOF system (Agilent Technologies Inc., Santa Clara, CA, USA) and identified by using Mascot software (Matrix Science Inc., Boston, MA, USA). The N-terminal sequence of each protein band, which was transferred to PVDF (Polyvinylidene difluoride) membranes with CAPS buffer (10 mM CAPS (pH 11.0), 10% (v/v) methanol), was analyzed using the PPSQ-31 A Protein Sequencer (Shimadzu Corp., Kyoto, Japan).

2.5. Protoplast preparation, transformation, fusion, and transfection

Protoplasts of the virus-infected and virus-free strains were prepared according to a method described by Eusebio-Cope and Suzuki (2015). Mycelia was harvested from a 4-day old liquid culture (PDB) and washed with 0.6 M $MgSO_4$, followed by digestion in a filter-sterilized enzyme osmoticum (1.2 M $MgSO_4$; 10 mM sodium phosphate (pH 5.8); 0.3% lysing enzyme (Sigma, St. Louis, MO, USA); 0.6% Bovine Serum Albumin (Sigma); 1% (w/v) beta-glucuronidase (Sigma)) at 28 °C overnight with gentle agitation. After filtering through nylon mesh, the protoplast solution was overlaid with trapping buffer (0.4 M sorbitol, 100 mM Tris − HCl (pH 7.0)) and subjected to centrifugation at 1200 × *g* for 15 min. at 4 °C. The protoplasts were collected at the interface, washed with 1 M sorbitol, then suspended in STC (1 M sorbitol, 100 mM $CaCl_2$, 100 mM Tris − HCl (pH 8.0)).

The protoplasts from the virus-free strain of *A. alternata*, Ally-12, were transfected with purified virus particles in a PEG-calcium chloride-mediated manner, as described by Hillman et al. (2004). Solid regeneration media was then used to regenerate the treated cells and colonies were then transferred to PDA. The virus-infected strain was used as a positive control for screening. Protoplasts of virus-free *A. alternata* strain Ally-12 were transformed with a vector, pCPXHY3, carrying a hygromycin resistance gene (hygromycin B phosphotransferase) as a selection marker, as previously described (Faruk et al., 2008). The protoplasts of the hygromycin-resistant, virus-free Ally-12 and virus-infected 4a strains, were fused by a method developed by Shahi et al. (2019). Virus-infected hygromycin-susceptible recipients were obtained after repeated co-culturing (hyphal anastomosis) with virus-free Ally-12 strain.

3. Results

3.1. Genome organization of Alternaria alternata botybirnavirus 1

The 4a strain manifested a grey colony growth typical of *A. alternata* on PDA (Fig. 1A) and was found to carry virus-like dsRNA agent (Fig. 1B). The dsRNA nature of this agent was confirmed after treatment with DNase I and SI nuclease (Fig. 1B). An NGS approach showed the presence of two virus-like contigs derived from different dsRNA moleules: contig numbers 21 and 114 with total-read counts of 237,090 and 146,884, and sizes of 5938 and 5551 bp, repectively. The larger fragment was designated dsRNA1 and smaller one as dsRNA2. The complete sequence of the two dsRNA segments was determined by a combination of NGS and RLM-RACE, as illustrated in the materials and methods. The dsRNA1 and dsRNA2 were 6127 and 5860 bp in length (Fig. 1C) and this dsRNA agent was tentatively named Alternaria alternata botybirnavirus 1 (AaBbV1). The complete dsRNA sequences were deposited in DDBJ/GenBank/EMBL with accession numbers LC437024 and LC437025, respectively. The 5′ untranslated regions (UTRs) of the positive strand of dsRNA1 and dsRNA2 were 395 and 408 nt in length, while the 3′ UTRs were 95 and 97 nt in length, respectively. Alignment of the 5′ and 3′ UTRs of the two AaBbV1 segments revealed a high degree of sequence identity with a strict conservation at the extreme terminals: 95.5% and 67.0% existed between the nucleotide sequences of the two segments at the 5′ and 3′ UTRs, respectively (Fig. S1). Furthermore, stem loop structures were predicted at the 5′ and 3′ terminal regions of both the AaBbV1 segments (Fig. 1D), which is thought to provide stability to the genome and may be involved in viral replication and assembly (Compel et al., 1999).

