New insights into reovirus evolution: implications from a newly characterized mycoreovirus

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Abstract
We performed molecular cloning and complete genome sequencing of a novel mycoreovirus, Sclerotinia sclerotiorum reovirus 1 (SsReV1), isolated from an isolate of the phytopathogenic fungus Sclerotinia sclerotiorum. SsReV1 has a genome of 28,055 bp and is composed of 11 double-stranded RNA segments. With a combination of unique molecular features, virion shape and composition, and phylogenetic analysis, SsReV1 is significantly distinct from all known reoviruses and defines a novel genus in the family Reoviridae. Interestingly, two conserved domains, double-stranded RNA binding motif (dsRBM, Pfam 00035) and reovirus sigma C capsid protein (Reo_σC, pfam04582), were identified in the genome of SsReV1, which are widespread in diverse virus lineages. Sequence comparison and phylogenetic analysis revealed that multiple cross-family horizontal gene transfer (HGT) events could occur between reoviruses and double-stranded DNA viruses, single-stranded RNA viruses and even cellular organisms. Interestingly, the dsRBM of SsReV1 was phylogenetically related to dsRNA-binding proteins of some insects, but not reoviruses. These results indicated that SsReV1 is a new taxonomic representative in Reoviridae, which provides new insights into the diversity and global ecology of reoviruses and other segmented double-stranded RNA viruses. More importantly, the present results provided evidence indicating that reoviruses indeed have HGT events with other virus lineages on a large scale and that HGT may serve as an important driving factor that plays a key role in the evolution of reoviruses.

INTRODUCTION

Mycoviruses (or fungal viruses) initially attracted attention as potential biological agents to control plant fungal disease [1]. Presently, mycoviruses are known to be widespread in virtually all major groups of fungi and most of them cause little or no symptoms in their fungal hosts [2]. In fact, as an essential evolutionary virus lineage from three kingdoms (plants, fungi and animals), mycoviruses not only play significant roles in understanding viral diversity and global ecology, but also provide new insights into the origin and evolutionary relationships of viruses. Therefore, it is critical to discover new mycoviruses, in particular those with unique molecular features and taxonomic considerations.

With the exceptions of rare DNA and single-stranded RNA (ssRNA) mycoviruses, most mycoviruses have double-stranded RNA (dsRNA) genomes, including monopartite genomes of totiviruses, bipartite genomes of megabirnaviruses, botybirnaviruses and partitiviruses, quadrpartite genomes of chrysoviruses and quadriviruses, and multipartite genomes of reoviruses [3]. Reoviruses form the largest dsRNA group and have a very diverse range of hosts, from higher mammals and insects, to plants and lower protists. A significant feature of reoviruses is their multi-segmented genome consisting of 9–12 dsRNA segments, and the strictly conserved terminal sequences at the termini of each dsRNA segment. Three mycoreoviruses (or fungal reoviruses) have so far been reported from two phytopathogenic fungi, Cryphonectria parasitica and Rosellinia necatrix [4–6]. However, whether reoviruses can infect other fungi remains unknown.

Although an important virus lineage, the origin and evolutionary relationship of reoviruses remain largely unknown. It is interesting and challenging to elucidate the evolutionary
relationships of reoviruses because of the current lack of sequence similarities and evolutionary implications. Reoviruses rarely have sequence similarities with other virus groups, even if using the most conserved RNA-dependent RNA polymerase (RdRp). In contrast, all other multiple-segmented dsRNA viruses are usually associated with some monopartite dsRNA viruses, suggesting their common origin [7].

Horizontal gene transfer (HGT) is the sharing of genetic material between non-parent–offspring organisms, and is widespread in prokaryotes and eukaryotes [8, 9]. Similarly, HGT events are also widespread in viruses and have a dramatic influence on the evolutionary dynamics and physiological functions of viruses and their hosts [10–13]. Although HGT events commonly occur in dsRNA and ssRNA viruses [7, 14–16], few HGT events have been reported in reoviruses [7].

