Virus Genes (2007) 35:705–712
DOI 10.1007/s11262-007-0144-6

Identification and phylogenetic analysis of orf virus from goats in Taiwan

Kun-Wei Chan · Jen-Wei Lin · Shu-Hwae Lee · Chih-Jung Liao · Meng-Chun Tsai · Wei-Li Hsu · Min-Liang Wong · Hung-Chang Shih

Received: 2 October 2006 / Accepted: 11 December 2006 / Published online: 8 August 2007

Abstract An outbreak of contagious ecthyma in goats in central Taiwan was investigated. The disease was diagnosed by physical and histopathologic examinations, and the etiology of the disease was identified as orf virus by electron microscopy and polymerase chain reaction (PCR) and sequence of major envelope protein (B2L) gene. The entire protein-coding region of B2L gene were cloned and sequenced. Phylogenetic analysis of B2L amino acid sequences showed that the orf virus identified in this outbreak was closer to the Indian ORFV-Mukteswar 59/05 isolate. This is the first report on the molecular characterization of orf virus in Taiwan.

Keywords Orf virus · B2L gene · Phylogenetic analysis

K.-W. Chan · J.-W. Lin · M.-L. Wong (✉)
Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung 402, Taiwan
e-mail: mlwong@dragon.nchu.edu.tw

S.-H. Lee
Animal Health Research Institute, Council of Agriculture, Tamsui 251, Taiwan

C.-J. Liao · M.-C. Tsai
Nantou Animal Diseases Control Center, Natou 540, Taiwan

W.-L. Hsu
Graduate Institute of Veterinary Public Health, College of Veterinary Medicine, National Chung-Hsing University, Taichung 402, Taiwan

H.-C. Shih
Department of Laboratory Medicine, China Medical University Hospital, Taichung 404, Taiwan

Introduction

Poxviruses are large DNA viruses containing a double-stranded DNA genome with closed hairpin loop ends. The family *Poxviridae* is divided into *Entomopoxvirinae* (insect poxviruses) and *Chordopoxvirinae* (vertebrate poxviruses) subfamilies; and the latter is further divided into eight genera, including parapoxviruses [1].

Genus parapoxvirus has four members, orf virus (ORFV), bovine popular stomatitis virus (BPSV), pseudocowpox virus (PCPV), and parapoxvirus of red deer in New Zealand (PVNZ). The orf virus is the prototype of the parapoxvirus genus. It can cause contagious ecthyma in goats, sheep, and other ruminants worldwide. The viruses are sometimes transmissible to humans due to direct contact [1–3].

Parapoxviruses are morphologically distinguished from other members of poxvirus genera by their ovoid shape, the crisscross pattern on the particle surface, and relatively small size [4–7]. The virus particles are ovoid, about 260 nm in length and 160 nm in width [4]. The viral genome consists of the linear double-stranded DNA (134–139 kb). Essential genes are located in the conserved central part of the genome, and variability is observed in the terminal ends [5]. Genes in near-terminal regions of the genome encode factors with important roles in viral–host interactions such as modulating host responses to infection and determining host range, but often not essential for growth in cultured cells [2, 8]. The B2L gene resides in the *Bam*HI B fragment of NZ-2 strain and encodes a major envelope protein of 42 kDa [9]. The B2L gene has been used for detection of orf virus by PCR [10–17]

Infected animals develop contagious ecthyma, which also has other synonyms as orf, infectious labial dermatitis, scabby mouth, contagious pustular dermatitis, or sore mouth [4, 18]. The disease is characterized by proliferative

lesions around the muzzle and lips (scabby mouth), and sometimes also affects the gums and tongue, especially in young lambs. Eyelids, feet, and teats can be affected occasionally. Rarely, the lesions may extend into the esophagus, stomach, intestines, or respiratory tract. The disease lasts for 3–4 weeks and usually resolves in 1–2 months, with mortality rates up to 10% and 93% in lambs and kids, respectively [19–21].

Zoonosis occurs through abrasions in the skin, most frequently during drenching, docking, shearing, lambing, or slaughtering [1, 4]. In humans, 2–4 days after onset of the symptoms, the lesions proceed from macular through popular to large painful nodules, sometimes became papillomatous. Hands, face, and arms are often the major sites of lesions. The lesions normally last 4–9 weeks, but usually 6–7 weeks [2].

The orf infection occurs endemically in Taiwan but is only occasionally reported, probably because of its low severity and low economic loss. In this work, we studied an outbreak of orf virus infection in goats and verified the identity of the virus by PCR. The full-length B2L gene of orf virus from field isolated in central Taiwan was cloned and completely sequenced. This is the first report on the phylogenetic analysis of orf virus found in Taiwan with other isolates worldwide.

