博士論文

Molecular Epidemiological Studies on Animal Papillomaviruses

（動物のパピローマウイルスに関する分子疫学的研究）

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General Introduction

1. The family *Papilomaviridae*

The family *Papillomaviridae* is non-enveloped double-stranded DNA virus of small-sized virion about 55 nm with icosahedral structure (Van Doorslaer et al., 2018). Papillomaviruses (PVs) have been isolated from wide vertebrae species including birds, fish, mammals and reptiles. The name *papilloma* derived from Latin "*papilla"* meaning "nipple" and the Greek suffix "*-oma"* meaning "tumor". Since the first discovery of DNA tumor virus, cottontail rabbit papillomavirus (CRPV), made by Dr. Richard Shope in the 1930s (Shope and Hurst, 1933), PVs have been studied for decades. Studies on animal PVs have enabled investigators to study their biologic and biochemical characteristics. Advancing technologies in genomic research, such as polymerase chain reaction (PCR), molecular cloning, and sequencing technologies have initiated more extensive studies of PVs. The discovery of the etiological association of human PVs (HPVs) and cervical cancer made by Dr. Harald zur Hausen was awarded a Nobel Prize in Physiology or Medicine in 2008. Papillomaviral studies throughout the 20th to 21st centuries have led to the development of HPV vaccines for humans. Behind this breakthrough and enhanced research on HPVs, we cannot disregard the contribution of animal PV studies. Research on animal PV is fundamental for both medical and veterinary fields, providing new insights into various research fields.

1.1 Genomic structure of papillomaviruses

The sizes of the circularized viral genomes of PVs vary from 5,748 (*Sparus aurata* papillomavirus type 1: SaPV1) to 8,607 (canine papillomavirus type 1: CPV1) base pairs (bp) with GC content between 36% and 59% (Van Doorslaer et al., 2018). The PV genome comprises of two late open reading frames (ORFs), six to seven early ORFs, and a non-coding region called the long control region (LCR) or upstream regulatory region (URR) (Fig. I-1) (Van Doorslaer and McBride, 2016). Two late ORFs, the L1 and L2 proteins code viral capsid. L1, the major capsid protein is the structural component of the viral capsid (Buck et al., 2013) and the minor capsid protein L2 is known to be active during the viral assembly and infection (Wang and Roden, 2013). Two early ORFs, the E1 and E2 are conserved in all PV types, but structures of the other early ORFs differ between PV types. E4 ORF is contained within the E2 and facilitates virus transmission (Doorbar, 2013). E5, E6, E7, and E10 are known as oncoproteins, as they have been shown to have transforming activity *in vitro* (Altamura et al., 2016; Van Doorslaer and McBride, 2016). The LCR is located between the end (3') of L1 and the beginning (5') of E6, known as a viral replication and transcription regulator (DeVilliers et al., 2004).

1.2 Classification

The classification of the family *Papillomaviridae* termed by the International Committee on Taxonomy of Viruses (ICTV), is based on the pairwise nucleotide sequence identity of the L1 ORF (Van Doorslaer et al., 2018). PVs are categorized by *Subfamily, Genera, Species, Type, Subtype,* and *Variant.* The novel PV is proposed when the sequence identities shared less than 45% (> 55% difference) for *Subfamily*, less than 60% (> 40% difference) for *Genera*, between 71% to 89% (11% to 29% difference) for *Species*, more than 89% (> 10% difference) for *Type*, between 90% to 98% (2% to 10% difference) for *Subtype*, and above 98% (< 2% difference) for *Variant* with the closest known PV (DeVilliers et al., 2004). The complete genome of the DNA sequence needs to be cloned when proposing a novel PV (DeVilliers et al., 2004). Based on this classification criteria, more than 200 types each of HPV and animal PV have been characterized ("PaVE: Papillomavirus genome database," n.d.; Van Doorslaer et al., 2017).

PVs are known to have high species specificities and proposed to have co-evolved with hosts that most vertebrae species carry their own set of PV types. A previous study noted that PV genus corresponded to their respective host taxa: *Alpha-* and *Betapapillomavirus*, *Primates*; *Delta-* and *Epsilonpapillomaviruses*, *Ruminantia*; *Lambdapapillomaviruses, Carnivora*; *Omikron-* and *Epsilonpapillomaviruses*, *Cetacea* (Gottschling et al., 2011b). PVs are considered to show slow evolution with an overall evolutionary rate of 1.95×10^{-8} nucleotide substitutions per site per year (Rector et al., 2007).

1.3 Viral life cycle

PV infects epithelial cells and the productive infection of cells by PVs are dependent on the differentiation state of the epithelial cell (Doorbar, 2005). In the squamous epithelium, the basal cell is the only cell which undergoes cell division. Therefore, the initial infection is established when the infectious virion particles enter the basal layer cells. The virion uncoating occurs by the disruption of intracapsomeric disulphide bonds within the cells letting the viral DNA to be transferred into the nucleus (Li et al., 1998). After the viral infection and uncoating process, the episomal PVs maintain as a low copy number by the regulation of early proteins such as E1 and E2 (You et al., 2004). The expression of the oncoproteins, E6 and E7 disrupts the normal cell differentiation by associating with cell cycle regulators. The suprabasal cell proliferation will be promoted by E6, binding to cellular PDZ via E6 PDZ-ligand domain (Nguyen et al., 2003; Thomas et al., 2002). E7 is known to bind retinoblastoma tumor suppressor protein (pRb) via E7 pRb-binding domain (Dahiya et al., 2000),

resulting in the release of the E2F transcription factors and the transactivation of E2F-associated genes, leading to cell-cycle progression (Zhang et al., 2006). The increase in E1 and E2 levels within the upper epithelial layers facilitates viral genome amplification (Ozbun and Meyers, 1998). Following the viral genome amplification, the two capsid proteins, L1 and L2 package the infectious viral particles in the superficial layers of the epithelium and sheds from the epithelial surface (Crosbie et al., 2013).

2. Human papillomaviruses (HPVs)

HPV has been known from the 1960s and was described as an oncogenic DNA virus named "Human papovavirus" (Rowson and Mahy, 1967). In that period, the viral infection has been assumed to be associated with cutaneous warts showing spontaneous regressions (Hellier, 1951). The studies on vaccine therapies by injecting wart-derived suspensions were also demonstrated in humans (Rowson and Mahy, 1967). Epidemiological, molecular, and clinical studies of HPVs have been conducted for over a half-century.