Here, we isolated and characterized a new mycoreovirus SsReV1 (Sclerotinia sclerotiorum reovirus 1) from Sclerotinia sclerotiorum strain SCH941. SsReV1 displays unique features in genome organization, virion composition, evolutionary relationship and biological effects on its host. These properties are markedly distinct from all known reoviruses and may define a new taxonomic unit in the family Reoviridae. More importantly, we also provided evidence based on sequence comparison and phylogenetic analysis that cross-family HGT events may have widely occurred in reoviruses.

RESULTS

Novel reovirus isolated from a hypovirulent S. sclerotiorum strain showing unique genomic features

Thirteen dsRNA segments were detected in the hypovirulent strain SCH941 and all their full cDNA sequences were determined. Sequence analysis suggested that 13 dsRNA segments represented the genomes of two unrelated dsRNA mycoviruses: a previously reported bipartite dsRNA virus SsBRV1 (Sclerotinia sclerotiorum bipartite dsRNA virus 1) [17] and a novel reovirus SsReV1 (Sclerotinia sclerotiorum reovirus 1). The complete genome of SsReV1 is composed of 28 055 bp with 42.6% GC content and contains 11 dsRNA segments designated segments 1–11 (S1–S11) based on their migration rate on 1% agarose gel (Fig. 1a). The whole genome sequence of SsReV1 was submitted to the GenBank database under accession numbers KU255424 to KU255434. All of these dsRNA segments were confirmed by Northern blot analysis using gene-specific probes for each dsRNA segment (Fig. 1d and Table S1, available in the online Supplementary Material). The genome size (28.06 kbp) of SsReV1 was obviously larger than those of all previously reported mycoreoviruses (23.4 kbp for mycoreovirus 1 and 24.8 kbp for mycoreovirus 3), but was similar to coltiviruses (29.2 kbp) (Table S2). Notably, the S1 segment of SsReV1 (4.45 kbp) was larger than those of mycoreoviruses (4.14 kbp) and coltivirus (4.35 kbp) (Table S2). The genome of SsReV1 exhibited a ‘3-2-3-2’ dsRNA profile on 1% agarose gel, which was distinct from those of reported members of the genera Coltivirus (4-6-1-1) and Mycoreovirus (3-3-5-6) (Fig. 1a and Table S2).

A single ORF was identified in each dsRNA segment (ORFs 1–11), with the exception that S6 and S11 encoded two ORFs (ORF6-1 and ORF6-2 in S6, ORF11-1 and ORF11-2 in S11) (Fig. 1b). Relatively short 5′-untranslated region (5′-UTR) and 3′-UTR exist in each dsRNA segment with the exception of the S4 segment with long 5′-UTR (373 bp) and 3′-UTR (249 bp) (Fig. 1b and Table S3). The 5′ and 3′ termini of each dsRNA segment are highly conserved and share the consensus sequence ‘5′-GAGWUKK- - -UGCA-GUC-3′, where ‘W’ represents ‘U’ or ‘A’, but ‘U’ is dominant, and ‘K’ represents ‘U’ or ‘G’, but ‘U’ is dominant (Fig. 1c). The conserved 5′-terminal sequences of SsReV1, as well as of other reoviruses, are ‘AU’-rich (Table S4). Interestingly, SsReV1 shared a stable 3′-terminal sequence ‘UGCA-GUC’ with coltivirus (Table S4).