Materials and methods

Disease outbreak

Materials were taken from an outbreak of orf virus in goats which occurred in a farm with 250 goats including 200 breeders and 50 kids in the Nantou County, Taiwan, in 2006. Fifteen out of 50 lambs in the flock developed "wart-like" lesions around the mouth. In young kids, the lesions appeared on the muzzle, lips, gums as well as on tongue. Two of the affected 2-week-old lambs died due to inanition, presumably resulting from the suckling difficulty caused by severe facial and oral lesions in lambs. The morbidity and the mortality of this disease were 15 in 250 (6%) and 2 in 250 (0.8%), respectively. The verrucose masses of ulcerative lesions were found along the muzzle. Lesions were nodular with various sizes ranging from approximately 2–6 mm in diameter. Among 15 infected animals, 13 of them recovered 15–20 days after clinical signs appeared.

Necropsy and histopathologic examination

Biopsies of the skin and lip of affected goat were sent for pathologic evaluation. Biopsy samples collected from the submitted carcasses were either fixed in 10% buffered formalin for histological examination, or were un-fixed for PCR analysis and for electron microscopic evaluation. Necropsy was performed on brain, heart, liver, kidney, lungs, skin, and spleen. Organ samples of the carcasses including lungs and skin were fixed as above. The samples were trimmed, dehydrated, embedded in paraffin and stained with hematoxylin and eosin (H&E) and examined by light microscope.

Electron microscopy

The specimen taken from lips of affected goat was negatively stained with 2% phosphor-tungstic acid followed by electron microscopy.

DNA isolation and PCR

The DNA of the skin samples collected from lip lesions of affected animals was extracted by QIAamp DNA Mini Kit (QIAGEN, TAIGEN Bioscience Corporation, Taiwan) according to the manufacturer's instructions. Briefly, vesicular lesions around lower lip from the affected goat were triturated in sterile 0.1 M phosphate buffered saline. The homogenized sample was then extracted with tissue lysis buffer containing proteinase K, and the mixture was incubated 56°C overnight. Finally, the mixture was passed through a column and DNA was purified from the column.

Diagnostic PCR employing PPP-1 and PPP-4 primers [10] was carried out using DNA extracted from skin lesions. For phylogenetic analysis, full-length B2L gene was also amplified from this DNA using OVB2LF1 and OVB2LR1 primers [17], and its sequence determined. The sequences of two sets of the PCR primers and their corresponding binding sites relative to the B2L gene in the viral genome are illustrated in Fig. 1.

PCR was carried out following the method described [17] with minor modifications. Briefly, reaction was performed in 50 µl of reaction mixture, which contained 5 µl of 10× buffer, 200 µM of each dNTP, 2 nmol of each oligonucleotide primer, 100 ng of each DNA sample, and 5 units of OptiPol DNA polymerase (GeneTeks BioScience Inc., Canada). PCR had initial denaturation step of 94°C for 3 min followed by 29 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 7 min. After amplification, 5 µl of PCR products was analyzed with 1% agarose gel that was subsequently stained with ethidium bromide for visualization of the expected product under UV light.

Cloning and sequencing of PCR products containing B2L gene

The PCR product of expected size (~1.2 kb) was purified from gel using MiniElute gel extraction kit (QIAGEN,

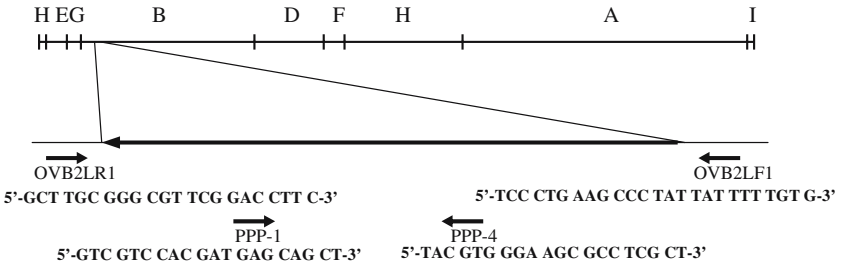

Fig. 1 The order of DNA fragments is as per the *Bam*HI digestion map of ORFV NZ-2 strain as described earlier [9]. The primers set OVB2LR1, OVB2LF1 and PPP-1, PPP-4 were designed according to those of Hosamani and colleagues [17] and of Inoshima and colleagues [10], respectively

TAIGEN Bioscience Corporation, Taiwan) according to manufacturer's procedures. Ten microliter of the purified fragment containing full-length of B2L gene was then cloned into the pTA cloning vector (pTA easy cloning kit, Genomics Corp, Taiwan) and transformed into host cell, *E. coli* strain Top10. The authenticity of resultant plasmids was initially determined by restriction endonuclease (*Eco*RI and *Bam*HI) digestion followed by agarose gel electrophoresis to confirm the existence of insert. Three positive clones were sent for automated sequencing using M13 forward and M13 reverse universal primers (Mission Biotech, Taipei, Taiwan). Complete Sequences of the B2L gene were submitted to the NCBI GenBank database and was assigned the accession number, DQ904351 (called the Nantou isolate).