2.1 HPV-associated diseases and molecular epidemiology

To date, more than 200 HPV genotypes have been characterized worldwide and are classified into five genera: *Alpha-, Beta-, Gamma-, Mu-,* and *Nu- papillomaviruses* (Bernard et al., 2010; Latsuzbaia et al., 2018; Van Doorslaer et al., 2017). *Alphapapillomavirus*-classified HPVs are known to harbor both mucosal and cutaneous tropisms and are associated with cervical cancers, anogenital warts, and cutaneous warts (DeVilliers et al., 2004). In cervical cancers, two common HPV types, 16 and 18 account for over 57% and 16%, respectively and are so-called 'high-risk HPVs' (Guan et al., 2012; Li et al., 2011). Besides HPV16 and HPV18, HPV types 31, 33, 45, 52, and 58 are similarly described as high-risk HPVs, as these types are also known to have etiological roles in cervical cancers (Guan et al., 2012). The other well-known HPV types classified as *Alphapapillomavirus* are HPV6 and HPV11, which have been identified from a large portion (about 90%) of anogenital warts (Schiffman et al., 2007). *Betapapillomavirus*-classified HPVs, for example, HPV5, HPV8, and HPV9 are known to be causative agents of cutaneous lesions such as epidermodysplasia verruciformis (EV) (Kremsdorf et al., 1983), but some types are also detected from mucosal sites (DeVilliers et al., 2004). Infections of HPV5 and/or HPV8 with immunosuppressed patients are described to be associated with squamous cell carcinoma (SCC) development (Barr et al., 1989). HPV types, 4, 60, and 65, classified as *Gammapapillomavirus* were characterized from cutaneous lesions developed on the foot soles and fingers (DeVilliers et al., 2004; Egawa et al., 1993).

Extensive HPV studies on pathogenicity and epidemiology have revealed the association between disease phenotype(s) and HPV genotype(s), and lead to the development of preventive strategies including prophylactic vaccines.

Detection and genotyping of HPVs, especially in cervical cancer-related high-risk types have been demonstrated worldwide. Previous literature described the variations of cervical cancer-associated HPV type proportions between geographical locations (Li et al., 2011). Among the high-risk HPV types, HPV16 and HPV18 were the top two prevalent types in cervical cancers worldwide, but the third prevalent type was HPV33 in Africa and Europe, HPV31 in North/South/Central America, and HPV58 in Asia (Clifford et al., 2006). Not only HPV type distributions but also genomic diversities within the same HPV type have been observed and shown to have relationships between geographic distributions (Eschle et al., 1992). Moreover, HPV LCR sequence variants derived from different geographical locations have shown to alter viral replication efficiencies (Walter G. Hubert, 2005). Molecular epidemiological studies on HPVs provide the basic knowledge contributing to further applied research including biological, etiological and vaccination studies.

2.2 HPV vaccine development and future vaccines

The development of a prophylactic HPV vaccine has led us to prevent from HPV-associated cervical cancers and anogenital wart development. The HPV vaccines are composed of the self-assembled L1 major capsid protein of the target HPV type expressed in eukaryotic cells, called as viral-like particles (VLPs) (Kirnbauer et al., 1992). The first commercially available HPV vaccine approved by the United States Food and Drug Administration (FDA) was the quadrivalent HPV vaccine named Gardasil (Merck & Co., Kenilworth, NJ, USA) in 2006, followed by the bivalent vaccine, Cervarix (GlaxoSmithKline Biologicals, Brentford, England, UK) approved by FDA in 2009 (de Oliveira et al., 2019). The latest nine-valent vaccine, Gardasil 9 extended the coverage against HPV types and shown to protect against 90% of cervical cancers (Yang and Bracken, 2016).

Before these successes in the development of HPV vaccines, as a surrogate model for humans, immunogenicity studies in animal PVs were demonstrated from the late 1930s (Shope, 1937). Early studies have investigated PV vaccinology using animal PV models. Among the animal PVs, CRPV and *Bos taurus* PVs (BPVs) have been widely investigated and the immunogenic properties of VLP were described (Christensen et al., 1996; Kirnbauer et al., 1992). As BPV4 infection in cattle has been associated with the papillomas of the alimentary canal mucosa showing malignant progression (Campo et al., 1994) resembling that of human mucosal infections, BPV4 has also been studied for vaccinology in addition to two classical BPV types, 1 and 2.

HPV vaccine studies are still undergoing to become more efficient and universal vaccines. Since the introduction of HPV vaccines, publications support the protective and positive effects of vaccinations against HPV infection (Brisson et al., 2009; Fesenfeld et al., 2013; Jit et al., 2014). However, the high vaccine cost becomes one of the obstacles to deliver HPV vaccines to world-wide populations. The commercialized HPV vaccines are generated by inoculating individually expressed L1 VLP of the target HPVs, making the vaccine expensive. Therefore, the current HPV vaccine studies are focusing on the development of cost- and long term-effective vaccines, for example, by targeting broadly protective L1/L2 epitope conserved within the wide range of HPVs (Boxus et al., 2016). Immunological studies on HPV also focus on immunotherapeutic vaccine development for HPV-driven cancers by stimulating cellular immune cells (Kenter et al., 2009). HPV vaccines still harbor potential to become more affordable and protective for the worldwide population.

3. Animal Papillomaviruses

Since the first discovery of animal PV noted in the 1930s (Shope and Hurst, 1933), numerous animal PVs (more than 200 types), have been characterized from various species ("PaVE: Papillomavirus genome database," n.d.). From the aspect of veterinary medicine, the author describes animal PVs focusing on large (cattle, horses) and small (cats, dogs) domestic animals, as PV-associated diseases have been described to become clinical issues.

3.1 Domestic Cattle

Cutaneous wart/papilloma lesions of cattle have been observed and reported from the 1950s (Bagdonas and Olson, 1953). The prevalence of bovine papillomatosis has been described by previous studies. In Egypt, the numbers of papilloma-bearing cattle were 13 out of 267 (4.86% prevalence) and female cattle showed higher prevalence rate (8 out of 267 cattle, 2.99%) than male cattle (5 out of 267 cattle, 1.87%) (Awadalla Salib and Ali Farghali, 2011). Another survey conducted in the United Kingdom described that 260 out of 721 examined cattle (36.1%) were affected with teat papillomatosis and this study also confirmed the higher prevalence in female than male cattle (Meischke, 1979). In Japan, an epidemiological study demonstrated in Hokkaido prefecture found that 283 cattle out of a studied population of 1085 (26.1%) showed teat/udder papillomatosis and higher prevalence was observed in female than male cattle (Kadohira et al., 2014). This survey showed that papilloma-bearing cattle were older (mean: 78-month-old) than papilloma-negative cattle (mean: 63-month-old), describing the significant association between age and sex with papillomatosis (Kadohira et al., 2014). Another study focusing on teat papillomatosis demonstrated in Japan showed that 80%

(560 out of 700 cattle) of Holstein Friesian heifers (560 out of 700 heifers) aged between 1 to 2 years, were affected by teat papillomatosis (Maeda et al., 2007). To the author's knowledge, significant data describing economic losses due to bovine papillomatosis is unavailable, however, the papilloma lesions become an obstacle while milking (Maeda et al., 2007; Watanabe et al., 2017), induce leather depreciation (Ugochukwu et al., 2019) and may decrease beef exports (Rojas-Anaya et al., 2016) leading to economic losses to livestock owners.

The complete genome of BPV1 sequence identified from bovine fibropapilloma was characterized in 1982 (Chen et al., 1982). Among the animal PVs, BPVs have been studied the most for many decades. To date, 29 types of BPVs classified into the genus, *Deltapapillomavirus*, *Dyokappapapillomavirus, Epsilonpapillomavirus* and *Xipapillomavirus* have been identified from bovine cutaneous/mucosal-derived samples/lesions worldwide (Table I-1).