The properties of the SsReV1 ORFs were analysed and are summarized in Table S3. ORF1 encodes the largest protein (virus protein 1, VP1). VP1 showed a significant sequence similarity to RdRps of other members within family Reoviridae (Table S5), suggesting VP1 is responsible for SsReV1 replication and maintenance. Although SsReV1 VP2, VP5 and VP8 had significant similarities to VP2, VP4 and VP10 of members within Coltivirus (Table S5), their roles were unidentified. No sequences from public databases were significantly similar to the remaining proteins of SsReV1. Intriguingly, a search of the publicly conserved domain database (CDD) revealed that a region of VP6 (aa 253–357) shared a significant sequence similarity (E-value=1.26e–03) with double-stranded RNA binding motif (dsRBM, pfam00035) (Fig. 1b). The CDD search further revealed that three regions (aa 296–396, 296–531, and 307–457) of VP7 were significantly similar to the reovirus sigma C capsid protein (Reo_{\sigma C}, Pfam04582, E-value=5.24e–05), UV radiation resistance protein and autophagy-related subunit 14 (Atg14, pfam10186, E-value=1.89e–03), and the mechanosensitive ion channel porin domain (MscS_{porin}, pfam12795, E-value=2.97e–03), respectively.

Identification of SsReV1 virion components

The virions of SsReV1 were successfully purified from mycelia of strain SCH941R117 (with the same genetic background as SCH941, infected with SsReV1 but lacking SsBRV1) and observed under a transmission electron microscope (TEM). The morphology of the SsReV1 virion is spherical and approximately 65 nm in diameter (Fig. 2a). The protruded turret substructure was not observed on the SsReV1 virion surface (Fig. 2a). dsRNA segments from virions showed the same dsRNA profile (11 dsRNA segments) as those from mycelia of strain SCH941R117 (Fig. 2b). The component proteins of the purified SsReV1 virions were subjected to SDS-PAGE analysis. The results showed that nine clear protein bands with approximate sizes of 165 (p165), 145 (p145), 102 (p102), 90 (p90), 65 (p65), 63 (p63), 57 (p57), 50 (p50), and 30 kDa (p30) were detected from the virion preparations (Fig. 2c). However, four protein bands (p102, p90, p57 and
p50) were also detected in the preparations for the SsReV1-free isogenic fungal strain SCH941R124 following the same method that was used for the SsReV1-infected strain SCH941R117. These four bands were thus likely to be host proteins co-fractionated with the virions (Fig. 2c). Although protein p57 was detected in both SsReV1-free and SsReV1-infected strains, it seemed to accumulate more in strain SCH941R117, indicating that p57 may be a potential structural component of SsReV1 virions (Fig. 2c).

Six potential structural proteins were separately subjected to polypeptide mass fingerprint–MS (PMF–MS) analysis. p165, p145, p65, p63, p57 and p30 respectively generated 26, 59, 10, 15, 26 and 13 peptide fragments, which respectively matched the amino acid sequence of proteins VP1, VP2, VP5, VP8, VP9 and VP9 of SsReV1 (Table S6). Therefore, the virion of SsReV1 comprises at least six component proteins that were individually encoded by ORF1, ORF2, ORF5, ORF8, ORF9 and ORF9. Also noteworthy is that the molecular weight of proteins p165, p145 and p57 were roughly identical to the predicted sizes of VP1, VP2 and VP9, but the proteins of p65, p63 and p30 were obviously smaller than predicted for VP5 (91.3 kDa), VP8 (69.8 kDa) and VP9 (57.3 kDa) (Table S3). Interestingly, both structural proteins p57 and p30 were encoded by ORF9 (Table S6).

**SsReV1 defines a new genus in the family Reoviridae**

All representative members of family Reoviridae, comprising 14 genera, were included in the phylogenetic analysis of SsReV1 (Table S7). A neighbour-joining phylogram was generated based on RdRp sequence of each member of reoviruses. The results suggested that SsReV1 is phylogenetically related to coltiviruses and mycoreoviruses, but formed a separate branch with a strong support value (98 %) (Fig. 3). Notably, coltiviruses were firstly identified in mosquitoes and can also infect mammals and humans [18]. SsReV1 was included in a super-clade which consisted of coltiviruses, mycoreoviruses, plant reoviruses (fijivirus and oryzavirus) and insect reoviruses (cypovirus, dinovernavirus and cimodo virus). The unique genomic features and phylogenetic analysis supported that SsReV1 is a new member of the family Reoviridae, but does not belong to any known genus. Thus, a new genus, Sclereovirus (Sclerotinia...
Identification and evolution of the dsRBM domain in diverse virus lineages