Two large single ORFs, ORF1 (nucleotide (nt) position 396 to 6032; molecular mass ˜211 kDa) and ORF2 (nt position 409 to 5763; molecular mass ˜197 kDa), of 1878 and 1784 aa in length were detected in the positive strand of dsRNA1 and dsRNA2, respectively (Fig. 1C). A proline-rich region was also present at the amino acid position 212 to 252 of the ORF1 protein (Fig. 1C, Fig. S2) corresponding to nt position 1032 to 1152, as reported for BpBRV1 ORF1 protein (Wu et al., 2012). No particular functional motif was found in the ORF2-encoded protein. The putative protein deduced from AaBbV1 ORF1 showed 61% sequence similarity with that of two botybirnaviruses (BpBRV1 and SsBRV3), and moderate (31–40%) or low levels of similarity (24–29%) were observed with that of other botybirnaviruses or several members of *Totiviridae, Megabirnaviridae, Chrysoviridae, Quadriviridae* and a few unassigned dsRNA mycoviruses under the BLASTp search (Table 1 and data not shown). The polypeptide encoded by AaBbV1 ORF2 shows 26–54% identity with the hypothetical proteins of BpBRV1 and other botybirnaviruses in BLASTp results (Table 1).

An RNA-dependent RNA polymerase (RdRp) superfamily domain (RdRp_4, pfam 02,123) was found to be present in the ORF1 protein at the amino acid position 1249–1570. All but Alternaria botybirnavirus 1 (ABRV1) encode RdRp on the larger segment dsRNA1. The RdRp domain of AaBbV1 contains all eight of the conserved RdRP motifs (including the GDD) that are observed in botybirnaviruses. (Fig. S3). Based on the predicted protein (cap-pol) sequence from dsRNA1 ORF1, a neighbor joining (NJ) (Fig. 2A) tree was generated. This phylogenetic analysis suggests a close relation of AaBbV1 to botybirnaviruses (Fig. 2A). A maximum likelihood tree displayed a similar phylogenetic relation (data not shown). In support of this notion, the phylogenetic analysis of the deduced ORF2 polypeptides from AaBbV1 and other botybirnaviruses also showed a similar phylogenetic relationship (Fig. 2B and see also the discussion section). Note that AaBbV1 ORF1 and ORF2 polypeptides showed the highest amino acid sequence identities (61% and 54%, respectively) with the corresponding proteins of the type member (BpBRV1) of the genus *Botybirnavirus* (Table 1 and Fig. 2B). In addition, both the 5′ and 3′ terminal sequences are highly conserved between AaBbV1 and BpBRV1 (Fig. S4). These results allowed us to conclude that AaBbV1 belongs to a new species of the genus *Botybirnavirus*.

3.2. Protein components of AaBbV1 particles

The purified virus particles of AaBbV1 were isometric, non-enveloped, and ~40 nm in diameter (Fig. 3A). The dsRNA from these particles had an identical agarose gel profile to the one extracted from mycelia (Fig. 3B). Virus particle preparations were obtained repeatedly from different fungal cultures. An SDS-PAGE pattern of one representative prepration is shown in Fig. 3C, which indicates that the AaBbV1 purified preparation contained at least five protein bands whose migration positions correspond to 110 kDa (p110), 80 kDa (p80), 73 kDa (p73), 70 kDa (p70) and 66 kDa (p66). Interestingly, the ratios

Table 1
Similarity between AaBbV1 and other botybirnavirueses.

Query	Virus Name	Coding segment	Accession Number	AA identity[a]	E-value[a]	Query cover[a]
AaBbV1	Botrytis porri botybirnavirus 1	dsRNA1	YP_006390636	61%	0.0	99%
ORF1 protein	Sclerotinia sclerotiorum botybirnavirus 3	dsRNA1	AWY10943	61%	0.0	99%
(dsRNA1)	Sclerotinia sclerotiorum botybirnavirus 1	dsRNA1	YP_009141011	31%	0.0	99%
	Bipolaris maydis botybirnavirus 1	dsRNA1	AVT42115	31%	0.0	98%
	Alternaria botybirnavirus 1	dsRNA2[b]	ARQ84133	31%	0.0	88%
	Sclerotinia sclerotiorum botybirnavirus 2	dsRNA1	AMT92139	40%	0.0	76%
	soybean leaf-associated botybirnavirus 1	n.d.[c]	ALM62244	40%	7e-170	40%
AaBbV1	Botrytis porri botybirnavirus 1	dsRNA2	YP_006390637	54%	0.0	99%
ORF2 protein	Sclerotinia sclerotiorum botybirnavirus 3	dsRNA2	AWY10944	55%	0.0	97%
(dsRNA2)	Sclerotinia sclerotiorum botybirnavirus 2	dsRNA2	AMT92140	27%	3e-98	68%
	Sclerotinia sclerotiorum botybirnavirus 1	dsRNA2	YP_009141012	27%	3e-83	68%
	Bipolaris maydis botybirnavirus 1	dsRNA2	AVT42114	26%	7e-81	66%
	Alternaria botybirnavirus 1	dsRNA1[b]	ARQ84132	27%	2e-77	68%
	soybean leaf-associated botybirnavirus 1	n.d.[c]	ALM62245	26%	3e-77	52%
	Sclerotinia sclerotiorum botybirnavirus 2	dsRNA1	AMT92139	32%	3e-10	7%