Two *Deltapapillomavirus*-classified BPV types 1 and 2 (BPV1/2) have been associated with (fibro)papillomas (Daudt et al., 2018). Detections have been reported from various geographical regions: Asia (Hamad et al., 2018; Hatama et al., 2011; Kumar et al., 2015a; Ogawa et al., 2018; Saveria Campo et al., 1981; Shimakura et al., 2018; Timurkan and Alcigir, 2017); Africa (El-Tholoth et al., 2020); Europe (Escudero et al., 2014; Grindatto et al., 2015; Saveria Campo et al., 1981); South America (Bertagnolli et al., 2020; Rojas-Anaya et al., 2016); Oceania (Munday et al., 2018). Typically, both BPV1/2 have an association with benign (fibro)papillomas of cutaneous tissues, such as body surfaces including the udder and teat (Hatama et al., 2011; Ogawa et al., 2004; Timurkan and Alcigir, 2017). Some previous case reports demonstrated the identification of BPV1/2 from (fibro)papillomas emerged on the mucosal sites, such as upper gastrointestinal tracts (Kumar et al., 2015b; Shimakura et al., 2018) and the anus (Munday et al., 2018) of cattle. Carcinogenesis of BPV1/2-associated lesions is uncommon. However, BPV1/2-associated invasive and/or papillary carcinomas of the bovine urinary bladder have been observed within the bracken fern (*Pteridium aquilinum*)-grazing cattle (Campo et al., 1992; Roperto et al., 2016a; Z. Yuan et al., 2007a), which is suggested to be triggered by immunosuppression via bracken fern-intake. Immunosuppressants contained in the bracken fern degrades circulating lymphocytes of cattle, leading to immunosuppression (Nasir and Campo, 2008). Besides BPV1/2, BPV13 and BPV14 are also *Deltapapillomavirus*-classified BPVs (Table I-1). In cattle, BPV13 has been associated with cutaneous fibro(papillomas) (Hamad et al., 2018) and urothelial bladder tumors (Roperto et al., 2016b). Similarly, BPV14 was also identified in urinary bladder tumors (Roperto et al., 2016a). Taken the species-specific characteristic of PVs, *Deltapapillomavirus*-classified BPVs have unique features showing cross-species infection to horses

(BPV1, BPV2, BPV13) and cats (BPV14) causing sarcoid lesions (Munday et al., 2015b; Nasir and Campo, 2008).

Among the 29-identified BPV types, twelve BPVs are classified into the genus, *Xipapillomavirus* (Table I-1). *Xipapillomavirus*-classified BPVs have been discussed to harbor epitheliotropic-features (Daudt et al., 2018). Among the *Xipapillomavirus*-classified BPVs, BPV3 (He et al., 2016), BPV6 (Maeda et al., 2007; Tozato et al., 2013), BPV9 (Tozato et al., 2013), and BPV10 (Tozato et al., 2013; Zhu et al., 2019b) have been identified in papilloma lesions of teat and udder. Among these BPV types, BPV6 is suggested to be the most common type related to bovine teat papillomas in Japan (Maeda et al., 2007). In the dairy cow industry, teat/udder papillomatosis may become an obstacle to milking. From this aspect of veterinary hygiene, detection of teat/udder associated BPV types have been conducted worldwide (Table I-1).

Xipapillomavirus-classified BPV types have also been identified in mucosal sites. BPV4 has been studied over decades, because its infection in cattle has been associated with papillomas of the upper gastrointestinal mucosa showing malignant progression (Campo et al., 1994), resembling human mucosal infections. The etiology of BPV4 infection and carcinogenesis progression has also been suggested to be caused by immunosuppression via bracken fern-intake (Campo et al., 1994). Besides BPV4, BPV10 and BPV12 have been identified from the mucosal sites, characterized from lingual papillomas of domestic cows (Zhu et al., 2014, 2012). In the recent studies, detection of *Xipapillomavirus*-classified BPVs (BPV15, BPV17, BPV20, BPV23, BPV24, BPV26, BPV28) have been reported from bovine papillomas emerged on the body surface (da Silva et al., 2016; Daudt et al., 2019a, 2016; Hu et al., 2020) (Table I-1).

These molecular epidemiological studies on BPVs have demonstrated the discoveries of novel BPV types and harbor the largest number of genotypes in the animal PVs. However, the association between infective tissue sites and BPV genotypes are not significantly defined than that of human studies. Moreover, the underlying phenomenon of the cross-species infections of *Deltapapillomavirus* 4-classified BPVs is still unraveled. Though BPVs have been studied for decades, many unknown aspects of BPVs remain.

3.2 Horses

In horses, PV-infection has been associated with the development of cutaneous/mucosal neoplasms. A retrospective study on equine neoplasms conducted from the 1970s showed that the prevalence of papilloma was between 3.4 to 5.5% (Bastianello, 1983; Knowles et al., 2016; Sundberg et al., 1977). However, the significant prevalence of wart/papillomatosis has not been well understood

due to limited publications. To date, nine types of *Equus caballus* PVs (EcPVs) have been characterized from benign and malignant tumors, mostly reported from Europe and North America (Table I-2). EcPVs are classified into the genus, *Zetapapillomavirus* (EcPV1), *Dyoiotapapillomavirus* (EcPV2, EcPV4, EcPV5), and *Dyorhopapillomavirus* (EcPV3, EcPV6, EcPV7). Two recently identified EcPV types 8 and 9 remain unclassified (Table I-2). EcPV1 (formerly EqPV) has been identified from classical papillomas/warts of the cutaneous sites (Dong et al., 2017; Ghim et al., 2004; O'banion et al., 1986). EcPV2 has been detected from equine genital benign/malignant neoplasms such as papillomas, SCCs, and carcinoma *in situ*, in Europe and North America (Ramsauer et al., n.d.; Scase et al., 2010; Sykora and Brandt, 2017; van den Top et al., 2015). In Japan, EcPV2 was recently identified in laryngeal papilloma and SCC (Hibi et al., 2019). From these reports, EcPV2 has been suggested to be associated with malignant mucosal neoplasms in horses. Previous studies described EcPV2 prevalence in both healthy and tumor-bearing horses. In healthy horses, EcPV2 DNA was detected between 1 to 18% of the studied population (Bogaert et al., 2012; Fischer et al., 2014; Lee et al., 2019). Serological studies on EcPV2 revealed that 15 to 36% of the examined horses harbored EcPV2 antibodies (Fischer et al., 2014; Schellenbacher et al., 2015). A recent epidemiological study conducted in Western Canada demonstrated that 29% (10 out of 35) genital SCC and 30% (8 out of 27) genital papilloma samples were positive for EcPV2 DNA (Greenwood et al., 2020). In the same study, the presence of EcPV2 DNA was not associated with breeding history nor overall survival period (Greenwood et al., 2020). EcPV types 3 to 6 have been associated with aural plaques (Lange et al., 2013b). EcPV7 was characterized from the penile mass lesion of a gelding (Lange et al., 2013b). EcPV8 was identified from generalized papillomatosis (Linder et al., 2017) and inguinal lesions diagnosed as viral plaques and SCCs (Peters-Kennedy et al., 2019). The latest characterized EcPV is type 9, identified in the semen taken from a thoroughbred stallion with a genital lesion (Li et al., 2019).