The dsRBM domain is a consensus sequence of 65–68 amino acids and is present in diverse groups of dsRNA-binding proteins in cellular organisms [19]. It was of interest that the conserved dsRBM domain was detected in the genome of mycoreovirus SsReV1. Although dsRBM proteins were present in diverse groups of organisms, there were still some conserved amino acids and consensus sequence length in conserved dsRBM domains (Fig. S1a). For example, the alanine (A) residue in the C-terminal region is strictly conserved and the leucine (L) residue in the N-terminal region is relatively conserved. Secondary structural analysis showed that this alignment profile had two α-helices and three β-sheets (αββαα) (Fig. S1a). A classical dsRBM secondary structure, widespread in both prokaryotes and eukaryotes, has no specific dsRNA recognition in ribonucleases [20]. Similarly, the predicted three-dimensional (3D) model of the dsRBM domain from SsReV1 resembled that from the *Saccharomyces cerevisiae* dsRNA binding domain (PDB, 2LBS_B) (Fig. S1b), indicating that the conserved domain has biological and physiological functions in SsReV1.

To clarify whether other viruses have the conserved dsRBM domain, an online hidden Markov model-based HMMER3 search was then conducted by using the above dsRBM alignment as the seed sequences against the virus database. The result showed that homologues of the dsRBM domain are present in diverse virus groups which had a wide host range of mammals, insects and protists (Table S8). In addition to SsReV1, the dsRBM domain was also present in four other reoviruses, including three seadornaviruses and a rotavirus that belongs to the subfamily *Sedoreovirinae*, which infect mammal and insect hosts, respectively (Table S8). Genome comparison revealed that the dsRBM domain was present in dsRNA segment S11 or S12 of seadornaviruses, S5 of adult diarrheal rotavirus, and S6 of SsReV1 (Table S8). Interestingly, homologues of the dsRBM domain are widespread in giant linear dsDNA viruses, including members of *Poxviridae*, *Phycodnaviridae*, *Iridoviridae* and *Mimiviridae* (Table S8). Notably, these dsRBM-containing viral proteins seemed to play important roles in diverse biological processes, because they occurred in dsRNA binding proteins, ribonuclease III, interferon resistance proteins and some uncharacterized proteins (Table S8).

The present results indicated that multiple independent recombination or HGT events may occur in different reoviruses. To elucidate the possible evolutionary relationships of these dsRBM homologues, a maximum-likelihood phylogenetic tree was constructed. The result revealed that the dsRBMs of poxviruses are grouped together and formed an independent lineage which covered the mammal hosts (Fig. 4). However, dsRBMs of the remaining viruses and cellular organisms are scattered in this phylogenetic tree and formed two independent groups (Fig. 4 and Table S8). The dsRBMs of reoviruses were also located in this supergroup, but scattered in different clusters (Fig. 4). SsReV1 dsRBM was grouped with the dsRNA-binding protein Staufen homologues of insects, which was also supported by the BLAST result (data not shown). The LNV dsRBM clustered with an interferon-inducible dsRNA-dependent protein kinase activator homologue of fish, while dsRBM of the remaining reoviruses formed two independent groups (Fig. 4 and Table S8). Importantly, these dsRBM-containing reoviruses have a wide range of hosts, including mammals, insects and fungi, in which no homologues of reovirus dsRBMs were detected. Therefore, these results are inconsistent with the evolutionary processes of their hosts, and indicate that the sporadic distribution of dsRBM homologues in viruses was more...
likely generated by HGT events rather than vertical inheritance. This conclusion is also supported by the sporadic distribution of iridoviruses in the phylogenetic tree. Although dsRBMs in members of 
Mimiviridae, Phycodnaviridae and Poxviridae (Fig. 4) tended to be grouped together, they were not consistent with the evolutionary processes of their hosts. The results revealed that multiple independent HGT events occurred in the ancestral viruses and are retained in the present viruses by vertical inheritance. Therefore, there might be wide HGT events among reoviruses, dsDNA viruses and cellular organisms.