[a] Detected by blastp search.
[b] The CP-pol polypeptide is encoded by the smaller dsRNA segment (dsRNA2).
[c] Not determined.

among these proteins varied depending on the purified virus preparation, suggesting the degradation of structural proteins during virus prufication (Fig. 3D). For example, while prepartion 2 predominantly contained p110, p80 and p66, preparation 3 contained p66 as the major constituent. PMF of preparation 2 (Fig. 3D) showed that while peptide fragments derived from p110 and p80 were mapped to dsRNA2- and dsRNA1-encoded ORFs, respectively, peptide fragments derived from p73, p70 and p66 each were mapped to both ORF1 and ORF2-coded proteins (Fig. 4). Trypsin-digested peptides ("IDYLR", "QKTPR") were mapped to the AaBbV1 ORF1 protein at amino acid positions 1001 to 1005 and 1104 to 1108, respectively (Fig. S5). Although their assignment had relatively high ion values, the peptides likely derived from host fungi for a few reasons. First, no other pepetide fragments were mapped between these fragments and the second or third most C-terminal peptides (map position 823 to 844) on ORF1-coded protein (Fig. 4), where the trypsin recognition residues K and R, exist (data not shown). Second, the densely mapped regions shown in Fig. 4, which do not extend to the peptide at 823 to 844, were consistent with the molecular mass values of the structural proteins detected in SDS-PAGE gel (Fig. 4).

The proteins, p73, p70 and p66 were subjected to N-terminal sequence analyses. The results (Fig. S6) suggested the presence of N-terminally different proteins, as expected from the PMF analyses (Fig. 4). The sequence of p73 was determined to be "?IPVEMP" and those of p70 and 66 were likely "EYNNSGH" (Fig. S6).

3.3. Biological properties of AaBbV1

To investigate the effect of AaBbV1 on *A. alternata*, we attempted to cure the 4a strain of the virus using a variety of methods, including hyphal tipping and single spore isolation in the presence or absence of cycloheximide. However, our attempts were unsuccessful. Thus, a different approach, protoplast fusion, was taken to artificially introduce AaBbV1 into the Ally-12 virus-free strain of *A. alternata*.

The Pakistani 4a strain is slightly different from the Japanese Ally-12 strain (Fig. 1A vs. Fig. 5A). Of 20 subcultures obtained from the protoplast fusion procedure (Shahi et al., 2019), two cultures designated as Ally-12-V1 and Ally-12-V2 were found to be AaBbV1-positive and had the same dsRNA profile (Fig. 5B). Both of them showed colony morphology indistinguishable from each other (Fig. 5A and data not shown). Importantly, no significant difference was observed between these virus-infected strains and AaBbV1-free Ally-12, as shown in Fig. 5A. Note that both recipient strains carried AaBbV1 dsRNA like the Pakistani strain 4a (Fig. 1B vs. Fig. 5B). Ally-12-V1 and Ally-12-V2 are believed to have the same nuclear genetic background as Ally-12.

4. Discussion

In this study, we biologically and molecularly characterized a novel bi-segmented dsRNA botybirnavirus designated Alternaria alternata botybirnavirus 1 (AaBbV1) that infects *A. alternata.* The genome organization, sequence information of deduced polypeptides and phylogenetic analysis of the putative ORF1 (cap-pol polyprotein) suggest that the virus is comprised of a genome that would classify it as a member of the genus *Botybirnavirus*. Botybirnaviruses are characterized by: 1) spherical virus particles, 2) bi-segmented dsRNA genomes, 3) multiple (two) structural proteins encoded by two dsRNA segments, 4) a single large ORF on each segment and 5) long 5′-UTRs conserved between genome segments. AaBbV1 possesses all of these attributes and shows sequence similarities to previously reported botybirnaviruses, particularly the BpRV1 isolated from *Botrytis porri*, rather than ABRV1 that was isolated from *Alternaria* sp. (Figs. 1–3, S3–S4). These observations, together with the phylogenetic relation (Fig. 2), confirmed AaBbV1 to be the member of a new species of the established genus *Botybirnavirus*. To our knowledge, this is the first report of a well-characterized mycovirus from South Asia.

