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Isolation and Characterization of a Mycovirus in *Lentinula edodes*

Hyo-Kyoung Won[1], So-Jung Park[1], Dong-Kyu Kim[1], Myeung Ju Shin[1], Nari Kim[1], Song-Hee Lee[1], Young-Chul Kwon[1], Han Kyu Ko[2], Hyeon-Su Ro[1*], and Hyun-Sook Lee[1*]

[1]*Department of Microbiology and Research Institute of Life Sciences, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[2]*Forest Mushroom Research Institute, Gyeonggi 469-803, Republic of Korea*

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A mycovirus was isolated from an edible mushroom, *Lentinula edodes*, that was suffering from a severe epidemic. Fractionation of the diseased cell extract by isopycnic centrifugation with 50% CsCl revealed that the diseased mushroom was infected by *Lentinula edodes* spherical virus (LeSV), a new spherical virus with a diameter of 55 nm. The particle of LeSV encapsidated the 12 kb RNA genome by a 120 kDa coat protein. BLAST analysis of the partially sequenced LeSV genome showed 95% sequence identity with a putative RNA-dependent RNA polymerase (RdRp) gene of the mycovirus HKB, which was previously reported as being a double-stranded RNA (dsRNA) element. In contrast to HKB, the RNA genome in LeSV is encapsidated by the 120 kDa coat protein. To confirm that the LeSV coat protein is encoded by the viral genome, the N-terminal amino acid sequence of the coat protein was determined. The resulting N-terminal amino acid sequence, N-SALDVAPVVPELYFXXLEV-C, was found to be located in the middle of the HKB ORF1, suggesting that the LeSV coat protein was indeed encoded by the virus. To detect LeSV in *L. edodes*, a primer set targeting the RdRp gene was designed based on the partial sequence of the LeSV genome. RT-PCR analysis showed that 56 of the 84 commercially available dikaryotic cultivars carry LeSV. The transmission pattern of the virus was determined by analysing basidiospores from LeSV-infected and LeSV-free fruiting bodies. Nine out of 10 basidiospores from the LeSV-infected cultivars contained the virus while the spores from the LeSV-free parent were free of LeSV, suggesting that vertical transmission is the primary mode of LeSV propagation.

***Keywords*:** detection, HKB, LeSV, mushroom, mycovirus

*For correspondence. E-mail: (H.S. Ro) rohyeon@gnu.ac.kr / (H.S. Lee) hslee@gsnu.ac.kr; Tel.: +82-55-772-1321; Fax: +82-55-759-0187

Introduction

Infection with a mycovirus (fungal virus) induces phenotypic and physiological changes in fungal cells and tissues. Mycoviruses such as CHV and SsHV1 cause hypovirulence in the plant pathogenic fungi *Cryphonectria parasitica* (Shapira *et al.*, 1991) and *Sclerotinia sclerotiorum* (Zhang *et al.*, 2009; Xie *et al.*, 2011), respectively. In mushrooms, mycoviruses often cause serious diseases with symptoms including degeneration of mycelium, premature fruiting body formation, deformed fruiting bodies, and reduced yield. In the button mushroom (*Agaricus bisporus*), La France disease is a well-known mycoviral disease and a double-stranded RNA (dsRNA) virus named La France isometric virus (LIV) was identified as the causative agent (Hollings, 1962; Goodin *et al.*, 1992; Van der Lende *et al.*, 1994). LIV and the mushroom virus X (MVX) (Grogan *et al.*, 2003; Rao *et al.*, 2007) are of major concern at commercial button mushroom farms. Various mycoviruses similar to LIV and MVX, including OMSV (Yu *et al.*, 2003), OMIV (Ro *et al.*, 2006), and PeSV (Ro *et al.*, 2007), have been discovered in the fruiting bodies of *Pleurotus* mushrooms. PeSV is a single-stranded RNA (ssRNA) virus isolated from *P. eryngii*. OMSV and OMIV are ssRNA and dsRNA viruses, respectively, which are found together in *P. ostreatus*. Infection by both OMSV and OMIV in a certain cultivar of *P. ostreatus* resulted in complete loss of fruiting body production, and production was recovered only by the removal of OMSV and OMIV from the infected mycelia (unpublished data).