Phylogenetic analysis

Comparison of the Nantou isolate sequences with those of parapoxviruses available in the Genbank database was performed by using the online BLAST program. Sequence identities of nucleotides as well as of amino acids were analyzed by the Clustal W method [22]. Deduced amino acid sequences were assembled into multiple sequence alignment. A phylogenetic tree derived from deduced amino acid sequences was constructed for the parapoxviruses by using neighbor-joining method of MEGA version 3.1 [23].

Results and discussion

Necropsy and microscopic analysis

The goats had papillary, verrucose, multifocal to coalescing, ulcerated, proliferative lesions in the epidermis of lips, muzzle, and tongue. The lips and muzzle were almost completely covered by a 1–2 cm thick, fissured crust of malodorous material masses (Fig. 2A).

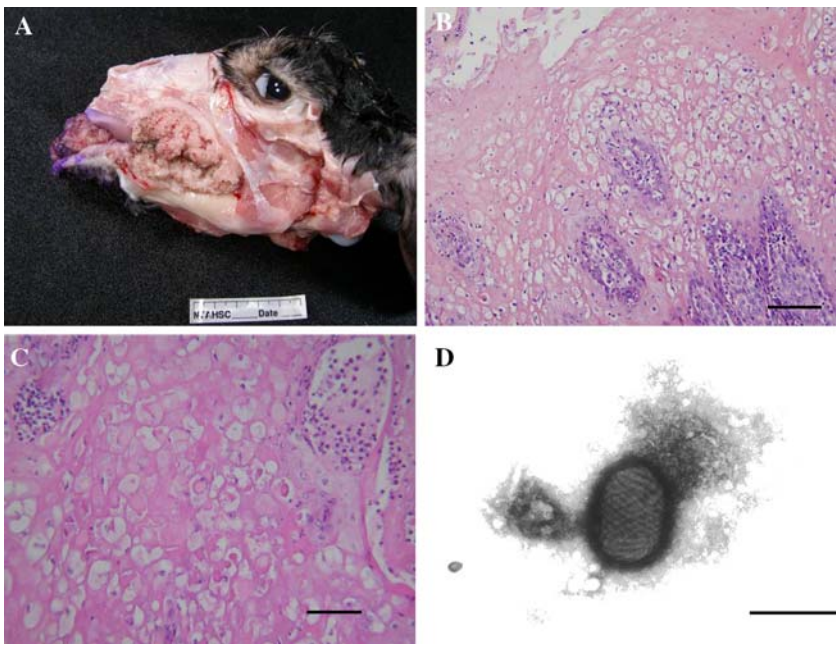

Fig. 2 Pathologic findings and electron microscopy study of orf. (**A**) Papillomatous lesions in lips and muzzle were noticed on necropsy. (**B**) Keratinocytes of the stratum spinosum show the changes including acanthosis, vacuolation, vesiculation, and ballooning degeneration. (H&E, 200×; bar = 100 μm). (**C**) Intracytoplasmic eosinophilic inclusion bodies in the acanthocytes indicated virus-induced lesions. (H&E, 400×; bar = 50 μm). (**D**) Electron microphotograph showing the characteristic morphology of an orf virion from the skin lesion of lip (bar = 100 nm)

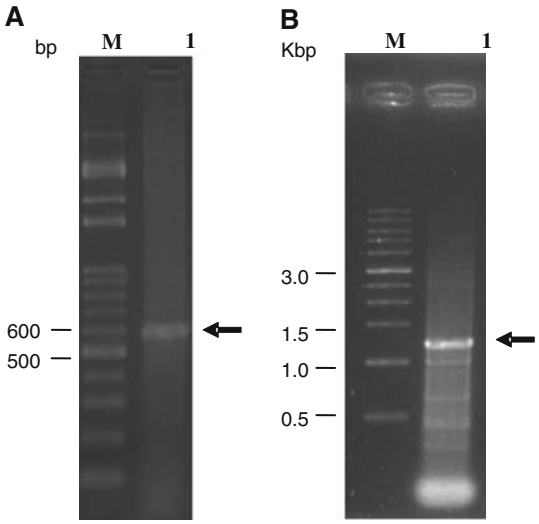

Fig. 3 Amplification of major envelope gene (B2L) gene by PCR. (**A**) Lane 1: PCR product (594 bp) containing partial portion of the B2L gene (arrowhead); M: DNA size markers (bp). The identity of 594 bp product was determined by DNA sequencing. (**B**) Lane 1: PCR product (1206 bp) containing the complete coding region of B2L gene (arrowhead); M: DNA size markers (kb). The identity of 1206-bp product was also determined by DNA sequencing

Microscopically, the lesions of lips, muzzle, and tongue were characterized by marked epidermal hyperplasia and hyperkeratosis (Fig. 2B). The epidermic lesions were multifocally ulcerated and covered by serocellular crusts containing numerous bacterial colonies. The superficial layers of the epidermis underwent degenerative changes consisting of vesiculation, and pustule formation. Keratinocytes of the stratum spinosum showed vacuolation and ballooning degeneration (Fig. 2B). Characteristic intracytoplasmic eosinophilic inclusion bodies were found in keratinocytes of lesions (Fig. 2C). Suppurative and a moderate to acute level of lobular bronchopneumonia were found in the lung, which might be caused by the difficulty in consuming food and water.