Recently, many fungal viruses, including dsRNA and positive-strand RNA viruses, have been shown to have internal ribosomal entry site (IRES) activities in their 5′-UTRs (Chiba et al., 2018). All botybirnaviruses sequenced thus far have long 5′-UTRs that are conserved between the two genomic segments of a botybirnavirus, as is the case for the megabirnaviruses (bi-segmented dsRNA genome) that have IRES activities in their extremely long 5′-UTR of 1.6 kb (A. Jamal, S. Chiba, and N. Suzuki, submitted elsewhere). In addition, the dsRNA1 and dsRNA2 segments of AaBbV1 have, respectively, 2 and 3 AUGs upstream of the AUG at position 396–398 and 409–411, which is the expected initiation codon for ORF1 and ORF2. These are the typical sequence signatures expected for an IRES. Further biochemical analyses are needed to demonstrate their IRES activities. In this regard, the recently developed single and dual-luciferase assay systems will be useful (Chiba et al., 2018; Guo et al., 2009).

To study the impact of AaBbV1 on its host *A. alternata*, we first attempted to cure the fungus of infection by several methods. The first attempt was made by treating the fungus by hyphal tipping with a protein synthesis inhibitor, cycloheximide, which was successful in elimination of some mycoviruses (Bhatti et al., 2011; Elias and Cotty, 1996). However, we failed to obtain virus-cured strains from the original AaBbV1-infected Pakistani *A. alternata* 4a strain using reported concentrations. Single spore isolation was also unsuccessful in producing virus-free isogenic lines of fungus (data not shown). The alternative approach is to introduce viruses of interest into virus-free strains,

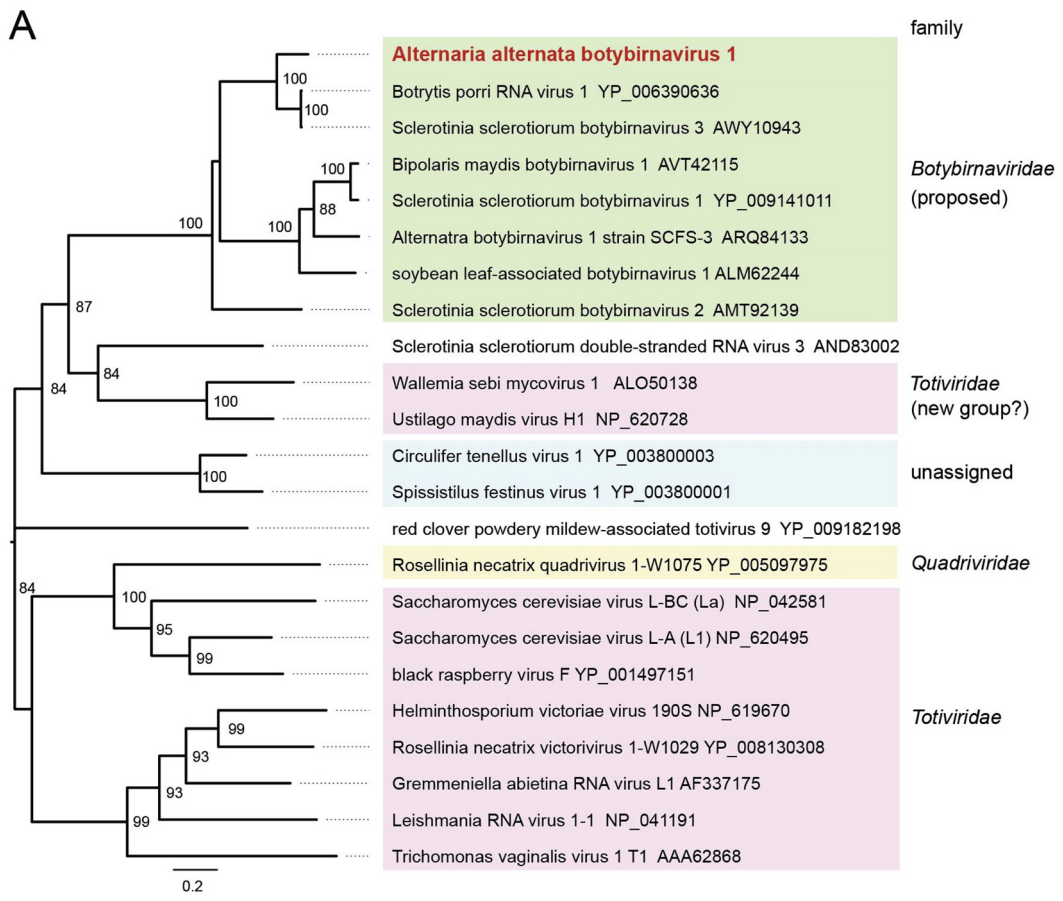

Fig. 2. Phylogenetic analysis of AaBbV1. Phylogenetic trees generated based on RNA-dependent RNA polymerase (A) and homologous regions encoded by dsRNA1 and two ORFs (B). Trees were made using the neighbor joining method with amino acid alignments of ORF1 polypeptides for (A) and ORF1 and ORF2 polypeptides for (B). Their alignments are available upon request. Numbers at the nodes are bootstrap values out of 1000 replicates. Bootstrap values lower than 70 are not shown in the figure.

