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Phylogenetic analysis of three orf virus strains isolated from different districts in Shandong Province, East China

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ABSTRACT. Orf virus (ORFV) is the causative agent of contagious ecthyma, which is a zoonotic disease that affects sheep, goats, wild small ruminants and humans. Shandong Province in East China is one of the main producing areas in China for sheep and goats. Here, we conducted epidemiological surveys in different areas in this Province, isolated three orf virus strains, SDLC, SDTA and SDJN, from goat flocks and then analyzed the genetic evolution of these strains. The *ORFV011*, *ORFV059, ORFV109*, *ORFV110* and *ORFV127* genes of these three strains were amplified, sequenced and analyzed. Phylogenetic analysis showed that *ORFV011* of the SDLC and SDTA strains cluster together with the Gansu, Liaoning, Shanxi, Nantou, Hoping and FJ-YX strains, while SDJN clusters with the FJ-GS and FJ-GO strains. *ORFV059* of the SDLC and SDTA strains cluster together with the FJ-YX strain, while SDJN clusters with the FJ-GS and FJ-GO strains. *ORFV059* and *ORFV127* of these three strains were similar to those of the OV-SA00 strain. The results suggested that SDLC, SDTA and SDJN originated from Fujian Province and formed a complex group of viruses in Shandong Province. As the role of *ORFV127* gene responsible for the immune evasion of ORFV, the pathogenesis of these three virus strains may similar to that of OV-SA00. These three strains first isolated in Shandong Province are novel ORFV strains, and the data reported here will be helpful for further research about ORFV and its comprehensive prevention and control.

KEY WORDS: East China, goat, Orf virus, phylogenetic analysis, Shandong Province

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Orf disease, also named contagious ecthyma (CE), is a zoonotic disease that affects sheep, goats, wild small ruminants and humans in all sheep-breeding countries [2, 11, 12]. Clinically, proliferative and self-limiting maculopapular and vesicular pustules occur in the epithelium of the lips, mouth, muzzle, nose, and even in the teat, udder, esophagus or other organs [2, 7, 11, 12]. Orf disease has high morbidity but usually low mortality, while it also shows high fatality due to secondary infection or extreme debilitating condition [12, 35]. Orf virus (ORFV) is the causative agent of this disease and is the prototype member of the genus Parapoxvirus, which includes pseudocowpox virus (PCPV), bovine papular stomatitis virus (BPSV), squirrel parapoxvirus (SPPV) and parapoxvirus of red deer in New Zealand (PVNZ) [12, 19, 35]. The genome of ORFV is a linear double-stranded DNA of 134–139 kb in length that encodes 132 putative genes including 89 highly conserved genes essential for viral structure and assembly located in the core region of the genome and variable genes responsible for virus virulence and pathogenesis at the terminal regions [8, 25].

Although the genomes of OV-IA82 and OV-SA00 in America [8], NZ2 in New Zealand [25], D1701 in Germany [24] and NA1/11 in China [21] have been completely sequenced, there is still little information on the characterization of endemic ORFV strains in mainland China given its large scale. To date, most genetic variation and molecular epidemiology of the orf virus has been based on the highly conserved genes of *ORFV011* (*B2L*), *ORFV059* (*F1L*) and/or *ORFV020* (*VIR*) [1, 17, 20, 31]. ORFV field strains have different levels of heterogeneity even in the same place [6], and the relationship between disease severity and the molecular characterization of ORFV strains has not been fully elucidated.

In spite of the fact that ORFV infections have been reported in Xiangjiang, Fujian, Gansu and Jilin Provinces in China [6, 20, 31, 35], there is a lack of genetic data on circulating ORFV strains in Shandong Province in East China, which is adjacent to Japan and Korea and the top mutton production area in China. Here, we isolated and identified ORFV strains in Shandong Province of China. Genetic diversity was also determined by comparing the full lengths of the *ORFV011*, *ORFV059*, *ORFV109*, *ORFV110* and *ORFV127* genes with reference strains in GenBank. This is the first phylogenetic analysis of orf virus strains isolated from different districts in Shandong Province, East China.