Electron microscopy

Electron microscopic examination of skin lesion material from goat revealed the presence of characteristic parapoxvirus virions. The ovoid-shape virion had a characteristic spiral crisscross pattern against an electron dense background core. The size of virion was approximately 125 nm in length and 80 nm in width (Fig. 2D).

PCR, cloning, and DNA sequencing

The methods routinely used in diagnosis of orf infection are based on clinic symptoms, pathologic examinations, and time-consuming virus isolation. In the past 15 years, polymerase chain reaction (PCR) has been widely used to amplify the desired genomic materials from tissue specimens and has become as a powerful tool for molecular diagnosis. A PCR method using primers to amplify B2L gene was developed. This method successfully detected DNA from four members in parapoxvirus genus and was able to diagnose the orf virus infection from field specimens of affected animals [10].

To confirm the causative agent, the first goal was to amplify a partial region of BL2 gene by PCR. The B2L

Table 1 B2L genes of parapoxviruses used in phylogenetic analysis

Virus	Species affected	Accession number	Country of isolation	Reference
ORFV-Nantou	Goat	DQ904351	Taiwan	This study
ORFV-Shanhjahnpur 82/04	Goat	DQ263303	India	Hosamani et al. [17]
ORFV- Mukteswar 59/05	Goat	DQ263304	India	Hosamani et al. [17]
ORFV-caprine isolate	Goat	AY278208	USA	Guo et al. [24]
ORFV-vaccine strain isolate from goats	Goat	AY278209	USA	Guo et al. [24]
ORFV-SA00	Goat	AY386264	USA	Delhon et al. [5]
ORFV-NZ2	Sheep	OVU06671	New Zealand	Sullivan et al. [9]
ORFV-Izatnagar 79/04	Sheep	DQ263306	India	Hosamani et al. [17]
ORFV-Mukteswar 67/04	Sheep	DQ263305	India	Hosamani et al. [17]
ORFV-ovine isolate	Sheep	AY424970	USA	Guo et al. [13]
ORFV-IA82	Sheep	AY386263	USA	Delhon et al. [5]
ORFV-takin isolate	Takin	AY424971	USA	Guo et al. [13]
ORFV-Musk ox	Musk ox	AY424969	USA	Guo et al. [13]
Pseudocowpox virus	Cow	AY424972	Not available	Guo et al. [13]
Bovine papular stomatitis virus	Calf	AY424973	Not available	Guo et al. [13]
Bovine papular stomatitis virus-AR02	Calf	AY386265	USA	Delhon et al. [5]

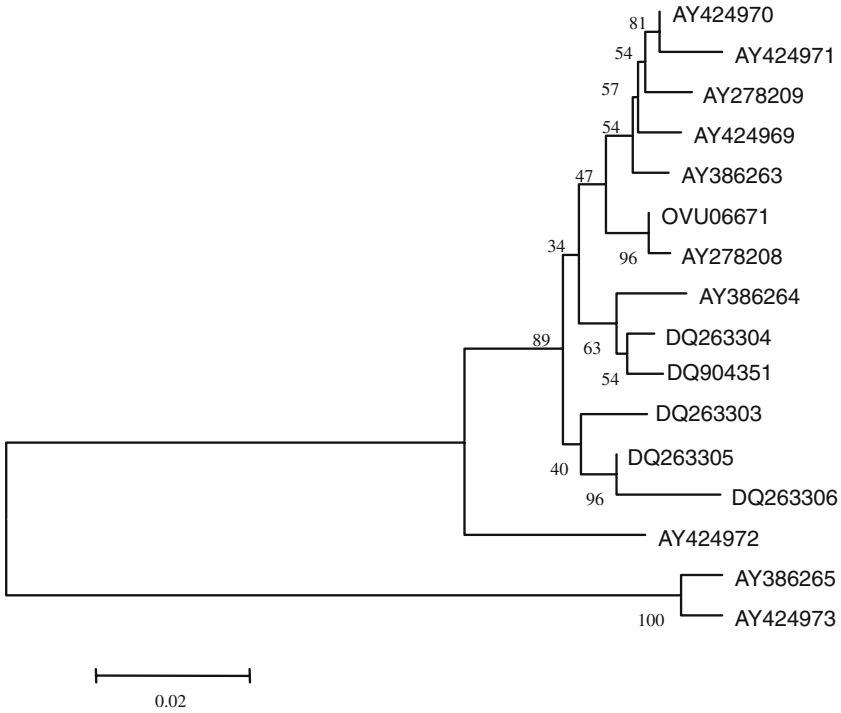

Fig. 4 Phylogenetic analysis of different parapoxviruses based on deduced amino acid sequence of complete B2L gene. The phylogenetic relationship was constructed by using the neighbor-joining program of MEGA version 3.1, and bootstrap analysis was performed with 1000 trials. The Nantou isolate has an accession number DQ 904351 in GenBank. The scale bar beneath the tree stands for amino acid substitution per site