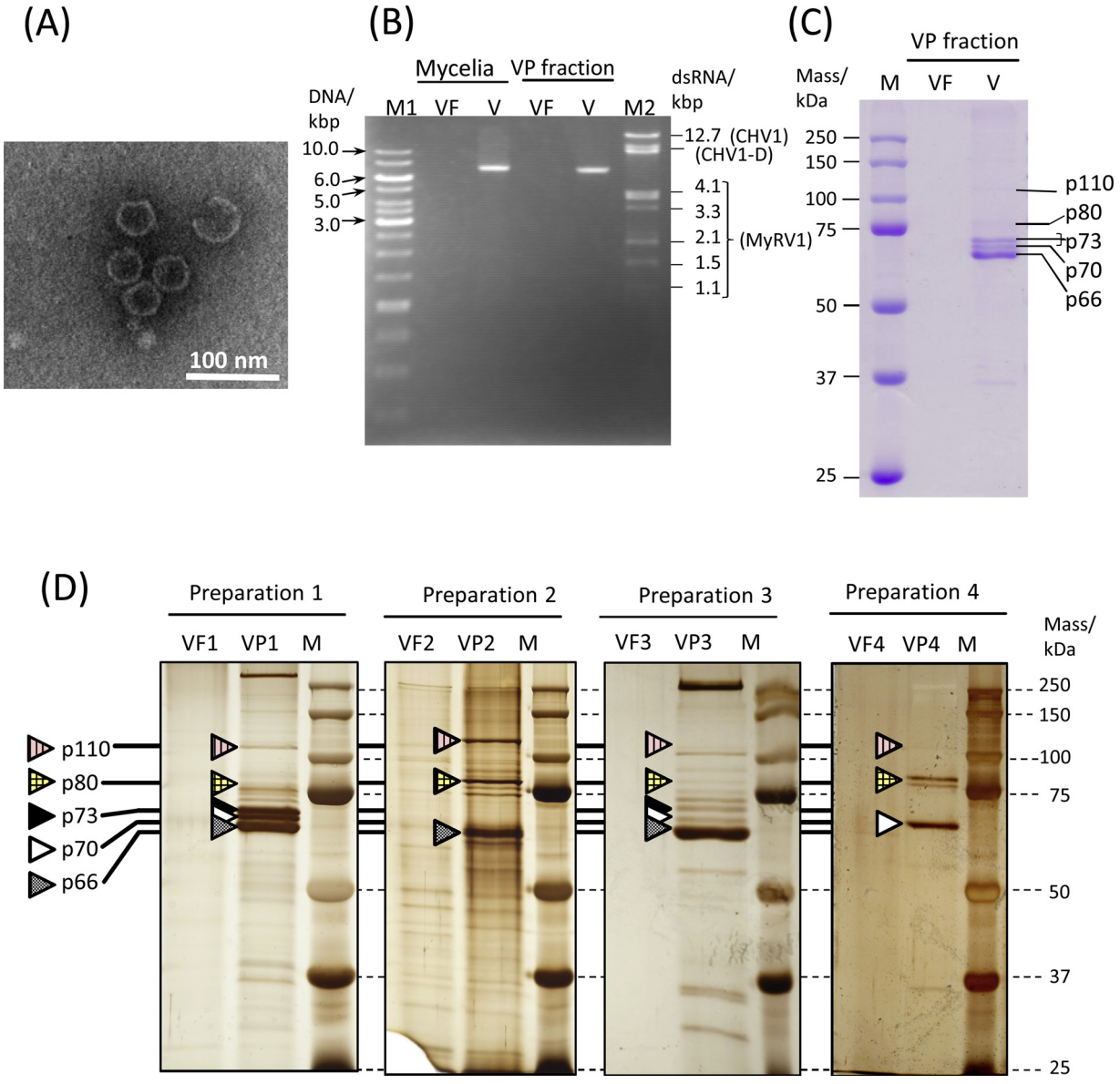

Fig. 3. Components of purified AaBbV1 particles. (A) Transmission electron micrograph of negatively stained AaBbV1 particles. (B) Agarose gel electrophoresis of dsRNAs extracted from mycelia and purified virus particles (VP) fractions. Virus particle and dsRNA fractions were obtained from the virus-free (VF) Ally-12 and AaBbV1-infected (V) 4a mycelia. M1: DNA size marker (GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific., Inc., Waltham, MA, USA)); M2: dsRNA size marker (mixture of dsRNA extracted from *Cryphonectria parasitica* strain EP155 infected with Cryphonectria hypovirus 1 (CHV1 and CHV1-D, a defective dsRNA segment) and mycoreovirus 1 (MyRV1)). (C) SDS-PAGE of viral proteins stained with Coomassie Brilliant Blue R-250. Proteins contained in VP fraction were denatured with modified Laemmli sample buffer containing 6% (v/v) 2-mercaptoethanol at final concentration and run on a 12% (w/v) polyacrylamide gel. The approximate size of viral proteins was estimated by relative mobility and is shown to the right of the gel image. (D) Instability of structural proteins of AaBbV1. AaBbV1 structural proteins were subjected to SDS-PAGE as above but strained by silver staining. The results of four independent preparations of AaBbV1 (preparations 1 to 4) are shown. Proteins contained in virus particle fractions purified from the AaBbV1-infected host (VP) and the virus-free (VF) control were run on 10% (w/v) polyacrylamide gels. The protein identifiers (p110, p80, p73, p70 and p66) shown on the left correspond to those in Figs. 3 and 4. The protein bands of each viral protein are indicated by triangles with different colors and/or patterns as shown on the left. The sizes of the standard protein markers (M: Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA)) are shown on the right (D) or left (C). to the left of the image.