In equids, PVs associated with equine cutaneous/mucosal neoplasms is not restricted to EcPVs. Infection of BPV1, BPV2, and BPV13 has been associated with the development of sarcoids (de Alcântara et al., 2015). Equine sarcoid is a common tumor in horses (Knowles et al., 2016), where they develop on any cutaneous site of the body (Nasir and Campo, 2008). Although equine sarcoids are termed benign tumors, they could be aggressive and impeditive to equine vital function. Previous surveillance showed that 12 to 43% of the skin tumors were sarcoids (Baker and Leyland, 1975; Bastianello, 1983; Knowles et al., 2016; Sundberg et al., 1977). Sarcoids could develop in any ages but typically observed in younger horses (mean age: 7.88 years) than other skin tumors such as SCCs (mean age: 15.79 years) and Lymphomas (mean age: 12.19 years) (Knowles et al., 2016). Another study showed that the risk of sarcoid development increased with age up to 15 years and no significant

difference between sex was observed for lesion development (Mohammed et al., 1992). Appaloosa, Quarter horse, and Arabian breeds were shown to harbor higher proportions of sarcoid cases than Standardbred horses (Mohammed et al., 1992). Another study demonstrated in the United States showed that the frequency of sarcoid development in Quarter horses was nearly twice that of thoroughbreds (Angelos et al., 1988). However, these epidemiological data on papilloma-associated diseases in horses are limited to Europe and North America. Thus, more epidemiological studies are needed to strengthen the risk factors associated with sarcoid development. To the author's knowledge, the significant data of economic loss due to sarcoid development is uncertain. However, since sarcoids have been more frequently observed in working donkeys than non-working donkeys (Gebre et al., 2018) and due to frequent lesion recurrence after treatment (Chambers et al., 2003a), sarcoid development becomes a financial burden to horse/donkey owners.

The association of BPV and the development of equine sarcoid was experimentally shown by BPV injection to a horse (Olson and Cook, 1951). A molecular study showed the sequence variants of BPV1, which were only detected from equine sarcoid lesions, and not from the bovine papilloma lesions (Nasir et al., 2007). On the other hand, a recent genomic study found that no specific sequence variants of BPV were observed between equine and bovine lesions (Koch et al., 2018). The viral pathogenesis of cross-species infection is still undetermined.

3.3 Dogs

In dogs, up to 23 genotypes of *Canis familiaris* PVs (CPVs) have been identified from cutaneous/oral neoplasms and are classified into three genera: *Lambda-, Tau-, Chi- papillomaviruses* (Table I-3). CPVs have been detected from benign neoplasms such as oral papillomas, pigmented viral plaques and also from malignant tumors including SCCs. A previous epidemiological study conducted in Germany described that among the analyzed epithelial tumors, 17.8% (95 out of 535 cases) were papillomas and 18.7% (100 out of 535 cases) were SCCs (Schwegler et al., 1997). In the same study, immunohistochemical staining of papillomavirus antigen was positive for 44.2% of 95 papilloma lesion samples and 27% of 100 SCC samples (Schwegler et al., 1997). A retrospective study demonstrated in Switzerland showed that among the papillomavirus-associated neoplasms, SCC was observed in 3.19% (375 out of 3284 cases) of the analyzed epithelial tumors followed by papilloma (2.84%, 333 out of 3284 cases) and basal cell carcinoma (BCC, 0.16%, 19 out of 3284 cases) (Graf et al., 2018). This study described that papillomas and SCC were likely to be found in dogs between ages 8 and 13, but papillomas were also frequently observed in young dogs between ages 1 and 2 (Graf et al., 2018). The significant association between papillomavirus-associated lesion development and

breeds is still uncertain, but pug dogs and schnauzers have been described to develop viral plaques and papillomas more likely than other breeds (Graf et al., 2018; Lange et al., 2016).

CPV1 also noted as canine oral PV (CoPV), classified as *Lambdapapillomavirus*, has been associated with oral papillomas (Chang et al., 2020a; Lange and Favrot, 2011; Regnard et al., 2016a; Reis et al., 2019). Although CPV1-associated oral papillomas are mostly benign, some studies identified CPV1 from oral SCCs (Regalado Ibarra et al., 2018; Reis et al., 2019). Another *Lambdapapillomavirus*-classified CPV type 6 is also associated with benign papillomas of the skin (Chang et al., 2020a; C. E. Lange et al., 2009). Eight CPV types (CPV2, CPV7, CPV13, CPV17, CPV18, CPV21, CPV22, CPV23) are classified as *Taupapillomaviruses* (Table I-3). Among these *Taupapillomavirus*-classified CPVs, CPV2, CPV7, and CPV17 have been identified from malignant tumors such as cutaneous and oral SCC lesions (Goldschmidt et al., 2006; C. E. Lange et al., 2009; Munday et al., 2016a; H. Yuan et al., 2007). *Taupapillomavirus*-classified CPVs have also been detected from benign oral papillomas (Lange et al., 2012a). Three CPV types, 21 to 23, were detected from nasal swabs collected from dogs with respiratory symptoms (Altan et al., 2019), but the significant etiological roles have not been identified yet. Among the 23 CPV types, 13 genotypes are classified as the genus, *Chipapillomavirus* (Table I-3). CPV3, CPV9, CPV12, CPV15, CPV16, and CPV18 have been identified in canine malignant neoplasms reported from Asia, North America, and Europe (Chang et al., 2020b, 2020a; Luff et al., 2016; Tobler et al., 2006; Yu et al., 2019). Related to benign tumors, *Chipapillomavirus*-classified CPVs, such as CPV4, CPV5, CPV8, CPV10, CPV11, and CPV14 have been associated with pigmented viral plaques (Lange et al., 2013a, 2012b; J. A. Luff et al., 2012; Yu et al., 2019; Zhou et al., 2016, 2014). CPV-associated diseases have been identified from various dog breeds. Interestingly, CPV4-related pigmented viral plaques have been observed in pug dogs and these similar findings have been reported from Europe, North America, and Japan (J. A. Luff et al., 2012; Tobler et al., 2008; Yu et al., 2019). However, the significant relationships between CPV type(s) and dog breed(s) are unraveled.

Based on the previous literature, diverse CPV types have been characterized worldwide. The etiological roles of CPVs have been described in both benign and malignant neoplasms in dogs, but the significant CPV type(s) associated with malignant tumors are not defined yet. For example, CPV9 has been detected from both benign and malignant cases (Chang et al., 2020b). Therefore, in terms of making a CPV vaccine for dogs, it may be challenging to consider which type(s) should be included in the vaccine at the moment.