Identification and evolution of the Reo_σC domain in diverse virus lineages

The Reo_σC domain was originally identified in the cell attachment protein encoded by the third ORF of the first small dsRNA (dsRNA segment 7, S7) of avian reovirus (ARV) [21, 22]. A multiple alignment revealed that there were some strictly conserved amino acid residues in the Reo_σC domain of SsReV1, while two small sequence deletions were observed in this region (Fig. S2). Secondary structural analysis revealed only α-helices present in these Reo_σC domains (Fig. S2) and no reliable 3D models were predicted in the Reo_σC domain of SsReV1. We then used the alignment of these five Reo_σC domains as the seed sequences against the virus databases. In addition to members of family 
Reoviridae, homologues of the Reo_σC domain were widespread in diverse linear dsDNA viruses (Table S9). There was also a homologue in a +ssRNA virus (Drosophila immigrans noravirus). Intriguingly, homologues of the Reo_σC domain were also detected in a hypothetical protein of 
Lachnocolstridium phytofermentans, formerly known as 
Clostridium phytofermentans (an anaerobic bacterium) [23]. Furthermore, multiple homologues were detected within a single protein in some viruses, such as three copies in faustovirus and two copies in 
Lactobacillus phage LdL1, tunivirus fontaine and iridovirus (Table S9).
To probe into the evolution of these Reo_σC domains, phylogenetic analysis was performed. These Reo_σC domains of orthoreoviruses are located in three independent clades (Fig. 5). The clade of mammalian orthoreovirus and mammalian orthoreovirus 3 is located at the base of the phylogenetic tree, indicating the relatively low evolutionary rate of these Reo_σC domains. SsReV1 was grouped together with four different avian reoviruses and an unclassified environmental halophage eHP-36, and formed an independent clade within the inner position of the phylogenetic tree (Fig. 5). Similarly, members of Baculoviridae, Myoviridae, Siphoviridae and Poxviridae tended to form independent clades, respectively. Considering the fact that these viruses shared a remote relationship and very diverse groups of hosts, it seems impossible that Reo_σC homologues originate from the same ancestor. Therefore, the wide presence of Reo_σC homologues in diverse virus lineages may result from HGT events, instead of vertical inheritance. Multiple homologues within a gene were usually clustered together (Fig. 5), reflecting their relative relationships and common origins. However, the three homologues in faustovirus were divided into two independent groups, possibly due to two independent HGT events. Furthermore, the Reo_σC domain could occasionally be transferred into the genome of cells, explaining the presence of a Reo_σC homologue in a hypothetical protein of the phytobacterium Lachnoclostridium phytofermentans (Fig. 5). Intriguingly, in addition to the Reo_σC domain, the region aa 307–396 of SsReV1 VP7 also showed significant sequence similarities to the Atg14 and MscS-porin domains – two important domains in cellular organisms. However, these
two domains were only detected in a few viruses (data not shown).