for which a few methods are available. Virus can also be introduced by the well-established protocol for protoplast transfection (Chiba et al., 2009; Hillman et al., 2004; Sasaki et al., 2006). Unlike BpBRV1, SsBRV1 and SsBRV2 (Liu et al., 2015; Ran et al., 2016; Wu et al., 2012), the virus particles of AaBbV1 failed to be transfected into the Japanese *A. alternata* strain, Ally-12. Protoplast fusion was then used in this study. Note that this method is useful for horizontal transfer of other viruses into any host fungus (Lee et al., 2011; Shahi et al., 2019). Subsequently, we were able introduce the virus in the hygromycin-resistant virus-free transformant of *A. alternata* via protoplast fusion, and later a hygromycin-susceptible, virus-free strain via co-culturing. In this study we confirmed the replication competency of AaBbV1 in a different strain, Ally-12, of the same fungal species, *A. alternata,* in which the virus showed asymptomatic infection (Fig. 5). Consequently, the virus was shown to be asymptomatic in contrast to other botybirnaviruses, such as BpBRV1 and SsBRV2, that cause hypovirulence in *B. porri* and *Sclerotinia sclerotiorum*, respectively (Liu et al., 2015; Wu et al., 2012). Symptomless infections by botybirnaviruses are not unusual, as exemplified by SsBRV1 in the absence of a satellite-like dsRNA segment (Liu et al., 2015). These results clearly demonstrate that symptom induction depends on virus-host combinations, as is the case for others (Chiba et al., 2013, 2016). It will be of interest to