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Table 1. Clinical and epidemiological information for the ORFV strains analyzed in this research

Strains	Species	Goats affected/total	Sites of lesions	Districts of Shandong Province	Year	Deposited Accession No. in GenBank
SDLC	Goat	4/30	Lips	Liaocheng City	2013	*ORFV011*, KP339952; *ORFV059,* KP339950; *ORFV109*, KP339948; *ORFV110,* KP339946; *ORFV127,* KP339944
SDTA	Goat	15/40	Lips, feet	Tai'an City	2014	*ORFV011,* KP339951; *ORFV059*, KP339949; *ORFV109*, KP339947; *ORFV110,* KP339945; *ORFV127*, KP339943
SDJN	Goat	7/60	Lips	Jining City	2014	*ORFV011*, KP336709; *ORFV059*, KP336710; *ORFV109*, KP336711; *ORFV110,* KP336712; *ORFV127*, KP336713

MATERIALS AND METHODS

Sheep herds and tissue collection: Skin biopsies with gross pathologic changes were collected from goat flocks that had typical clinical features of orf disease, including papules, pustules and scabs, in Shandong Province from 2013 to 2014. Clinical and epidemiological information of ORFV isolates analyzed in this research are shown in Table 1. Samples were collected as described in our previous reports [19, 22] and stored in sterile tubes at −80°C for virus isolation and polymerase chain reaction (PCR). From 2013 to 2014, 26 samples from these flocks were collected, and three virus strains were isolated from them.

DNA extraction, polymerase chain reaction (PCR) and sequencing: DNA extraction, PCR, sequencing and isolation of orf virus using primary ovine fetal turbinate (OFTu) cells were performed as described in our previous reports [19, 22, 26]. Briefly, total DNA was extracted from 200 μl of cell culture suspensions using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To amplify the entire open reading frame of the two highly conserved genes *ORFV011* (*B2L*) and *ORFV059* (*F1L*), and three variable genes, *ORFV109*, *ORFV110* (*EEV*) and *ORFV127* (*vIL-10*), five sets of primer pairs were designed and synthesized based on the ORFV genomic sequences [8, 22]. The sequences of the primers were as follows: *ORFV011*, 5′- ATGTGGCCGT TCTCCTC-TATC-3′ (forward) and 5′-TTAATTTATTGGCTTGCAG-3′ (reverse); *ORFV059,* 5′-ATGGATCCACCCGAAATCAC-3′ (forward) and 5′-TCACACGAT GGCCGTGACCAG-3′ (reverse); *ORFV109*, 5′- ATGGCACATAACACGTTC-3′ (forward) and 5′-CTAACCAGACACACAAA-3′ (reverse); *ORFV110*, 5′- ATGGGTTGCTGTAAGGT C- 3′ (forward) and 5′-TTATCCGTGCATCTCCTTC-3′ (reverse); and *ORFV127*, 5′-CAATT GGAATGTCGAAGAAC-3′ (forward) and 5′-AGCAGAACGATACGAGAATCCGAAC-3′ (reverse). The genes were amplified by PCR in a 50 μl reaction volume under the following conditions: 36 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec for *ORFV011* and *ORFV059*, and at 58°C for 40 sec for *ORFV109*, *ORFV110* and *ORFV127*, and extension at 72°C for 90 sec, with a final PCR ending with one extension cycle at 72°C for 10 min. The amplified DNA products were purified with a Gel Extraction Kit (Takara Biotech, Dalian, China), subcloned into pMD18-T vector (Takara Biotech) and sequenced.

Phylogenetic analysis: The nucleotide sequences of *ORFV011*, *ORFV059*, *ORFV109*, *ORFV110* and *ORFV127* of SDLC, SDTA, SDJN and other isolated strains used for phylogenetic analysis in this research were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and are listed in Table 2. Sequence editing, alignment and comparison of the sequences were performed by using Clustal W (http://www.clustal.org/clustal2/). Phylogenetic trees were constructed with the MEGA 5 software using the Kimura-2 parameter evolution model and the neighbor-joining method with 1,000 bootstrap replicates to calculate pairwise distances.

RESULTS

Products of PCR amplification: The full sequences of *ORFV011*, *ORFV059*, *ORFV109*, *ORFV110* and *ORFV127* were successfully amplified by PCR. The sizes of the amplicons were 1137, 1029, 498, 480 and 558 bp, respectively. The amplified products were purified and ligated into pMD18-T vectors. At least three different clones of each gene were amplified and sequenced bidirectionally. The sequences were edited, aligned and deposited in GenBank. Fifteen accession numbers for *ORFV011*, *ORFV059*, *ORFV109*, *ORFV110* and *ORFV127* of these three ORFV strains are listed in Table 1.