**SsReV1 has limited biological effects on S. sclerotiorum**

To separate the two mycoviruses SsBRV1 and SsReV1 from strain SCH941, protoplasts of strain SCH941 were prepared, and 150 regenerated isolates were obtained. Both dsRNA and reverse transcription PCR (RT-PCR) detection suggested that strains SCH941R15 and SCH941R117 merely encompassed this reovirus, while strains SCH941R124 and SCH941R155 were cured (Fig. S3e, f). Biological assay showed that there were no significant differences between SsReV1-alone infected strains and SsReV1-free strains in colony morphology, lesion size on detached leaves of rape-seed plants and growth rate (Fig. S3a–d). SsReV1 virions were successfully introduced into the protoplasts of a virulent strain Ep-1PNA367, which was confirmed by dsRNA extraction and RT-PCR detection (Fig. S4e, f). Transfectants (Ep-1PNA367-PT12 and Ep-1PNA367-PT13) showed no significant difference from strain Ep-1PNA367 in biological properties (Fig. S4a–d). These results suggested that SsReV1-alone produced symptomless infection in the fungal host S. sclerotiorum.

**DISCUSSION**

In the present study, a novel mycoreovirus SsReV1 was isolated and characterized from the fungal plant pathogen S. sclerotiorum. SsReV1 is a new member of the family Reoviridae and possesses common properties of reoviruses including a multipartite dsRNA genome, conserved terminal sequences,
Spherical virions and mostly monocistronic ORFs. SsReV1, in addition, exhibits some unique characteristics that are distinct from all previously reported reoviruses.

**SsReV1 defines a new genus of the family Reoviridae**

Phylogenetic analysis revealed that SsReV1 was located at the base of the coltivirus and mycoreovirus group, but not within the genus *Mycoreovirus* with a strong support (Fig. 3). Moreover, SsReV1 has a closer relationship to coltiviruses than to mycoreoviruses based on phylogenetic analysis, genome attributes and *BLAST* results (Fig. 3, Tables S4 and S5). Notably, projections from the surface of virions were present in members of *Coltivirus* and *Mycoreovirus*, which were not observed in SsReV1 (Fig. 2). Additionally, SsReV1 has a slight effect on *S. sclerotiorum* (Figs S3 and S4), while mycoviruses *MyRV1* and *MyRV3* caused hypovirulence-related phenotypes on their fungal hosts [4, 5]. Therefore, SsReV1 may represent a new evolutionary lineage of reoviruses, and a new genus, *Sclereovirus* (*Sclerotinia* reovirus), is proposed to accommodate SsReV1. In fact, even if sharing the same host, some reoviruses are still divided into different genera. The plant reoviruses of rice were divided into three genera, *Fijivirus*, *Oryzavirus* and *Phytoreovirus*, since the members within three three genera significantly differ in molecular characteristics, virion morphology and composition, and propagative vectors [6]. Moreover, fijiviruses and oryzaviruses have large projections on their virion surfaces and belong to the subfamily *Spinareoviridae*, while phytoreoviruses lack projections on the surface of virions and belong to the subfamily *Sedoreoviridae* [6, 24, 25]. Therefore, it is not surprising that SsReV1, MyRV1 and MyRV3 belong to different genera of *Reoviridae* even though they all have fungal hosts.

**Reoviruses have widespread gene-communications with other virus lineages**

As a large group of dsRNA viruses, the phylogenetic relationships between reoviruses and other dsRNA viruses are still unclear. In fact, even though among different genera of family *Reoviridae*, the RdRps showed only 10–20% amino acid identities [26]. Moreover, few HGT events in reoviruses have been reported. There are only sporadic HGT events restricted to among species of rotaviruses and phytoreoviruses [27–29]. The S7 domain of phytoreoviruses is the sole reported example of HGT events occurring between reoviruses and other dsRNA virus groups [7]. It seems that reoviruses are a chaotic group in the phylogeny of dsRNA viruses and all virus lineages.