Lentinula edodes, commonly known as the shiitake mushroom, is the second most cultivated edible mushroom in the world. A few reports on the discovery of mycovirus in *L. edodes* have been published (Ohta *et al.*, 2008; Magae, 2012). The mycovirus HKB, the first virus isolated from *L. edodes*, was discovered in a polyethyleneglycol precipitant of diseased *L. edodes* cell lysate (Ohta *et al.*, 2008). It was characterised as an unencapsidated dsRNA element by the direct visualisation of the nucleic acid using atomic force microscopy and comparative analysis of the HKB RNA-dependent RNA polymerase (RdRp) gene sequence with that of other viruses (Ohta *et al.*, 2008 ; Magae, 2012). The 11 kb long HKB viral genome (GenBank ID: 307746861) contains two open reading frames (ORFs), one encoding a hypothetical protein (1975 aa) and the other an RNA-dependent RNA polymerase (RdRp, 1426 aa). Subsequently, a similar sequence was determined in China (GenBank ID: 329568072).

In this report, a new mycovirus, LeSV, was discovered from a diseased *L. edodes*. The partial sequence of the RNA genome showed high similarity with that of HKB. The transmission pattern of the virus was also evaluated by reverse transcription-PCR (RT-PCR) analysis and showed that the virus is transmitted through basidiospores.

Materials and Methods

Mushroom strain and culture conditions

The diseased fruiting bodies of *L. edodes* San704 strain were collected from a commercial farm in Korea. To obtain virus particles from the diseased mushroom, internal parts of the mushroom basidiocap tissue were sliced aseptically and cultured on a solid potato dextrose agar (PDA, Difco) plate at 25°C for 12 days. The mycelia developed on the plate were cut to a diameter of 1 cm and moved to a new PDA plate. For the liquid culture, 5–8 mycelia blocks from the solid medium were inoculated into a potato dextrose broth and incubated for 20 days at 25°C. The dikaryotic and monokaryotic strains of *L. edodes* used for the detection of the virus were obtained from the Forest Mushroom Research Institute (FMRI), Korea.

Purification of mycovirus and transmission electron microscopy analysis

Frozen fruiting bodies of *L. edodes* (100 g) were homogenised in a Waring Blender for 3 min in 2 volumes (w/v) of TE buffer containing 50 mM Tris·HCl and 1 mM EDTA (pH 8.0). Cell debris was removed by centrifugation at 10,000×*g* for 20 min. The supernatant was precipitated with 10% polyethylene glycol 6000 and 0.6 M NaCl. The precipitate was collected by centrifugation at 10,000×*g* for 20 min, resuspended in TE buffer and clarified by an additional centrifugation step. The supernatant was then subjected to ultracentrifugation at 105,000×*g* for 90 min. The pellet was resuspended in 1 ml of TE buffer and clarified by low-speed centrifugation. The supernatant was layered onto 1.585 g/cm^3 cesium chloride (CsCl) solution prepared in TE buffer, and isopycnic centrifugation was performed in a Beckman Type 90Ti rotor at 130,000×*g* for 20 h. Fractions of 750 µl were collected and dialysed against 3 L of TE buffer for 24–36 h with two changes. The virus particles were precipitated by centrifugation at 130,000×*g* for 90 min. The precipitated virus particles were resuspended in TE and frozen at -20°C. Transmission electron microscopy was performed on a JEOL transmission electron microscope (Model 200) with the virus particles incubated on formvar-coated 400-mesh copper grids stained with 2% (w/v) uranyl acetate (pH 4.5).

Extraction and sequencing analysis of the viral nucleic acid, and N-terminal sequencing of the coat protein

The viral nucleic acids were extracted from the purified virus with phenol and phenol/chloroformisoamyl alcohol (25:24:1, v/v/v) and then precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in deionized water. Synthesis of cDNA from the purified RNA was carried out using a cDNA synthesis kit (Clonetech, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using a tagged random primer (5′-ACTAGCC ATGCAGCCCTTNNNNNNNNN-3′). The amplified cDNA products were cloned into the pGEM-T Easy vector (Promega, USA) for sequencing. Sequences were analyzed using the ClustalW and BLAST programs.

The N-terminal amino acid sequencing of the virus capsid protein was performed using purified virus particles and a commercial protein sequencing service (Genomine Co., Korea).