Phylogenetic analysis: To analyze and determine the phylogenetic evolutionary relationship of SDLC, SDTA and SDJN with other ORFV strains, including PCPV and BPSV, the corresponding sequences obtained from GenBank were used in this study, and they are presented in Table 2.

The *ORFV011* and *ORFV059* sequences of the three isolated Orf viruses shared 97.1–98.4% and 95.8–97.5% nucleotide identities and 97.6–99.2% and 95.3–98.8% deduced amino acid identities with published reference ORFV strains (Table 3). Furthermore, *ORFV127* also showed high identity, that is, 95.5–98.2% at the nucleotide level and 91.9–98.4% at the amino acid level.

Phylogenetic consensus trees were constructed by MEGA 5.0 based on the alignment results of nucleotides of *ORFV011*, *ORFV059* and *ORFV127*. *ORFV011* of the SDLC and SDTA strains clustered together with those of the Gansu, Liaoning, Shanxi, Nantou, Hoping and FJ-YX strains. *ORFV011*

Table 2. Strains of parapoxvirus used in phylogenetic analysis

Accession No. in GenBank	Strains	Country	Host species	Genes	Reference
JQ619903/JQ619904	NA1/11	China	Sheep	*011/059/127*	Li *et al.* [22]
KC568400/KC568411	FJ-ZX	China	Goat	*011/059*	Chi *et al.* [6]
KC568399/KC568410	FJ-YX	China	Goat	*011/059*	Chi *et al.* [6]
KC568398/KC568409	FJ-SL	China	Goat	*011/059*	Chi *et al*. [6]
KC568394/KC568405	FJ-GT	China	Goat	*011/059*	Chi *et al*. [6]
KC568393/KC568404	FJ-GS	China	Goat	*011/059*	Chi *et al.* [6]
KC568392/KC568403	FJ-GO	China	Goat	*011/059*	Chi *et al*. [6]
KC568391/KC568402	FJ-FQ	China	Goat	*011/059*	Chi *et al*. [6]
KC568390/KC568401	FJ-DS	China	Goat	*011/059*	Chi *et al.* [6]
KM583893/KM583894	GDQY	China	Goat	*011/059*	Duan *et al.* [9]
DQ184476	NZ2	New Zealand	Sheep	*011/059/127*	Mercer *et al.* [25]
HM133903	D1701	Germany	Sheep	*011/059/127*	McGuire *et al.* [24]
AY386263	OV-IA82	U.S.A.	Lamb	*011/059/127*	Delhon *et al.* [8]
AY386264	OV-SA00	U.S.A.	Sheep	*011/059/127*	Delhon *et al.* [8]
FJ808074	Jilin	China	Sheep	*011*	Zhao *et al.* [35]
KC485343	Gansu	China	Human	*011*	Zhang *et al.* [32]
HQ694773	LiaoNing/2010	China	Goat	*011*	Unpublished
GU320351	HuB/2009	China	Goat	*011*	Zhang *et al.* [34]
HQ694772	GanSu/2009	China	Sheep	*011*	Unpublished
KF703747/KC291656	Xinjiang	China	Goat	*011/059*	Unpublished
GU903501	JS04	China	Sheep	*011*	Liu *et al.* [23]
HQ202153	Shanxi	China	Goat	*011*	Unpublished
JN613809	MT-05	Brazil	Sheep	*011*	Unpublished
GQ328006	2009/Korea	South Korea	Goat	*011*	Oem *et al.* [28]
JQ904797	CQ/WZ	China	Goat	*011*	Zhang *et al.* [33]
GU139356	Mukteswar/09	India	Sheep	*011*	Unpublished
DQ263305	67/04	India	Sheep	*011*	Hosamani *et al.* [15]
DQ263306	79/04	India	Sheep	*011*	Hosamani *et al.* [15]
DQ263304	59/05	India	Goat	*011*	Hosamani *et al.* [15]
DQ263303	82/04	India	Goat	*011*	Hosamani *et al.* [15]
EU327506	Taiping	Taiwan	Goat	*011*	Chan *et al.* [5]
DQ904351	Nantou	Taiwan	Goat	*011*	Chan *et al*. [4]
EU935106	Hoping	Taiwan	Goat	*011*	Chan *et al*. [3]
AY424969	Musk ox	U.S.A.	Musk ox	*011*	Guo *et al.* [11]
JN613810	NE1	Brazil	Goat	*011*	Unpublished
JN088051	NE2	Brazil	Goat	*011*	Unpublished
AY424972	PCPV TQ	U.S.A.	Cow	*011*	Guo *et al.* [11]
GQ329670	PCPV VR634	New Zealand	Reindeer	*011/059*	Hautaniemi *et al.* [13]
GQ329669	PCPV F00.120R	Finland	Reindeer	*011/059*	Hautaniemi *et al.* [13]
JF773692	PCPV F07.798R	Finland	Reindeer	*011*	Hautaniemi *et al.* [14]
JF773694	PCPV F05.990C	Finland	Bovine	*011*	Hautaniemi *et al.* [14]
JF773695	PCPV F10.3081C	Finland	Bovine	*011*	Hautaniemi *et al.* [14]
AY424973	BPSV RS	U.S.A.	Calf	*011*	Guo *et al.* [11]
AY386265	BPSV BV-AR02	U.S.A.	Calf	*011/059*	Delhon *et al.* [8]
JQ271535	Jilin-Nongan	China	Sheep	*059*	Wang *et al.* [29]
JX142183	GanS/2010	China	NA	*059*	Unpublished
AY040083	OV/C2	Italy	Sheep	*059*	Unpublished
AY040082	OV/mi-90	Italy	Sheep	*059*	Unpublished
AY040081	OV/Torino	Italy	Sheep	*059*	Unpublished
AY040085	OV/20	Italy	Sheep	*059*	Unpublished
	SDLC	China	Goat	*011/059/127*	Present study
	SDTA	China	Goat	*011/059/127*	Present study
	SDJN	China	Goat	*011/059/127*	Present study