3.4 Cats

To date, six genotypes of *Felis catus* PVs (FcaPVs) have been characterized from feline benign/malignant tumors, such as viral plaques, oral papillomas, Bowenoid *in situ* carcinomas (BISCs), and SCCs (Table I-4). Epidemiological studies on PV-associated neoplasms showed that cutaneous SCCs account for 11 to 15% of cutaneous neoplastic diseases (Ho et al., 2018; Miller et al., 2012). Other PV-associated diseases such as viral plaques and BISCs develop on 8- to 14-year-old cats (Munday et al., 2019), but these two neoplasms have been considered to be uncommon in cats (Munday et al., 2016b; Munday and Peters-Kennedy, 2010). Development of viral plaques has been associated with immunodeficient status such as feline immunodeficiency virus (FIV) infection (Egberink et al., 1992; Munday et al., 2019). Devon Rex and Sphynx have been described to develop BISCs in younger ages than other breeds and are also considered to show malignant and aggressive forms of the lesions, which may progress into SCCs (Munday et al., 2019).

FcaPV type 1 (FcaPV1) was the first PV genotype identified in domestic cats, detected in a hyperkeratotic cutaneous lesion of a Persian cat (Tachezy et al., 2002). Also, FcaPV1 was detected in oral lesions, such as papilloma of the tongue (Munday et al., 2015a) and SCCs (Munday and French, 2015) of domestic cats. FcaPV2 has been identified in viral plaques (Munday and Peters-kennedy, 2010), Bowenoid *in situ* carcinomas (BISCs) (Lange et al., 2009), and cutaneous SCCs (Munday et al., 2011), whereas FcaPV2 detection was also confirmed in dermatologically healthy cats (Geisseler et al., 2016). FcaPV3 was detected in viral plaque (Munday and Peters-kennedy, 2010), SCC (Munday et al., 2012), and BISC lesions (Munday et al., 2013). FcaPV4 was identified in ulcerative gingivitis (Dunowska et al., 2014) and SCCs (Munday et al., 2013). FcaPV5 has been associated with viral plaque (Kok et al., 2019; Munday et al., 2017a) and BISC (Kok et al., 2019). Recently, the sixth FcaPV genotype, FcaPV6 was detected from nasal cavity lymphoma and recurrent SCC of the nasal planum in a cat (Carrai et al., 2020). The previous studies demonstrate the association of FcaPV infections with cutaneous and/or mucosal tumor development in domestic cats. When compared with CPV-associated tumors in dogs, FcaPV, especially type 2, has been detected from malignant lesions than benign tumors. Thus, from the aspect of veterinary medicine, establishing preventive and treatment strategies of FcaPV-associated neoplasms would become valuable.

Table I-1 Geographical regions and source of the respective papillomaviruses identified from domestic cattle.

Genera/Species	Genotype	Source	Site	Cutanous and/or Mucosal	Benign and/or Malignant	Geographical Regions	Reference
Deltapapillomavirus 4	BPV1	Fibropapilloma, papilloma	Body, udder, teat, upper gastrointestinal tract, anus	Cutaneous and mucosal	Benign	Africa, Asia, Europe, South America	(Bertagnolli et al., 2020; Ellson Y. Chen et al., 1982; El-Tholoth et al., 2020; Hamad et al., 2018; Hatama et al., 2011; Kumar et al., 2015a; Peng et al., 2019; Rojas-Anaya et al., 2016; Saveria Campo et al., 1981; Shimakura et al., 2018; Timurkan and Alcigir, 2017), This study
		Carcinoma	Bladder	Mucosal	Malignant	Europe	(Z. Yuan et al., 2007b)
Deltapapillomavirus 4	BPV2	Fibropapilloma, papilloma	Body, udder, teat, anus, rumen, vagina	Cutaneous and mucosal	Benign	Asia, Europe, South America, Oceania	(Bertagnolli et al., 2020; Escudero et al., 2014; Hamad et al., 2018; Hatama et al., 2011; Kumar et al., 2015a; Munday et al., 2018; Rojas-Anaya et al., 2016), This study
		Carcinoma	Bladder	Mucosal	Malignant	Asia, Europe	(Campo et al., 1992; Dagalp et al., 2017; Roperto et al., 2016a)
Xipapillomavirus 1	BPV3	Papilloma	Body, teat	Cutaneous	Benign	Asia	(Dagalp et al., 2017; Hatama et al., 2011; Ogawa et al., 2004; Zhu et al., 2019)
Xipapillomavirus 1	BPV4	Papilloma	Body, udder, teat, upper gastroitntestinal tract	Cutaneous and mucosal	Benign	Asia, Europe	(Hatama et al., 2011; Tsirimonaki et al., 2003)
		Carcinoma	Upper gastrointestinal tract	Mucosal	Malignant	Europe	(Jarrett et al., 1978)
Epsilonpapillomavirus 1	BPV5	Papilloma	Skin, rumen	Cutaneous and mucosal	Benign	Asia	(Hatama et al., 2011; Kumar et al., 2015a)
Xipapillomavirus 1	BPV6	Papilloma	Body, udder, teat	Cutaneous	Benign	Asia, South America	(Bertagnolli et al., 2020; Dagalp et al., 2017; Hatama et al., 2011)
Dyoxipapillomavirus 1	BPV7	Papilloma	Body, teat	Cutaneous	Benign	Asia, Europe	(Dagalp et al., 2017; Ogawa et al., 2007; Savini et al., 2016; Tozato et al., 2013)
Epsilonpapillomavirus 1	BPV8	Papilloma	Body, udder	Cutaneous	Benign	Asia, South America	(Lunardi et al., 2016; Tomita et al., 2008)
Xipapillomavirus 1	BPV9	Papilloma	Body, teat	Cutaneous	Benign	Asia, South America	(Dagalp et al., 2017; Hatama et al., 2011; Tozato et al., 2013)
Xipapillomavirus 1	BPV10	Papilloma	Body, teat, tongue	Cutaneous and mucosal	Benign	Asia, South America	(Dagalp et al., 2017; Hatama et al., 2011; Tozato et al., 2013; Zhu et al., 2019, 2014)
Xipapillomavirus 1	BPV11	Fibropapilloma, papilloma	Body, teat	Cutaneous	Benign	Asia, South America	(Carvalho et al., 2012; Hatama et al., 2011)
Xipapillomavirus 2	BPV12	Papilloma	Tongue	Mucosal	Benign	Asia	(Zhu et al., 2012)
Deltapapillomavirus 4	BPV13	Fibropapilloma, papilloma	Body, urinary bladder	Cutaneous and mucosal	Benign	Asia, Europe	(Hamad et al., 2018; Knowles et al., 2016)
		Carcinoma	Urinary bladder	Mucosal	Malignant	South America	(Roperto et al., 2016b)
Deltapapillomavirus 4	BPV14	Papilloma	Urinary bladder	Mucosal	Benign	South America	(Roperto et al., 2016a)
		Carcinoma	Urinary bladder	Mucosal	Malignant	Europe	(Roperto et al., 2016a)
Xipapillomavirus 1	BPV15	Papilloma	Body	Cutaneous	Benign	Asia	(Hu et al., 2020)
Dyokappapapillomavirus	BPV16	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Xipapillomavirus	BPV17	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Dyokappapapillomavirus	BPV18	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Unclassified	BPV19	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Xipapillomavirus	BPV20	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Unclassified	BPV21	Papilloma	Body	Cutaneous	Benign	South America	(Daudt et al., 2016)
Dyokappapapillomavirus	BPV22	Vulvovaginitis, swabs	Vagina	Mucosal		North America	(Bauermann et al., 2017)
Xipapillomavirus	BPV23	Papilloma	Skin	Cutaneous	Benign	South America	(da Silva et al., 2016)
Xipapillomavirus	BPV24	Papilloma	Skin	Cutaneous	Benign	South America	(Daudt et al., 2019b)
Epsilonpapillomavirus	BPV25	Papilloma	No information	No information	No information	South America	
Xipapillomavirus	BPV26	Papilloma	No information	No information	No information	South America	
Unclassified	BPV27	Swabs	Genital tract (healthy dairy cattle)			Asia	(Ling et al., 2019)
Xipapillomavirus	BPV28	Cutaneous wart	Face	Cutaneous	Benign	Asia	This study
		Papilloma	Vulva	Mucosal	Benign	Asia	This study
Xipapillomavirus	BPV29	Papilloma	Vulva	Mucosal	Benign	Asia	This study