RT-PCR primer design for the detection of LeSV

LeSV in the mycelia of *L. edodes* was detected by RT-PCR. A specific RT-PCR primer set was designed using the available partial sequence of the LeSV RdRp gene. The primer set (Forward: 5′-ATG GTT TTC CAA AGA CGC TA-3′, Reverse: 5′-AAA GGA GTG GCA TAC AAC CC-3′) yielded a 409 bp PCR product. For RT-PCR analysis, total RNA was isolated from mushroom mycelia by Trizol extraction, and cDNA was synthesised using the cDNA synthesis kit. PCR was performed using the synthesised cDNA under the following conditions: 94°C for 5 min; 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; 72°C for 10 min.

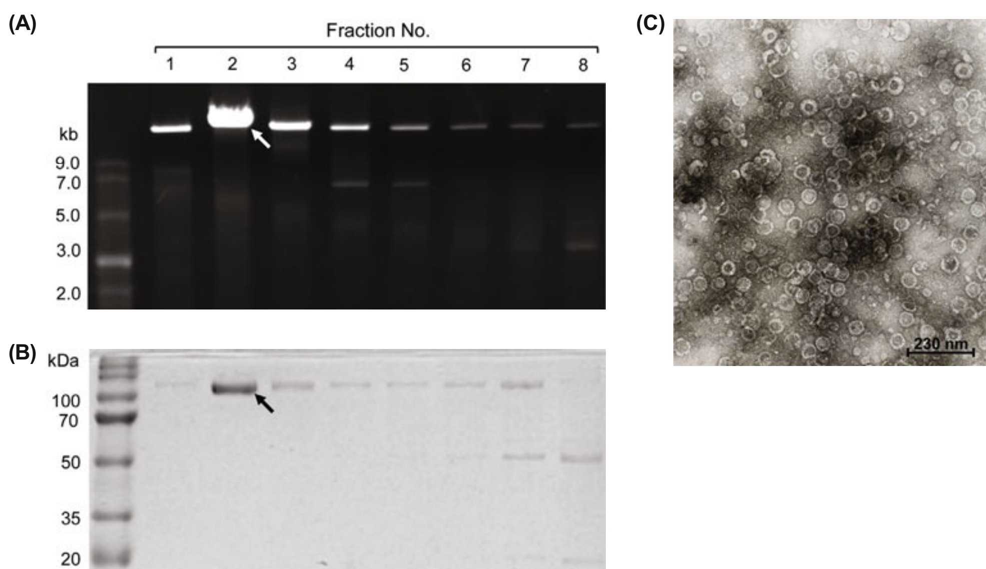

Fig. 1. Fractionation of LeSV from a diseased mushroom fruiting body using equilibrium centrifugation in 50% cesium chloride. (A) Analysis of nucleic acids by agarose gel electrophoresis. (B) Analysis of proteins by SDS-PAGE with Coomassie Blue staining. Fraction No. 2 contained both viral nucleic acid (12 kb) and a capsid protein (120 kDa). Arrows indicate the viral nucleic acid or the coat protein. (C) Transmission electron microscopy analysis of LeSV from fraction No. 2.

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LeSV         1 VDHLERMYRTSDMHGGATLASDYADFNFLHTIPDMQKFWRMIRASAEQFTGPGEWAGTNY 60
HKB RdRp   855 VDHLERMYRTSDMHGGATLASDYADFNFLHTIPDMQKFWRMIRASAEQFTGPGEWAGTNY 914

LeSV        61 AGHVVKLCVWLEEALFSMYVRETSTTGTFIHLLRGLWSGWRTTSVINNVFNEVYGKVLSR 120
HKB RdRp   915 AGHVVKLCVWLEEALFSMYVRETSTTGTFIHLLRGLWSGWRTTSVINNVFNEVYGKVLSR 974

LeSV       121 VCKDILGYDPIELARRNGDDEDARAKSVVASLLYLAMMTYSRLDAQPAKQKIGFTESEYT 180
HKB RdRp   975 VCKDILGYDPIELARRNGDDEDARAKSVVASLLYLAMMTYSRLDAQPAKQKIGFTESEYT 1034

LeSV       181 RVTYAKGVAYNPIARGIASFTSSDLQAPVIDVSPEYVRGTNEALHALIRRGADEERIEML 240
HKB RdRp  1035 RVTYAKGVAYNPIARGIASFTSSDLQAPVIDVSPEYVRGTNEALHALIRRGADEERIEML 1094