Table 3. The identity of nucleotide and amino acid sequences of SDLC, SDTA and SDJN with the references ORFV strains

Strain and its Geninfo Identifier (gi)	SDLC		SDTA		SDJN	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
ORFV011						
NZ2 (74230714)	97.4	97.6	97.3	97.1	97.4	97.9
OV-IA82 (40019122)	98.3	98.1	98.2	97.6	98.3	98.4
OV-SA00 (40019123)	98.2	98.7	98.0	98.1	98.3	98.9
D1701 (325073632)	98.2	98.4	98.1	97.9	98.4	99.2
NA1/11 (KF234407)	97.4	98.4	97.2	97.9	97.1	98.1
ORFV059						
NZ2 (74230714)	97.3	97.3	97.3	97.0	96.8	96.7
OV-IA82 (40019122)	97.2	97.3	97.2	97.0	96.6	96.7
OV-SA00 (40019123)	97.5	98.8	97.5	98.5	97.1	97.7
D1701 (325073632)	96.0	95.9	96.0	95.6	95.8	95.3
NA1/11 (KF234407)	97.1	97.3	97.1	97.0	96.6	96.7
ORFV109						
NZ2 (74230714)	89.0	85.0	88.6	83.8	89.0	85.0
OV-IA82 (40019122)	77.0	74.1	77.0	72.8	77.4	74.1
OV-SA00 (40019123)	57.4	49.1	57.4	49.1	57.4	49.1
D1701 (325073632)	35.6	10.8	35.4	10.8	35.6	10.8
NA1/11 (KF234407)	77.0	74.1	77.0	72.8	77.4	74.1
ORFV110						
NZ2 (74230714)	96.0	96.4	96.2	95.2	97.0	98.2
OV-IA82 (40019122)	85.1	81.8	85.7	81.8	86.1	83.6
OV-SA00 (40019123)	56.9	44.8	57.3	44.8	57.9	46.1
D1701 (325073632)	39.4	18.2	38.9	17.6	38.7	18.2
NA1/11 (KF234407)	94.6	95.2	94.8	93.9	95.6	97.0
ORFV127						
NZ2 (74230714)	97.0	98.4	96.8	97.8	96.4	98.4
OV-IA82 (40019122)	96.1	94.6	95.9	94.1	95.5	94.6
OV-SA00 (40019123)	98.2	97.8	98.2	97.3	97.8	97.8
D1701 (325073632)	95.9	91.9	95.9	91.9	95.5	92.4
NA1/11 (KF234407)	97.1	97.3	97.0	96.8	96.6	97.3