PCR has been developed for laboratory diagnosis of orf virus infection [2, 10]. Primers PPP-1 and PPP-4, described as universal primers to amplify most species of parapoxviruses [10, 15], were used in this study. A specific product of expected size (594 bp) representing the region (157–750 nt) of BL2 gene was amplified from the sample extracted from lip lesions of affected goat (Fig. 3A). The 594-bp DNA fragment was automated sequenced, and the nucleotide sequence data showed it was orf virus sequences.

Table 2 The percent identities and divergence of deduced amino acid sequences of B2L gene from different parapoxvirus strains/isolates by clustal W

Divergence	Percent identity																	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**		
1	■	97.3	97.2	97.5	98.2	98.0	85.0	97.5	97.5	97.1	94.4	84.7	97.2	98.2	97.9	97.5	**1**	DQ904351
2	2.8	■	99.8	98.8	98.3	97.9	84.7	98.5	98.5	98.2	95.3	84.7	96.9	97.4	98.2	97.8	**2**	QVU06671
3	2.9	0.2	■	98.6	98.2	97.7	84.6	98.3	98.3	98.0	95.1	84.6	96.8	97.3	98.0	97.6	**3**	AY278208
4	2.6	1.2	1.4	■	99.0	98.1	85.0	99.0	99.1	98.8	95.3	85.0	97.1	97.6	97.9	97.6	**4**	AY278209
5	1.9	1.7	1.9	1.0	■	98.0	85.5	99.0	99.0	98.6	95.0	85.3	97.8	98.3	98.5	98.0	**5**	AY386263
6	2.1	2.1	2.3	2.0	2.1	■	85.0	98.0	98.0	97.6	95.2	85.0	97.1	98.0	97.9	97.7	**6**	AY386264
7	16.9	17.1	17.4	16.8	16.3	16.9	■	85.0	85.0	84.7	86.1	98.3	84.5	84.9	85.2	85.0	**7**	AY386265
8	2.6	1.5	1.7	1.0	1.1	2.1	16.8	■	99.7	99.3	95.0	84.9	96.9	97.6	97.8	97.5	**8**	AY424969
9	2.6	1.5	1.7	0.9	1.1	2.1	16.8	0.4	■	99.5	95.0	85.0	96.9	97.6	97.8	97.5	**9**	AY424970
10	3.0	1.9	2.1	1.2	1.4	2.4	17.2	0.7	0.5	■	94.7	84.5	96.6	97.3	97.5	97.1	**10**	AY424971
11	5.9	4.9	5.1	4.9	5.2	5.0	15.5	5.2	5.2	5.6	■	85.9	94.5	94.7	95.4	95.1	**11**	AY424972
12	17.2	17.1	17.4	16.8	16.5	16.9	1.7	17.0	16.9	17.5	15.7	■	84.3	84.8	85.0	85.0	**12**	AY424973
13	2.9	3.2	3.3	3.0	2.2	3.0	17.5	3.2	3.2	3.5	5.8	17.8	■	97.6	97.7	97.5	**13**	DQ263303
14	1.9	2.6	2.8	2.4	1.8	2.1	17.0	2.4	2.4	2.8	5.6	17.1	2.5	■	98.2	97.9	**14**	DQ263304
15	2.1	1.9	2.1	2.1	1.5	2.1	16.6	2.2	2.2	2.6	4.8	16.8	2.3	1.9	■	99.1	**15**	DQ263305
16	2.6	2.2	2.4	2.5	2.1	2.3	16.9	2.6	2.6	3.0	5.1	16.9	2.6	2.1	0.9	■	**16**	DQ263306
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**		