Abbreviations: BPV, Bovine papillomavirus.

Table I-2 Geographical regions and source of the respective papillomaviruses identified from horses.

Species (or Genera)	Genotype (formerly)	Source	Site	Cutanous and/or Mucosal	Benign and/or Malignant	Geographical Regions	Reference
Deltapapillomavirus 4	BPV1	Sarcoid	Body	Cutaneous	Benign	Africa, Asia, Ocania, Europe, North America, South America	(Carr et al., 2001; Chambers et al., 2003; de Alcântara et al., 2015; Nasir et al., 2007; Savini et al., 2015; Szczerba-Turek et al., 2011; Trewby et al., 2014; Wilson et al., 2013), This study
Deltapapillomavirus 4	BPV2	Sarcoid	Body	Cutaneous	Benign	Asia, Europe, North America, South America	(Carr et al., 2001; Chambers et al., 2003; de Alcântara et al., 2015; Wilson et al., 2013; Wobeser et al., 2010), This study
Deltapapillomavirus 4	BPV13	Sarcoid	Body	Cutaneous	Benign	South America	(de Alcântara et al., 2015)
Zetapapillomavirus 1	EcPV1 (EqPV)	Papilloma	Body	Cutaneous	Benign	Asia, North America	(Dong et al., 2017; Ghim et al., 2004)
Dyoiotapapillomavirus 1	EcPV2	Swab	Penile plaque	Cutaneous		Europe	(Lange et al., 2013b)
		Papilloma	External genitals, larynx	Cutaneous and mucosal	Benign	Asia, Europe	(Hibi et al., 2019; Lange et al., 2011)
		SCC	External genitals, larynx	Cutaneous and mucosal	Malignant	Asia, Europe, North America, Oceania	(Hibi et al., 2019; Scase et al., 2010)
Dyorhopapillomavirus 1	EcPV3	Aural plaue	Ear pinna	Cutaneous		Europe	(Lange et al., 2011)
Dyoiotapapillomavirus 2	EcPV4	Aural plaque	Ear pinna	Cutaneous	Benign	South America	(Taniwaki et al., 2013)
		Swab	Vulva/inguina plaque	Cutaneous, mucosal		Europe	(Lange et al., 2013b)
		SCC	Ear pinna	Cutaneous	Malignant	North America	(Peters-Kennedy et al., 2020)
Dyoiotapapillomavirus 2	EcPV5	Swab	Aural plaque	Cutaneous		Europe	(Lange et al., 2013b)
Dyorhopapillomavirus 1	EcPV6	Swab	Aural plaque	Cutaneous		Europe	(Lange et al., 2013b)
Dyorhopapillomavirus 1	EcPV7	Swab	Penile mass	Cutaneous		Europe	(Lange et al., 2013b)
Unclassified	EcPV8	Viral plaque, papilloma, SCC	Inguinal	Cutaneous	Benign and malignnt	North America	(Peters-Kennedy et al., 2019)
		Generalized papillomatosis	Body	Cutaneous	Benign	North America	(Linder et al., 2018)
Unclassified	EcPV9	Semen	Penis (with wart lesion)			Oceania	(Li et al., 2019)

Abbreviations: BPV, Bovine papillomavirus; EcPV, *Equus caballus* papillomavirus; SCC, squamous cell carcinoma.

Table I-3 Geographical regions and source of the respective papillomaviruses identified from dogs.