of SDJN shared 99.1% nucleotide identity with those of the FJ-GS and FJ-GO strains (Fig. 1A). *ORFV059* of the SDLC and SDTA strains clustered together with that of the FJ-YX strain. *ORFV059* of SDJN shared 97.4% nucleotide identity with those of the FJ-GS and FJ-GO strains. *ORFV059* and *ORFV127* of SDJN, SDTA and SDLC were similar to those of the OV-SA00 strain (Fig. 1B and 1C).

DISCUSSION

Orf disease is a zoonosis that exists worldwide and has been reported in the past three years [9, 16, 18, 22, 27, 32]. In China, more than 20 provinces have reported orf disease breakouts during the past few years, and statistics from epidemiological surveys by our group show that there were 51 cases in China in 2014 (data not shown). Shandong Province, which is adjacent to Korea, Japan and other East Asian countries, is famous for sheep and goat breeding and export of mutton products. From 2013 to 2014, goat flocks in three different districts exhibited classic lesions of orf disease (Table 1). As there have been very few breakouts of this disease in Shandong Province, we were eager to know its origin. To date, there has been no report of phylogenetic analysis for the orf virus in Shandong Province. Here, we conducted the epidemiological surveys in different areas in this province, isolated three orf viruses and analyzed the genetic evolution of the coding genes.

The sequences of *ORFV011* and *ORFV059* are usually used for phylogenetic analysis of orf virus, and these two genes are regarded as epidemiologically relevant sequences [6, 13, 35]. Other genes, like *ORFV020* (*VIR*), are also used in molecular epidemiological studies of ORFV as a marker [3, 27]. Usually, phylogenetic analysis indicates a hypothetical origin of virus strains, and we also wish to determine the ORFV origin information based on *ORFV011* and *ORFV059*. In the present study, the ORFV strains of SDLC, SDTA and SDJN were most similar to isolates from Fujian Province based on the *ORFV011* and *ORFV059* gene sequences. We hypothesized that the orf infection in Shandong Province originated in Fujian Province. Detailed survey of trajectory formation for goat sales will be conducted in our future work. According to the phylogenetic relationships based on *ORFV011*, the SDLC and SDTA strains cluster together with the Gansu, Liaoning, Shanxi, Nantou, Hoping and FJ-YX