To gain further information of the viral sequences, we performed another PCR reaction to obtain the full-length B2L gene. Primer design was based on the sequences reported previously [17]. Using DNA purified from lip lesions of affected goat as template and the pair of OVB2LF1/OVB2LR1 primers, PCR resulted in the

```
DQ904351 MWPFSSIPVG ADCRVVETLP AEVASLAQGN MSTLDCFTAI AESAKKFLYI CSFCCNLSST KEGVDVKDKL   70
DQ263303 .......... .......... .......... .......... T......... .......... ..........   70
DQ263304 .......... .......... .......... .......... .......... .......... ..........   70
AY278208 ........L. .......... .......... .......... .......... .......... ..........   70
AY278209 ....YF..L. .......... .......... .......... .......... .......... ..........   70
AY386264 .......... .......... .......... .......... .......... .......... ..........   70
OVU06671 ........L. .......... .......... .......... .......... .......... ..........   70
AY386263 ........L. .......... .......... .......... T......... .......... ..........   70
AY424970 ....F...L. .......... .......... .......... .......... .......... ..........   70
DQ263305 .......... .......... .......... .......... .......... .......... ..........   70
DQ263306 .......... ..R....... .......... .......... .......... .......... ..........   70
AY424971 ....F...L. .......... .......... .......... .......... .......... ..........   70
AY424969 ........L. .......... .......... .......... .......... .......... ..........   70
AY386265 .......... .E........ ...S..QV.. ...FE..S.. V...T..... A......G.. R.........   70
AY424973 .......... .E........ ...S..QV.. ...FE..S.. V...T..... A......G.. R.........   70
AY424972 .......... .......... .......... .......... .......... .......... ..........   70

DQ904351 CTLAKEGVDV TLLVDVQSKD KDADELREAG VNYYKVKVST REGVGNLLGS FWISDTGHWY VGSASLTGGS  140
DQ263303 ........N. .......... .......... .......... .......... ..L....... ..........  140
DQ263304 .......... .......... .......... .......... .......... ..L..A.... ..........  140
AY278208 .......... .......... .......... .......... K......... ..L..A.... ..........  140
AY278209 ........N. .......... .......... .......... K......... ..L..A.... ..........  140
AY386264 .......... .......... .......A.. .......... K......... ..L....N.. ..........  140
OVU06671 .......... .......... .......... .......... K......... ..L..A.... ..........  140
AY386263 ........N. .......... .......... .......... .......... ..L..A.... ..........  140
AY424970 ........N. .......... .......... .......... K......... ..L..A.... ..........  140
DQ263305 .......... .......... .......... .......... .......... ..L..A.... ..........  140
DQ263306 .......... .......... .......... .......... .......... ..L..A.... ..........  140
AY424971 ........N. .......... .......... .......... K.......D. ..L..A.... .D........  140
AY424969 ........N. .......... ...N...... .......... K......... ..L..A.... ..........  140
AY386265 .....S..N. .V........ R..E...... .........G .DD....... ....NA.Q.. ..........  140
AY424973 .....S..N. .V........ R..E...... .........G .DD....... ...CNA.Q.. ..........  140
AY424972 .....D..N. .I........ .......... .......... K......... ..L..A.Q.. ..........  140

DQ904351 VSTIKNLGLY STNKHLAWDL MNRYNTFYSM IVEPKVPFTR LCCAVVTPTA TNFHLNHSGG GVFFSDSPER  210
DQ263303 .......... .........P .......... .......... .......... .......... ..........  210
DQ263304 .......... .......... .......... .......... .......... .......... ..........  210
AY278208 .......... .......... .......... .......... ....I..... .....D.... ..........  210
AY278209 .......... .......... .......... .......... .......... .....D.... ..........  210
AY386264 .......... .......... .......... .......... .......... .......... ..........  210
OVU06671 .......... .......... .......... .......... ....I..... .....D.... ..........  210
AY386263 .......... .......... .......... .......... .......... .....D.... ..........  210
AY424970 .......... .......... .......... .......... .......... .....D.... ..........  210
DQ263305 .......... .......... .......... .......... ....I..... .......... ..........  210
DQ263306 .......... .......... .......... .......... ....I..... .......P.. ..........  210
AY424971 .......... .......... .......... .......... .......... .....D.... ..........  210
AY424969 .......... .......... .......... .......... .......... .....D.... ..........  210
AY386265 I.N.....V. ...R...T.. .......... ........S. ....MI.... .D...D.A.. ......A..K  210
AY424973 I.N.....VC ...R...T.. .......... ........S. ....MI.... .D...D.A.. ......A..K  210
AY424972 .......... .......A.. .......... .......... ....I..... .D........ ..........  210

DQ904351 FLGFYRTLDE DLVLHRIENA KNSIDLSLLS MVPVIKHAGA VEYWPRIIDA LLRAAIDRGV RVRVIITEWK  280
DQ263303 .......... .......... .......... ........S. .....Q.... ......N... ..........  280
DQ263304 .......... .......... .......... .......... .......... .......... ..........  280
AY278208 .......... .......... .......... ........S. .....Q.... ......N... ..........  280
AY278209 .......... .......... .......... ........S. .....Q.... .......... ..........  280
AY386264 .......... .......... .......... .......... .......... ......N... ..........  280
OVU06671 .......... .......... .......... ........S. .....Q.... ......N... ..........  280
AY386263 .......... .......... .......... ........S. .......... .......... ..........  280
AY424970 .......... .......... .......... ........S. .......... .......... ..........  280
DQ263305 .......... .......... .......... ........S. .....Q.... ......N... ..........  280
DQ263306 .......... .......... .......... ....V...S. .....Q.... .....TN... ..........  280
AY424971 .......... .......... .......... ........S. .......... .......H.. ..........  280
AY424969 .......... .......... .......... ........S. .......... .......... .........C.  280
AY386265 .......... .......DS. .......... L....R..DR .......M.. .........S. .....V....  280
AY424973 .......... .......DS. .......... L....R..DR .......M.. .........S. .....V....  280
AY424972 .......... .......... N......... ........S. .....Q.... ......N... ...I.T....  280
```