The pervasive presence of dsRBM and *Reo_σC* domains in diverse virus groups provides important clues to clarify the evolutionary relationship of reoviruses. Although the dsRBM is an important RNA-binding domain and widely occurs in both prokaryotes and eukaryotes [20], dsRBM homologues in viruses have not been well identified and analyzed until now. Here, we have found that HGT may be a major factor to generate viral dsRBM homologues, but not vertical inheritance. Moreover, the HGT direction of the dsRBM should be initially transferred from cellular organisms to viruses and viral dsRBMs can also be transferred by HGT. The viral dsRBM homologues may result from multiple independent HGT events, because they are not consistent with the evolutionary process of their hosts. Unlike the dsRBM domain, the *Reo_σC* domain may initially occur in viruses considering its wide presence in viruses but rarely in cellular organisms. Moreover, *Reo_σC* has more frequent cross-family HGT events among diverse virus groups, because *Reo_σC* homologues in the same family are often located in independent phylogenetic branches. We also discovered that HGT events of the *Reo_σC* domain may also occur in +ssRNA virus *Drosophila* immigrans *Nora* virus (Table S9); thus they are not limited to dsRNA and dsDNA viruses.

dsRBM can be widely found in various proteins, ranging from non-structural RNA-binding protein 34 of porcine and bovine rotavirus to ribonuclease III of *Escherichia coli*, dsRNA-specific editase of *Drosophila melanogaster* and dsRNA-activated protein kinase p68 of *Homo sapiens* [20]. Thus, dsRBM has different roles in biological and physiological processes. In the case of rotavirus A, dsRBM is present in the RNA-binding protein NSP3, which can bind the viral mRNA 3' end during rotavirus infection and hijack the human eukaryotic initiation factor 4GI (eIF4GI), interfering with the function of cellular mRNA [30]. The *Reo_σC* domain was first identified in the protein sigma C and plays a critical role during the process of avian reovirus infection and pathogenesis [31]. Protein sigma C is a homotrimer in its native state and anchors the inner capsid via a short N-terminal sequence, and its protruding globular C-terminal domain is responsible for the interaction with host cells [32]. Recently, a unique gene of bat-derived coronavirus has been reported to originate from the p10 gene of a bat orthoreovirus through cross-family recombination and can induce the formation of cell syncitia [33]. Therefore, HGT events between reoviruses and other virus lineages are more common than previously recognized and could shed light on the function, diversity, ecology and evolution of reoviruses.

**SsReV1 reveals a new biological significance of reoviruses**

Although viruses have long been recognized as pathogens, some viruses that are beneficial to their hosts provide an opportunity to understand the essential functions, evolution and biological significance of viral entities [34]. Similarly, all previously known reoviruses are harmful to their hosts and some even cause serious diseases in humans, animals and plants [6]. As far as we have investigated, SsReV1 is the first member of the reoviruses that does not exert detrimental effects on its natural host (Figs S1 and S2). Therefore, symptomless or good reoviruses may be widespread in the environment and need to be further discovered. These reoviruses may play vital roles in producing novel insights into understanding the diverse biological significances and evolutionary hints of reoviruses. In addition, SsReV1 virions...
have been identified with six protein components and have no obvious spike structure on the surface, which are distinct from the mycoreoviruses MyRV1, MyRV3 and all other reoviruses, and which may be responsible for the unique biological functions of SsReV1. However, the structure and function of the SsReV1 virion need to be further investigated. As the largest dsRNA viruses, the genome of reoviruses has multiple dsRNA segments that may contribute to the adaptation for various living environments and successful infection of numerous hosts. Unlike other segmented dsRNA viruses, the phylogeny of reoviruses and their multiple dsRNA genomes is still a challenging problem in the long term. Undoubtedly, the presence of widespread HGT events in reoviruses provides fresh clues and insights into the evolution of reoviruses.

METHODS

Fungal strains and culture conditions

*S. sclerotiorum* strain SCH941 was isolated from a sclerotium collected from Yingjing County, Sichuan Province, PR China. Strains SCH941R15, SCH941R117, SCH941R124 and SCH941R155 were regenerated from protoplasts of strain SCH941. Strain Ep-1PNA367 is a single-ascospore virulent strain generated from strain Ep-1PN [35]. All *S. sclerotiorum* strains were cultured on potato dextrose agar (PDA) at 20–22 °C.