Species (or Genera)	Papillomavirus type (formerly)	Source	Site	Cutanous and/or Mucosal	Benign and/or Malignant	Geographical region	Reference
Lambdapapillomavirus 2	CPV1 (CoPV)	Papillloma	Oral cavity, skin	Cutaneous and mucosal	Benign	Africa, Asia, Europe, South America	(Chang et al., 2020a; Lange and Favrot, 2011; Regnard et al., 2016b; Reis et al., 2019)
		SCC	Oral cavity	Mucosal	Malignant	North America	(Reis et al., 2019)
Taupapillomavirus 1	CPV2 (CfPV2)	Papillloma	Body	Cutaneous	Benign	Asia, North America	(Chang et al., 2020a; H. Yuan et al., 2007)
		SCC	Body	Cutaneous	Malignant	North America	(Goldschmidt et al., 2006; H. Yuan et al., 2007)
Chipapillomavirus 1	CPV3	Pigmented viral plaque	Body	Cutaneous	Benign	Europe, North America	(J. A. Luff et al., 2012; Tobler et al., 2006)
Chipapillomavirus 2	CPV4	Pigmented viral plaque	Body	Cutaneous	Benign	Asia, Europe, North America	(J. A. Luff et al., 2012; Tobler et al., 2008; Yu et al., 2019)
Chipapillomavirus 1	CPV5	Pigmented viral plaque	Body	Cutaneous	Benign	Europe, North America	(C. E. Lange et al., 2009; J. A. Luff et al., 2012)
Lambdapapillomavirus 3	CPV6	Papilloma	Body	Cutaneous	Benign	Asia, Europe	(Chang et al., 2020a; C. E. Lange et al., 2009)
Taupapillomavirus 1	CPV7	Papilloma	Body	Cutaneous	Benign	Europe	(C. E. Lange et al., 2009)
		SCC	Body	Cutaneous	Malignant	Europe	(C. E. Lange et al., 2009)
Chipapillomavirus 3	CPV8	Pigmented viral plaque	Body	Cutaneous	Benign	Europe, North America	(Lange et al., 2012b; J. A. Luff et al., 2012)
Chipapillomavirus 1	CPV9	Pigmented viral plaque	Body	Cutaneous	Benign	Asia, Europe, North America	(Chang et al., 2020b; Lange et al., 2013a; Yuan et al., 2012)
		Papilloma	Body	Cutaneous	Benign	Asia	(Chang et al., 2020a)
		SCC	Body	Cutaneous	Malignant	Asia	(Chang et al., 2020b)
Chipapillomavirus 3	CPV10	Pigmented viral plaque	Body	Cutaneous	Benign	North America	(J. Luff et al., 2012)
Chipapillomavirus 1	CPV11	Pigmented viral plaque	Body	Cutaneous	Benign	North America	(Zhou et al., 2014)
Chipapillomavirus 1	CPV12	Pigmented viral plaque	Body	Cutaneous	Benign	North America	(Zhou et al., 2016)
		SCC	Body	Cutaneous	Malignant	North America	(Luff et al., 2016)
Taupapillomavirus 2	CPV13	Swab	Oral papilloma	Mucosal		Europe	(Lange et al., 2012a)
Chipapillomavirus 3	CPV14	Pigmented viral plaque	Body	Cutaneous	Benign	Europe	(Lange et al., 2013a)
Chipapillomavirus 3	CPV15	Verrucous SCC	Body	Cutaneous	Malignant	Asia	(Chang et al., 2020a)
Chipapillomavirus 2	CPV16	Pigment viral plaque, progressed to SCC	Body	Cutaneous	Malignant	North America	(Luff et al., 2019)
		Squamous epithelium dysplasia	Oral cavity	Mucosal	Pre-malignant	Asia	(Chang et al., 2020a)
		SCC	Body	Cutaneous	Malignant	Asia, North America	(Chang et al., 2020a; Luff et al., 2016)
Taupapillomavirus	CPV17	SCC	Oral cavity	Mucosal	Malignant	Oceania	(Munday et al., 2016)
Chipapillomavirus	CPV18	Basal cell tumor	Body	Cutaneous	Malignant	Asia	(Yu et al., 2019)
		Pigmented viral plaque	Body	Cutaneous	Benign	Asia, North America	(Lange et al., 2016; Yu et al., 2019)
Taupapillomavirus	CPV19	Papilloma	Oral cavity	Mucosal	Benign	North America	(Tisza et al., 2016)
Chipapillomavirus	CPV20	No infofmation					Unpublished, (GenBank accession number: KT901797)
Taupapillomavirus	CPV21	Swab	Nasal cavity	Mucosal		North America	(Altan et al., 2019)
Taupapillomavirus	CPV22	Swab	Nasal cavity	Mucosal		North America	(Altan et al., 2019)
Taupapillomavirus	CPV23	Swab	Nasal cavity	Mucosal		North America	(Altan et al., 2019)

Abbreviations: CPV, *Canis familiaris* papillomavirus; SCC, squamous cell carcinoma.

Table I-4 Geographical regions and source of the respective papillomaviruses identified from cats.

Species (or Genera)	Papillomavirus type (formerly)	Source	Site	Cutanous and/or Mucosal	Benign and/or Malignant	Geographical Regions	Reference
Deltapapillomavirus 4	BPV14	Sarcoid	Body	Cutaneous	Benign	Europe, North America	(Kiefer et al., 2017; Munday et al., 2015b)
Lambdapapillomavirus 1	FcaPV1 (FdPV1)	Hyperkeratotic lesion, viral plaque	Body	Cutaneous	Benign	Europe	(Tachezy et al., 2002)
		Papilloma	Oral cavity (tongue)	Mucosal	Benign	Oceania	(Munday et al., 2015a)
		Gingivitis	Oral cavity (gingiva)	Mucosal		Oceania	(Munday and French, 2015)
		SCC	Oral cavity (gingiva)	Mucosal	Malignant	Oceania	(Munday and French, 2015)
		BISC	Body	Cutaneous	Malignant	Europe	(Mazzei et al., 2017)
Dyothetapapillomavirus 1	FcaPV2 (FdPV2)	Viral plaque	Body	Cutaneous	Benign	Europe, Oceania	(Munday and Peters-Kennedy, 2010)
		BISC	Body	Cutaneous	Malignant	Europe, North America	(Mazzei et al., 2017; O'Neill et al., 2011)
		SCC	Body	Cutaneous	Malignant	Europe, North America, Oceania	(Lange et al., 2009; Munday et al., 2011; O'Neill et al., 2011)
		BSC	Body	Cutaneous	Malignant	Asia	(Oh et al., 2018)
Taupapillomavirus 3	FcaPV3	Viral plaque	Body	Cutaneous	Benign	Oceania	(Munday and Peters-Kennedy, 2010)
		BISC	Body	Cutaneous	Malignant	Oceania	(Munday et al., 2013)
		SCC	Body	Cutaneous	Malignant	Asia, Oceania	(Munday et al., 2013), This study
Taupapillomavirus 3	FcaPV4	Gingivitis	Oral cavity (gingiva)	Mucosal		Oceania	(Dunowska et al., 2014)
		SCC	Body	Cutaneous	Malignant	Asia, Oceania	(Munday et al., 2013), This study
		BISC	Body	Cutaneous	Malignant	Europe	(Vascellari et al., 2019)
Taupapillomavirus	FcaPV5	Viral plaque	Body	Cutaneous	Benign	Asia, Oceania	(Kok et al., 2019; Munday et al., 2017)
		BISC	Body	Cutaneous	Malignant	Asia	(Kok et al., 2019)
Unclassified	FcaPV6	Lymphoma and SCC	Nasal cavity	Mucosal	Malignant	Oceania	(Carrai et al., 2020)

Abbreviations: BISC, Bowenoid *in situ* carcinoma; BPV, Bovine papillomavirus; BSC, basosquamous carcinoma; FcaPV, *Felis catus* papillomavirusSCC, squamous cell carcinoma

Fig. I-1

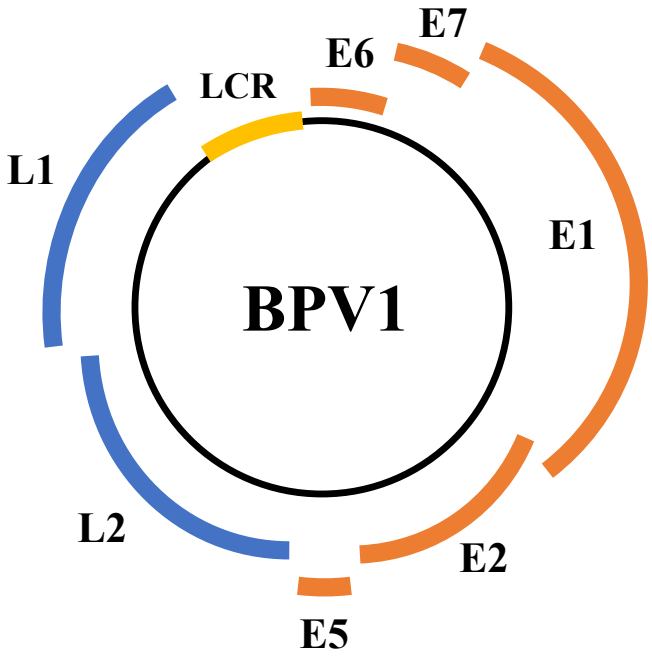


Fig. I-1

Schematic view of *Bos taurus* papillomavirus type 1 (BPV1) genome.