Fig. 5 Clustal W multiple alignment of deduced amino acid sequences of B2L from different isolates of parapoxviruses

```
DQ904351 NADPLSVSAA RSLDDFGVGS VDMSVRKFVV PGRDDAANNT KLLIVDDTFA HLTVANLDGT HYRYHAFVSV  350
DQ263303 .......... ...N...... .......... .......... .......... .......... ..........  350
DQ263304 .......... .......... .......... .......... ....E..... .......... ..........  350
AY278208 .......... .......... .......... ..G....... .......... .......... ..........  350
AY278209 .......... .......... .......... .......... .......... .......... ..........  350
AY386264 .......... .......... .......... .......... .......... .......... ..........  350
OVU06671 .......... .......... .......... .......... .......... .......... ..........  350
AY386263 .......... .......... .......... .......... .......... .......... ..........  350
AY424970 .......... .......... .......... .......... .......... .......... ..........  350
DQ263305 .......... ...N...... .......... .......... .......... .......... ..........  350
DQ263306 .......... ...N.....G .......... .......... .......... .......... ..........  350
AY424971 .......... .......... .......... .......... .......... .......... ..........  350
AY424969 .......... .......... .......... .......... .......... .......... ..........  350
AY386265 .......... .T.N...... I.I.T.L.SI .......... .......... .V....M... ..K.......  350
AY424973 .......... .T.N...... I.I.T.L.SI .......... .......... .V....M... ..K.......  350
AY424972 ......L... ...N...... .........I .......... .......... .V........ ..........  350

DQ904351 NAEKGDIVKD LSAVFERDWR SEFCKPIN 378
DQ263303 .......... .......... ........ 378
DQ263304 .......... .......... ........ 378
AY278208 .......... .......... ........ 378
AY278209 .......... .......... ........ 378
AY386264 .......... .......... ........ 378
OVU06671 .......... .......... ........ 378
AY386263 .......... .......... ........ 378
AY424970 .......... .......... ........ 378
DQ263305 .......... .......... ........ 378
DQ263306 .......... .......... ........ 378
AY424971 .......... .......... ........ 378
AY424969 .......... .......... ........ 378
AY386265 ........NQ .......... .QY..... 378
AY424973 ........NQ .......... ........ 378
AY424972 .......... R......... ........ 378
```

Fig. 5 continued

amplification of B2L gene with an expected DNA fragment (1206 bp) (Fig 3B). The identification of PCR product containing B2L gene was initially determined by restriction enzyme. As expected, two fragments with sizes of ~1000 bp and ~200 bp were generated by enzyme *Sac*I digestion (data not shown), supporting the presence of *Sac*I located at conserved region of B2L gene. The purified PCR products were then cloned into a TA vector and the sequences of three clones were identical and the sequence data was submitted to GenBank.

Phylogenetic analysis

DNA sequences comparisons of the full length of B2L obtained by PCR amplification were conducted to determine the phylogenetic relationship (Table 1). Using nucleotide sequences of entire B2L gene in the GenBank, phylogenetic relationship between the Nantou isolate and other isolates was constructed. Phylogenetic relationship was obtained by neighbor-joining method and bootstrap analysis. Numbers of the tree branches are the bootstrap support calculated per 1000 bootstrap replicates (Fig. 4). Results of amino acids sequences comparison of B2L demonstrated that the Nantou isolate was closer to the ORFV-Mukteswar 59/05 (DQ263304) isolated from India (Fig. 4). We obtained similar results from nucleotide sequences analysis of B2L gene (data not shown). The sequences of the Nantou isolate and the ORFV-Mukteswar 59/05 show 98.2 and 98.9% similarities at the nucleotide and amino acid level, respectively. As shown in Table 2, analysis of the amino acid sequences of B2L among ORFV, PCPV, and BPSV showed high identity (>82.8%). It was found that the central genome region (open reading frame 009 to 111) in parapoxvirus is generally more conserved in comparison with two terminal regions, open reading frame 001 to 008 and 112 to 134, respectively [5]. The B2L is located in the open reading frame 011 and is also conserved in different isolates.

It was demonstrated that many substitutions of amino acids dispersed along the B2L polypeptides of different isolates [17]; and the B2L of Nantou isolate also showed similar feature. Multiple alignment of amino acid sequences of the Nantou isolate revealed three unique substitutions (I123, T126, and V325) that were different from those (L123, A126, and E325) of the ORFV-Mukteswar 59/05. In addition, the Nantou isolate contains a unique isoleucine substitution at position 123, which is leucine in other orf virus isolates (Fig. 5).