LeSV       241 RTELLMTFSQQSYTEAGVKHVVTLHNWRMLYVPVEQGGFGVTRYGSSEPYKLKTAIPWDT 300
HKB RdRp  1095 RTELLMTFSQQSYMEAGIKHVVTLRNWRMLYVPVEQGGFGVTRYGSSEPYKLKTAIPWDT 1154

LeSV       301 VRPLWELPGSMHHGIASLENHLKDKFTKARITHEHVETVVVDATSIASQNVDTGHNEQWV 360
HKB RdRp  1155 VRPLWELPGSMHHGIASLENHLKDKFTKARITHEHVETVVVDATSIASQNVDTGHNEQWV 1214

LeSV       361 EYARKQRADHLVKLNATQCYPTSTSSDYSTTIPGLNALVYKCIDEILSLHPDKVRSYKAP 420
HKB RdRp  1215 EYARKQRADHLVKLNAAQCYPTSTSSDYSTTIPGLNALVYKCIDEILSLHPDKVRSYKAP 1274

LeSV       421 NLEKTLDSYVGKALGLASVTRDILYDLRDVSTNIKLKPWDALARLTGSSVASQQVANQIG 480
HKB RdRp  1275 NLEKTLDSYVGKALGLASVTRDILYDLRDVSTNIKLKPWDALARLTGSSVASQQVVNQIG 1334

LeSV       481 VMTASLSGNYPEALVEAFINRRLKWNRNTYGVMPNFCLPIVDHLER 526
HKB RdRp  1335 VMTASLSGNYPEALVEAFINRRLKWNRNTYGVMPNFCLPIVDHVHR 1380
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Fig. 2. Comparison of the amino acid sequence obtained from the partial genomic sequence of LeSV with the mycovirus HKB RdRp protein sequence. Arrowheads indicate the positions where the amino acid residues differ.

Results

Isolation of a mycovirus from diseased *L. edodes*

Shiitake mushrooms with disease symptoms were collected from a commercial farm located in the southern region of Korea. The symptoms appeared mainly on the caps of the fruiting bodies, resulting in under-developed, curled, or cracked morphologies. These types of morphological irregularities in mushrooms have been attributed to either environmental factors, including humidity and ventilation, or virus infection. To determine whether the observed symptoms were a result of virus infection, the cell lysate was fractionated by isopycnic centrifugation with 50% CsCl in TE buffer. The nucleic acids and proteins of each fraction were analysed by agarose gel electrophoresis and SDS-PAGE, respectively. The agarose gel electrophoresis analysis revealed that a 12 kb nucleic acid appeared in all of the fractions but was particularly concentrated in fraction No. 2 (Fig. 1A). Interestingly, a 120 kDa protein was found to be highly concentrated in the same fraction (Fig. 1B). TEM analysis of fraction No. 2 showed that this fraction contained spherical virus particles with a diameter of 55 nm (Fig. 1C).

Partial sequence determination and analysis

To determine the primary sequence of the RNA genome, genomic ssRNA cDNA libraries were made using tagged random primers. The cDNA clones were sequenced in both directions. Because the cDNAs obtained by tagged random priming could not cover the full length of the viral genome, only partial sequences were obtained. The sequence of 1,579 bases of the LeSV genome was deposited in GenBank under the accession number JQ687140. BLAST analysis of this LeSV sequence showed 95% nucleotide and 99% amino acid sequence identity with the amino acid sequence (Fig. 2) of the 3′-terminus of the ORF2 gene, which encodes the RdRp protein of mycovirus HKB (Fig. 3).

N-terminal amino acid sequence determination of LeSV coat protein

To further analyse the 120 kDa protein obtained in fraction No. 2 (Fig. 1B), protein from purified virus particles was subjected to N-terminal amino acid sequencing. A 19-mer peptide with the following sequence was identified: N-SALDV APVVPELYFXXLEV-C. Sequence comparison analysis re-