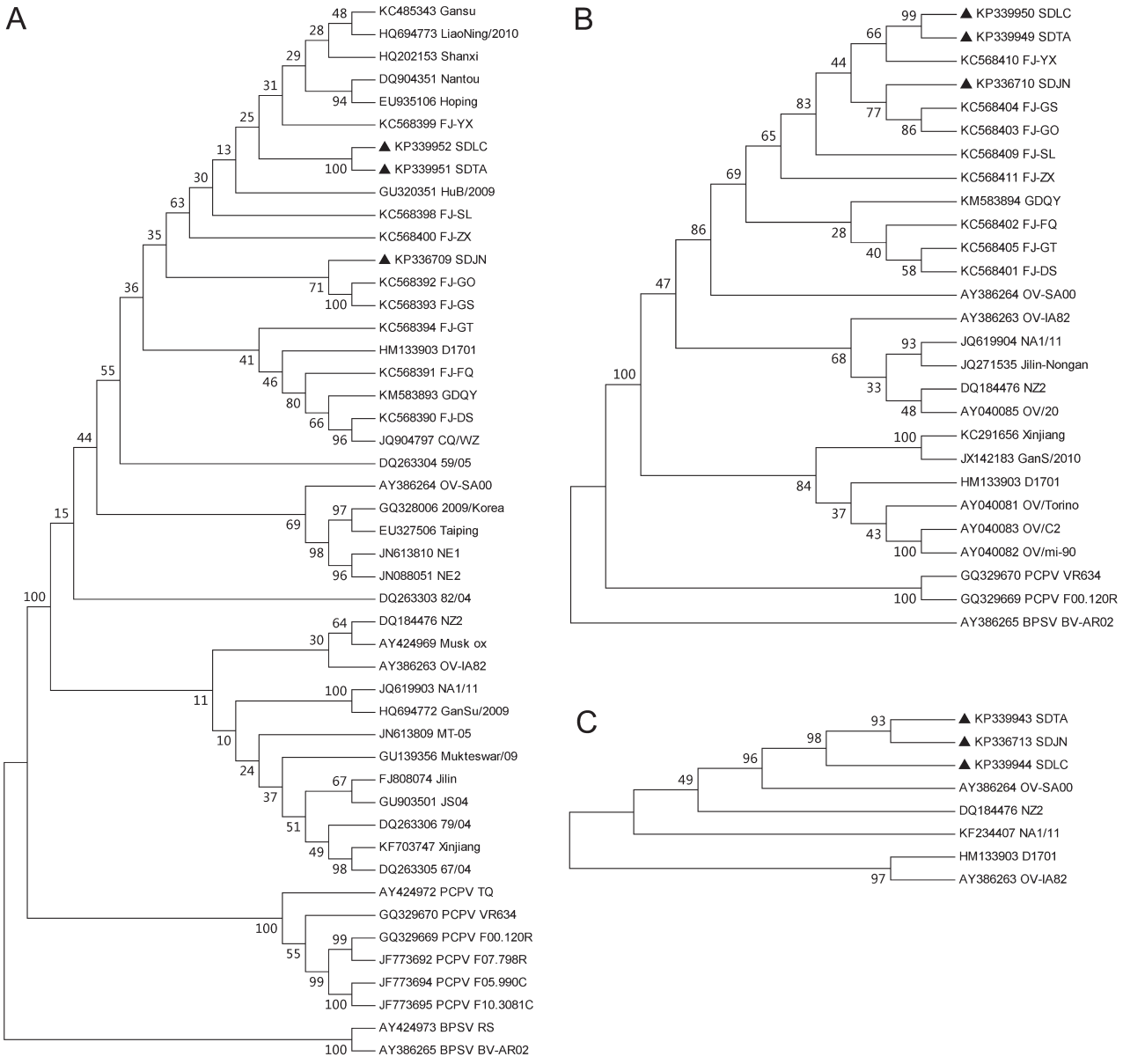

Fig. 1. Phylogenetic analysis based on nucleotide sequences of *ORFV011* (A), *ORFV059* (B) and *ORFV127* (C). The phylogenetic relationship was constructed by the neighbor-joining algorithm using the MEGA 5.0 software. Bootstrap values (%) are represented at each tree node. Black triangle, strains isolated from Shandong Province.

strains. *ORFV011* and *ORFV059* of SDJN shared 99.1% and 97.4% nucleotide identities with those of the FJ-GS and FJ-GO strains, respectively. *ORFV059* of the SDLC and SDTA strains cluster together with that of FJ-YX. This implies that the SDLC and SDTA strains might originate from the FJ-YX strain and that SDJN may derive from the FJ-GS and FJ-GO strains. We hypothesized that these three orf virus strains originated in Fujian Province and formed a complex group of viruses in Shandong Province.

Because of the role of IL-10 in limitation and termination of the inflammatory response during tissue repair, *ORFV127,* which encoded an IL-10-like protein, is also regarded as having a function related to immune evasion in ORFV infection [10, 30]. The phylogenetic analysis of the *ORFV127* genes of these three isolated viruses indicated that they were similar to that of the OV-SA00 strain. We hypothesized that the pathogenesis of these three virus strains may be similar to that of OV-SA00 based on the function of *ORFV127*. *ORFV109* and *ORFV110* encode envelope type II glycoproteins, and these two genes are highly variable and unsuitable for virus orthological analysis [9]. We will continue to isolate and characterize ORFV strains from different locations

in Shandong Province to obtain more detailed information.

In summary, we isolated three orf virus strains from different districts in Shandong Province. Based on the sequences of the *ORFV011*, *ORFV059* and *ORFV127* genes, they were novel ORFV strains, and phylogenetic analysis revealed that they clustered together with Fujian strains and formed a complex group of viruses in Shandong Province. Taken together, new phylogenetic information about ORFV strains in Shandong Province were obtained, and the data reported here will be helpful for further research about ORFV and its comprehensive prevention and control in East China.

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