Orf is endemic occasionally in Taiwan and no vaccination program was performed to control the disease. The methods routinely used in the diagnosis of orf virus infection are electron microscopy and PCR (e.g. 140-bp

DNA of viral RNA polymerase gene) in Taiwan [2]. The morbidity and economic losses are not significant because of improvement of management and practicing disinfection regularly. Although zoonosis had been reported in literature, but the workers in this orf outbreak did not display the clinical symptoms. In this study, we used the full length of B2L gene to analyze the genetic relationship in the other orf isolates. We conclude that Taiwan orf virus is phylogenetically close to other orf viruses reported worldwide, especially the ORFV-Mukteswar 59/05 isolated in India.

Acknowledgement We thank Dr. Shih-Chieh Chang (Department of Veterinary Medicine, National Chung-Hsing University) for helpful discussion. This work was supported in part by a grant from The Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Council of Agriculture, Taiwan.

References

1. F.A. Murphy, E.P.J. Gibbs, M.C. Horzinek, M.J. Studdert, in *Veterinary Virology*, ed. by F.A. Murphy, E.P.J. Gibbs, M.C. Horzinek, M.J. Studdert (Academic Press, New York, 1999), pp 277–291
2. E.G. Torfason, S. Gunadottir, J. Clin. Virol. **24**, 79 (2002)
3. A.J. Robinson, A.A. Mercer, Virology **208**, 812 (1995)
4. F. Fenner, J.J. Esposito, in *Fields Virology*, ed. by D.M. Knipe, P.M. Howley, D.E. Griffin, M.A. Martin, R.A. Lamb, B. Roizman, S.E. Straus (Lippincott, Williams and Wilkins, Philadelphia, PA, 2001), pp 2885–2921
5. G. Delhon, E.R. Tulman, C.L. Afonso, Z. Lu, A. de la Concha-Bermejillo, H.D. Lehmkuhl, M.E. Piccone, G.F. Kutish, D.L. Rock, J. Virol. **78**, 168 (2004)
6. C. Kottaridi, K. Nomikou, R. Lelli, P. Markoulatos, O. Mangana, J. Virol. Methods **134**, 119 (2006)
7. C. Kottaridi, K. Nomikou, L. Teodori, G. Savini, R. Lelli, P. Markoulatos, O. Mangana, Vet. Microbiol. **116**, 310 (2006)
8. A.A. Mercer, N. Ueda, S.M. Friederichs, K. Hofmann, K.M. Fraser, T. Bateman, S.B. Fleming, Virus Res. **116**, 146 (2006)
9. J.T. Sullivan, A.A. Mercer, S.B. Fleming, A.J. Robinson, Virology **202**, 968 (1994)
10. Y. Inoshima, A. Morooka, H.J. Sentsui, Virol. Methods **84**, 201 (2000)
11. Y. Inoshima, K. Murakami, T. Yokoyama, H. Sentsui, J. Gen. Virol. **82**, 1215 (2001)
12. M.K. Tikkanen, C.J. McInnes, A.A. Mercer, M. Buttner, J. Tuimala, V. Hirvela-Koski, E. Neuvonen, A. Huovilainen, J. Gen. Virol. **85**, 1413 (2004)
13. J. Guo, J. Rasmussen, A. Wunschmann, A. de la Concha-Bermejillo, Vet. Microbiol. **99**, 81 (2004)
14. Y. Kanou, Y. Inoshima, T. Shibahara, Y. Ishidawa, K. Kadota, S. Ohashi, K. Morioka, K. Yoshida, S. Yamad, JARQ **39**, 197 (2005)
15. J. Klein, M. Tryland, Virol. J. **2**, 79 (2005)
16. A. Nitsche, M. Buttner, S. Wilhelm, G. Pauli, H. Meyer, Clin. Chem. **52**, 316 (2006)
17. M. Hosamani, V. Bhanuprakash, A. Scagliarini, R.K. Singh, Vet. Microbiol. **116**, 317 (2006)
18. H.W. Reid, in *Disease of Sheep*, ed. by W.B. Martin, I.D. Aitken (Oxford, Blackwell scientific publications, London, 1991), pp 265–269
19. C. Mazur, R.D. Machado, Vet. Rec. **125**, 419 (1989)
20. R.C. Gumbrell, D.A. McGregor, Vet. Rec. **141**, 150 (1997)
21. B. Mondal, A.K. Bera, M. Hosamani, P.A. Tembhurne, S.K. Bandyopadhyay, Vet. Res. Commun. **30**, 531 (2006)
22. J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. **22**, 4673 (1994)
23. S. Kumar, K. Tamura, M. Nei, Brief Bioinform. **5**, 150 (2004)
24. J. Guo, Z. Zhang, J.F. Edwards, R.W. Ermel, C. Taylor Jr., A. de la Concha-Bermejillo, Virus Res. **93**, 169 (2003)