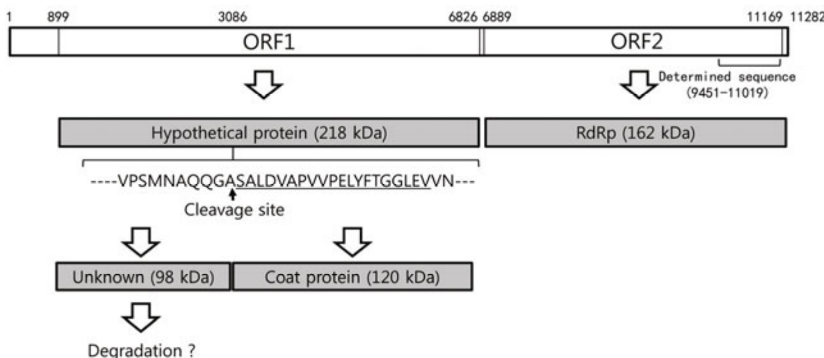

Fig. 3. Comparative analysis of the mycovirus HKB genome with the amino acid sequence data obtained for LeSV. The mycovirus HKB contains two open reading frames (ORFs), one encoding a 218 kDa hypothetical protein and another encoding a putative RNA-dependent RNA polymerase (RdRp). Partial sequencing of the LeSV genome showed 95% identity in nucleotide sequence and 99% identity in amino acid sequence with the 3′-region (9451-11019) of HKB ORF2. N-terminal amino acid sequencing of the LeSV capsid protein (120 kDa) identified the peptide SALDVAPVVPELYFTXXLEV located in the middle of the hypothetical protein encoded by HKB ORF1.

Table 1. **RT-PCR detection of LeSV in *L. edodes* strains**

No.	Stock No.	LeSV	Origin	No.	Stock No.	LeSV	Origin	No.	Stock No.	LeSV	Origin
1	F0092	+	W	29	F1058	+	C	57	F0037	+	W
2	F0107	+	W	30	F1059	-	W	58	F0053	+	W
3	F0259	-	P	31	F1076	+	C	59	F0060	-	W
4	F0264	+	P	32	F1077	+	C	60	F0064	+	W
5	F0329	+	P	33	F1078	-	W	61	F0066	+	W
6	F0659	-	P	34	F1080	-	C	62	F0070	-	W
7	F0731	+	W	35	F1086	+	C	63	F0073	-	W
8	F0733	+	W	36	F1087	+	W	64	F0080	+	W
9	F0948	+	W	37	F1088	+	W	65	F0088	-	W
10	F0961	+	P	38	F1089	+	C	66	F0098	+	W
11	F0962	+	P	39	F1096	-	W	67	F0101	-	W
12	F0963	+	P	40	F1098	+	C	68	F0299	+	P
13	F1004	-	W	41	F1099	+	C	69	F0313	+	P
14	F1009	-	W	42	F1100	+	C	70	F0315	+	P
15	F1012	+	P	43	F1102	+	C	71	F0339	+	P
16	F1038	+	P	44	F1106	-	C	72	F0356	-	P
17	F1040	+	C	45	F1112	+	C	73	F0364	+	P
18	F1041	+	C	46	F1113	+	C	74	F0367	-	P
19	F1042	+	C	47	F1114	+	C	75	F0386	+	P
20	F1043	+	C	48	F1115	+	C	76	F0540	-	P
21	F1044	+	C	49	F1117	+	C	77	F0589	+	W
22	F1045	-	C	50	F1118	+	C	78	F0592	-	W
23	F1046	+	C	51	F1119	-	C	79	F0723	+	W
24	F1047	+	C	52	F1120	+	C	80	F0724	-	W
25	F1048	+	C	53	F1121	-	C	81	F0725	-	W
26	F1049	+	C	54	Chama	-	C	82	F0726	-	W
27	F1056	+	W	55	San111	-	C	83	F0727	-	W
28	F1057	+	W	56	San704	+	C	84	F0728	+	W

W, Wild strain ; P, Parental strain for breeding; C, Cultivated strain

vealed that this N-terminal peptide sequence was located in the middle of a 218 kDa hypothetical protein encoded by ORF1 of HKB (Fig. 3). This finding suggested that HKB might generate the 120 kDa coat protein from the 218 kDa hypothetical protein by post-translational cleavage.