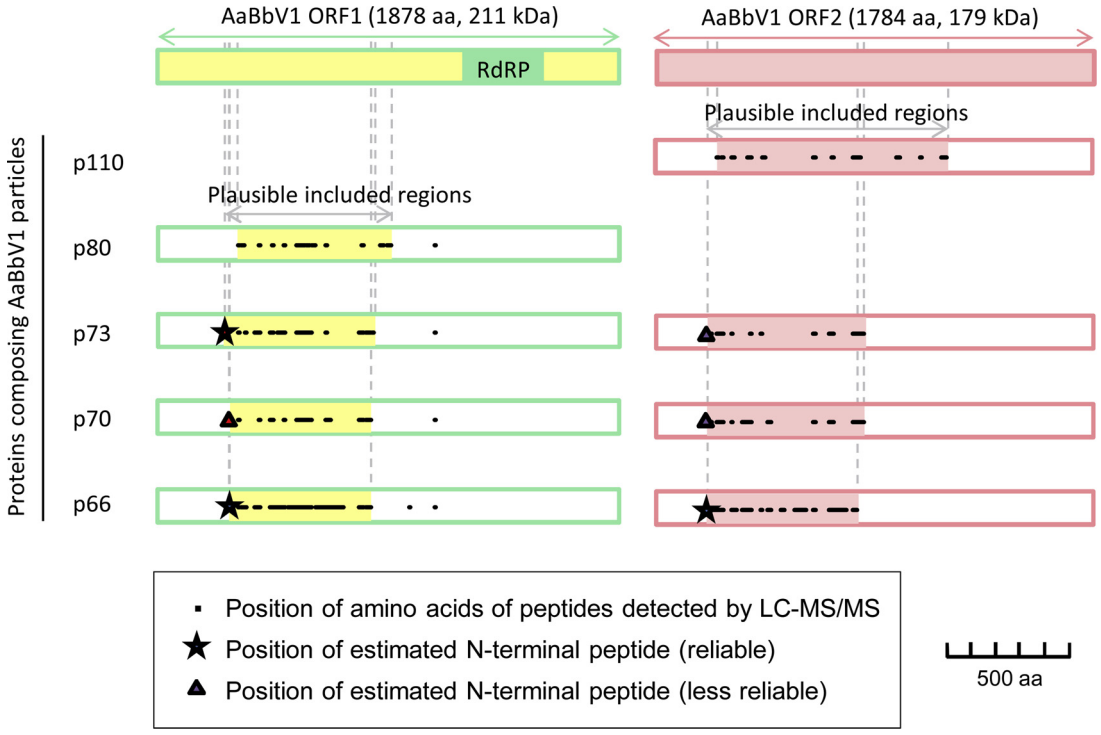

Fig. 4. Mapping of the coding region of AaBbV1 structural proteins. Proteins in purified virus particle fractions were subjected to in-gel tryptic digestion followed by LC–MS/MS analysis. Predicted coding regions for p110, p80, p73, p70 and p66 (see also Fig. 3C and D for each protein band) are shown in the diagram for the AaBbV1 ORFs. The yellow boxes with green frames and the pink boxes with deep pink frames represent ORF1 (dsRNA1) and ORF2 (dsRNA2) of AaBbV1, respectively. The green box in ORF1 shows the position of the RdRp domain. Dots in boxes indicate the positions of peptide fragments detected by LC–MS/MS (see Fig. S5 for their sequences). The predicted N-terminal positions of p73, p70 and p66 are indicated by stars or triangles, which express relatively reliable or less reliable N-terminal positions, respectively. Plausible coding regions for polypeptides p73, p70 and p66 are colored with yellow (ORF1) or pink (ORF2). The N-terminal sequencing and LC–MS/MS analyses suggest that p73, p70 and p66 each contained proteins encoded by the two segments.

examine the host range of the botybirnavirus, given the fact that there is no report on systematic investigation of their host ranges. In this regard, it is of note that BpBRV1 (SsBRV3) isolated from *B. porri* was also identified in an Australian isolate of *S. sclerotiorum* closely related to the genus *Botrytis* (Mu et al., 2017).

The virus particles of AaBbV1 are ~40 nm in diameter, which was similar to closely related ABRV1 (~40 nm particle size) and BpBRV1 (~35 nm), and to the members of the families *Chrysoviridae* and *Totiviridae* (both of which have isometric particles of 30–40 nm in diameter with multi-segmented and unsegmented dsRNA genomes, respectively). All fungal icosahedral viruses are considered to have $T = 1$ lattice composed of 60 asymmetrical homodimers (Luque et al., 2018; Sato et al., 2018). The capsid of chrysoviruses are exceptionally made up by 60 capsid protein monomers, with two duplicated intramolecular helical domains (Caston et al., 2003; Gomez-Blanco et al., 2012). Another exception is quadriviruses (45 nm isometric particles with quadripartite dsRNA genome, family *Quadriviridae*) that have a $T = 1$ capsid with 60 heterodimers (Luque et al., 2016; Mata et al., 2017). There are some similarities between botybirnaviruses and quadriviruses. Viruses of both groups appear to undergo proteolytic cleavage and purified virus preparations contain multiple protein products derived from two virally encoded large ORFs (Lin et al., 2012, 2013; Liu et al., 2015; Ran et al., 2016; Wu et al., 2012) (Figs. 3 and 4). Even unprocessed structural proteins correspond to about half or slightly less than that of the entire polyprotein (Liu et al., 2015 and this study). Meanwhile, as is the case for quadriviruses, the capsids of botybirnaviruses are likely composed of 60 heterodimer capsid proteins; however, further structural analyses are necessary to demonstrate this.

An interesting insight into the evolution and phylogeny of botybirnaviruses was provided by this study. Liu et al. (2015) noted for the first time that there is a homologous region between the two structural proteins of SsBRV1. An alignable region was also detectable between the two putative domains of the AaBbV1 structural proteins (Fig. S7). Phylogenetic trees generated based on the ORF1 and ORF2 polyprotein alignments displayed a similar topology, although SsBRV2 was placed differently between the trees (Fig. 2A and B). A closer look at the two major groups of the putative structural protein-based tree (Fig. 2B) shows a very similar clustering of botybirnaviruses, in which SsBRV2 is placed into different subgroups. These strongly suggest that the common ancestor of botybirnaviruses might have undergone one single duplication event of the structural protein domain. Neither recombination nor reassortment may have occurred during the course of evolution, as far as known botybirnavirus are concerned.