Biological assays

Biological assays including colony morphology, growth rate and virulence test were performed according to the methods of Liu et al. [17]. At least three replicates for each treatment were conducted for statistical analysis and observation.

dsRNA isolation, molecular cloning and sequence analysis

dsRNA isolation, molecular cloning, terminal sequence determination and sequencing analysis of SsReV1 were conducted as described by Liu et al. [17]. Random PCR was used to generate sequence information for all the 11 dsRNA segments. The terminal sequences of each dsRNA segment were determined based on the known sequences previously obtained by a modified RACE method [17]. The genome of SsReV1 was confirmed by RT-PCR and re-sequencing. Each nucleotide of the SsReV1 genome was sequenced at least three times. Sequence assembly and ORF findings were analysed using DNAMAN software.

Homologue search and phylogenetic analysis

All of the 11 dsRNA sequences were analysed using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment was performed using the online M-Coffee server (http://tcoffee.crg.cat/apps/tcoffee/do: mcoffee) [36] and visualized with ClustalX 2.0. The phylogram was generated using the neighbour-joining method based on RdRp sequences and refined with MEGA5.0 [37]. Conserved domains were detected using the online Conserved Domain Search Service (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [38]. Homologue search of the conserved domain was conducted with the online HMMER3 tool (http://toolkit.tuebingen.mpg.de/hmmer3) [39] and the virus nr database was selected. The secondary structure prediction was analysed by the online Jpred 4 server (www.compbio.dundee.ac.uk/jpred) [40] and the 3D model was analysed using the Phyre2 server (www.sbg.bio.ic.ac.uk/ phyre2/html/page.cgi?id=index) [41]. The best-fit model (RtREV+G+F and LG+I+G+F) of dsRBM was obtained using ProtTest server based on Akaike’s information criterion (AIC) (http://darwin.uvigo.es/software/prottest2_server.html) [41]. The phylogenetic tree of dsRBM was created by PhyML 3.0 [42] and refined by MEGA5.0 [37]. The phylogenetic tree of the Reo_σC domain was created by PhyML-mixtures based on phylogenetic mixture models [43] and refined by MEGA5.0 [37].

Virus purification and characterization of viral structural proteins

Purification of virions and characterization of structural proteins of SsReV1 were performed following previously reported methods on *Sclerotinia sclerotiorum* bipartite dsRNA virus 1 (SsBRV1) [17]. Approximately 30 g wet weight mycelia of strain SCH941R117 grown in potato dextrose broth (PDB) for 7 days was used for isolation and virus purification. The virus-cured strain SCH941R124 was used as negative control for analysis of the structural proteins of SsReV1. The virions were stained with 2 % (w/v) phosphotungstic acid (PTA) and observed by using a transmission electron microscope (Model Tecnai G2 20; FEI Company). SDS-PAGE gel (9 %) was used to separate the structural proteins of virions, and the specific protein bands were individually cut and were subjected to polypeptide mass fingerprint–MS (PMF–MS) analysis by BGI.

Protoplasts preparation and virus transfection

Protoplast preparations of strain Ep-1PNA367 and polyethylene glycol (PEG-mediated transfection using purified virus particles were as described previously [17]. SsReV1 transfectants were confirmed by RT-PCR analysis using virus-specific primers Rev1S1-F/Rev1S1-R (listed in Table S1) for SsReV1 and specific primers BRV1S1-F/BRV1S1-R (listed in Table S1) for SsBRV1.

Northern blot analysis

Northern blot analysis of SsReV1 was conducted as reported previously [17]. Eleven gene-specific probes labelled with digoxigenin were individually prepared for the detection of each dsRNA segment. All the primers used for probes are listed in the Table S1.

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Conflicts of interest
All authors declare that there are no conflicts of interest.

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

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