Detection of LeSV in various *L. edodes* strains

Because LeSV was found to be widespread in the mushroom farms, 84 strains of *L. edodes* were evaluated by RT-PCR using a LeSV-specific primer set. The strains included 33 wild strains collected from various part of Korea, 18 parental strains maintained for breeding, and 33 cultivated strains collected from mushroom farms. The RT-PCR results are shown in Fig. 4A and summarised in Table 1. Overall, 56 of the 84 *L. edodes* strains (66.7%) were found to be infected with LeSV. Among the wild strains, 18 were infected with LeSV (54.5%), while infection in cultivated strains was more widespread. Only 7 strains were free of LeSV. The

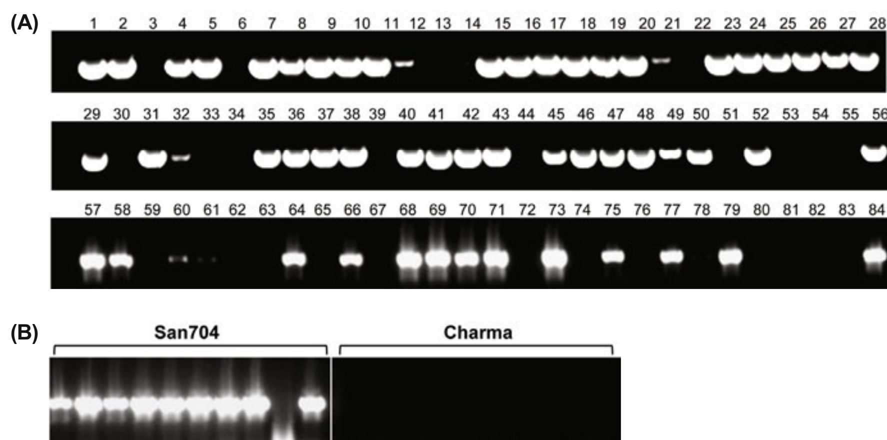

Fig. 4. **RT-PCR analysis of LeSV infection in various strains of *L. edodes*.** (A) Dikaryotic strains including 33 wild strains, 18 parental strains, and 33 cultivated strains. The data are summarised in Table 1. (B) LeSV infection in basidiospores. San704 is an LeSV(+) cultivated strain and Charma is an LeSV(-) cultivated strain. Only spores from the LeSV(+) strain were found to be predominantly LeSV(+).

78.8% infection rate in the cultivated strains was probably a result of the high infection rate (72.2%) in the breeding parental strains (13/18). These results indicate that LeSV transmission occurs via spores (vertical transmission), and the selection of an LeSV-free parental strain is essential for generating new virus-free cultivars.

Transmission of LeSV

To confirm the vertical transmission of LeSV, basidiospores of LeSV-infected [LeSV(+)] and LeSV-free [LeSV(-)] commercial mushroom strains were subjected to RT-PCR. San704, an LeSV(+) strain, is one of the most cultivated commercial strains during summer. This strain was generated by mating F0339 [LeSV(+)] and F1038 [LeSV(+)] strains. As shown in Fig. 4B, 9 out of 10 basidiospores from the San704 strain were LeSV(+), which suggests that basidiospores from the LeSV(+) parent mostly become LeSV(+) and that LeSV may be transmitted during sporogenesis. Only 10% of spores have a chance to be LeSV(-). Strain Charma, an LeSV(-) strain generated by mating F0339 [LeSV(+)] and F0367 [LeSV(-)] strains, is a popular mushroom for autumn and winter harvests. As expected, all the spores from this strain were LeSV(-) (Fig. 4B).

Discussion

In this study, a mycovirus was isolated from the diseased fruiting bodies of *L. edodes*. This virus, designated as LeSV, is a spherical virus as determined by TEM analysis (Fig. 1C). A partial sequence of its RNA genome matched with the RdRp gene of the *L. edodes* mycovirus HKB with 95% identity at the nucleotide level and 99% identity at the amino acid level, implying LeSV may be a variant or, at least, related virus of HKB. This was further supported by the fact that the N-terminal sequence of the viral coat protein of LeSV was found in the middle of the ORF1 gene of HKB.

Mushroom cultivation generally occurs under well-controlled environmental conditions. Therefore, mycovirus infection is a rare cause of severe epidemics. However, a mushroom strain like *L. edodes*, which is cultivated under near-natural conditions, can become a victim of mycoviral infection upon drastic environmental changes. The symptoms in *L. edodes* presented in this study are typical of virus infection. Bacterial or fungal diseases are generally confined to the local boundary of the mushroom field, whereas a viral disease is more widespread because it is inherited from spawn. RT-PCR analysis of the collected strains of *L. edodes* revealed that 78.8% of the cultivated strains were infected with LeSV compared with only 54.5% infection in the wild strains, suggesting that LeSV most likely spreads during the breeding process. Moreover, LeSV(+) strains used as a parental strain mostly result in LeSV(+) offspring, as confirmed by the high transmission rate of LeSV to basidiospores.

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