Conflict of interest

The authors declare that they have no conflict.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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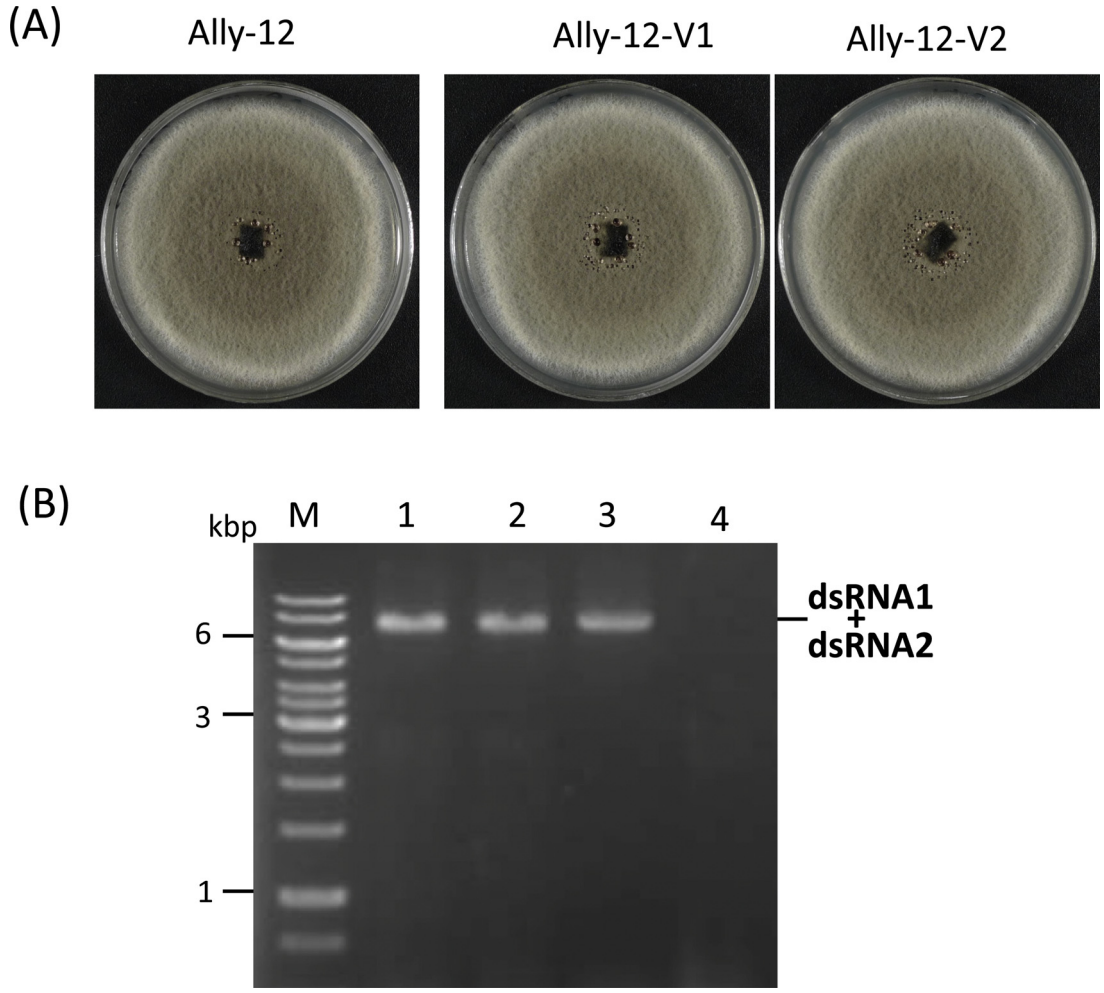

Fig. 5. Infection of a Japanese *Alternaria alternata* Ally-12 strain by AaBbV1. (A) Colony morphologies of Japanese strains of *A. alternate*, uninfected (Ally-12) and infected by AaBbV1 (Ally-12-V1 and -V2). (B) Agarose gel electrophoresis of dsRNA. Protoplasts of virus free Ally-12 were fused with those of AaBbV1-infected 4a to transfer the virus. Virus infection in Ally-12-V1 and V2 (lanes 1 and 2) was confirmed by dsRNA extraction and subsequent gel electrophoresis. The strain 4a (lane 3) and Ally-12 (lane 4) samples were treated in parallel. M refers to DNA standards (DNA ladders, Thermo Fisher Scientific.).

generous gift of *A. alternata* strain Ally-12, and Dr. Ida Bagus Andika for fruitful discussion. The authors are also grateful to Ms. Tsugumi Shiokawa and Dr. Hiroko Tada at Division of Instrumental Analysis, Okayama University for the protein analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.01.006.

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