BPV1 is composed of genomes coding early proteins (E1, E2, E5, E6, E7), capsid proteins (L1, L2) and non-coding region (LCR: long control region).

The complete genome of the reference BPV1 is 7946 bp in length (GenBank accession number: X02346.1)

4. Aim of the present study

In contrast to HPV studies, the significant association between PV infection and disease development in animals is uncertain. Moreover, no effective preventive measures and treatment of PV-associated diseases are available in the veterinary field. In terms of animal PV vaccine development, the vaccine should inoculate PV genotype(s) which is cost-effective, universally applicable and preventive against life-threatening diseases. Based on these backgrounds, this study aimed to clarify the etiological roles of animal PVs in cutaneous/mucosal neoplasms by molecular epidemiological approach, as the findings on molecular epidemiology provide valuable knowledge on both basic and applied research fields of PVs leading to the establishment of treatment and preventive strategies including vaccines. The study was performed focusing on three topics as follows.

Chapter 1

In human studies, etiological roles of HPV infection in anogenital neoplasms have been well studied. On the other hand, in animals, there is limited information on anogenital-associated PVs. In cattle, previous findings on anogenital neoplasms have been reported, but few attempts have been made to detect BPVs. Anogenital neoplasms in cattle has become problematic for breeding routine and lesion outbreaks may occur via vertical viral transmission. Therefore, this chapter aimed to evaluate the etiological roles and genomic features of BPVs in anogenital neoplasms of cattle.

Chapter 2

Regarding to the papillomaviral characteristics, BPV types, 1 and 2 are unique that they show cross-species infection to both cattle and horses. Based on the previous findings, the phylogenetic-trait characteristics of BPV1/2 have been described to be affected by either of the two classifiers, species or geographic origins. Based on these two hypotheses, this chapter intended to clarify the underlying phenotype(s) of BPV1/2 sequence differences by comparing BPV1/2 sequences between isolates derived from bovine/equine hosts and different geographic origins.

Chapter 3

Squamous cell carcinomas are one of the life-threatening and common diseases in senior aged cats. Although previous studies described the etiological roles of FcaPVs in feline SCC development, the significant pathogenicity and type(s) of FcaPV(s) is uncertain because these reports

have been restricted to limited numbers and locations. Therefore, the objective of this study was to define the associated type(s) and sequence properties of FcaPVs in SCCs of cats kept in Japan.

Chapter 1

Genomic characterization of Bovine papillomaviruses associated with anogenital tumors of cattle

Introduction

Infection of PVs has been associated with the development of mucosal and cutaneous neoplasia in humans and animals (DeVilliers et al., 2004; VanDoorslaer et al., 2018). In humans, anogenital-associated HPVs have been widely studied, as they can be sexually transmitted, causing anogenital neoplasms including malignant cancers. Molecular epidemiological studies on HPVs revealed the genotypes accounting for the majority of cervical cancers and anogenital warts. The association between disease phenotype(s) and HPV genotype(s) has been determined, and clinical studies have led to the development of prophylactic HPV vaccines.

On contrary to human studies, genomic characteristics on PVs associated with anogenital neoplasms in animals as well as disease-associated hosts factors are not well understood. Previous reports describing the identification of anogenital-associated animal PVs in nonhuman primates (Bergin et al., 2013; Chen et al., 2009; Ostrow et al., 1990), cetaceans (Cortés-Hinojosa et al., 2019; Gottschling et al., 2011a; Rector et al., 2008; Rehtanz et al., 2006; Robles-Sikisaka et al., 2012; Rodrigues et al., 2018; Van Bressem et al., 2007), domestic animals (Bauermann et al., 2017; Lange et al., 2013b; Li et al., 2019; Munday et al., 2018; Saveria Campo et al., 1981; Scase et al., 2010; van den Top et al., 2015), rodents (Cladel et al., 2015; Ingle et al., 2011; Joh et al., 2011; Nafz et al., 2008), and bats (Wu et al., 2012) are available, however, the studies are mostly limited to case reports that we should make an effort to further research on this topic. Studies of animal PVs will contribute not only to the veterinary field, but also to human medicine, as animals are also regarded as animal models for human diseases. In the 1930s, following the discovery of cotton tail rabbit PV (CRPV) (Shope and Hurst, 1933) and rabbit oral PV (ROPV) (Parsons et al., 1936), researchers have demonstrated to infect PV to its original host (animal) in order to mimic the PV-induced anogenital neoplasms of humans (Harvey et al., 1998). However, taken the biological characteristic of comparatively strong species- and tissue-specific properties of PVs, it is challenging to conduct *in vivo* studies on HPVs with laboratory animals. Regarding to these backgrounds, extending the studies of anogenital-associated PVs in animals have important roles to discover the biological, genomic, and pathogenicity of PVs contributing to both medical and veterinary fields.

In nonhuman primates, pathogenetic roles of PVs have been described in the benign and malignant neoplasms of the anogenitals. *Macaca mulatta* PV 1 (MmPV1), *Papio hamadaryas* PV 1 (PhPV1) (Bergin et al., 2013), *Macaca fuscata* PV 2 (MfuPV2) (Van Doorslaer et al., 2017), all classified into the genus, *Alphapapillomavirus*, has been characterized from anogenital tumors such as cervical intraepithelial neoplasia (CIN) and benign penile lesions. In marine animals, various PV types have been identified from papilloma/wart lesions, normal mucosa, and collected fecal swabs of

the anogenitals; *Delphis delphis* papillomavirus 1 (DdPV1), *Phocoena phocoena* PVs (PphPVs) (Gottschling et al., 2011a), *Phoconea spinpinnis* PV 1 (PsPV1) (Van Bressem et al., 2007), and nine types of *Tursiops truncatus* PVs (TtPVs) (Cortés-Hinojosa et al., 2019; Gottschling et al., 2011a; Rector et al., 2008; Rehtanz et al., 2006; Robles-Sikisaka et al., 2012; Rodrigues et al., 2018). In horses, the etiological roles of *Equus caballus* PV 2 (EcPV2) in equine genital neoplasms including malignant tumors such as SCCs have been described and widely been studied especially in Europe (Lange et al., 2013b; Scase et al., 2010). In rodents, two anogenital-associated PVs, *Mastomys coucha* PV 2 (McPV2) (Nafz et al., 2008) and *Mus musculus* PV 1 (MmuPV1) (Ingle et al., 2011) have been characterized. The discovery of anogenital-associated PVs in small rodents extends *in vivo* PV studies involving laboratory mice.

In animal kingdom, papillomas occur more commonly in cattle than any other animals, and diverse types of BPVs exist. Tumors of the bovine genitals have been reported worldwide from the 1960s (Anderson and Sandison, 1969; Bastianello, 1982; Burdin, 1964; Cotchin, 1960; McEntee and Nielsen, 1976; Meyers and Read, 1990; Murray, 1968; Shortridge and Shortridge, 1971; Wettimuny, 1974). Moreover, an outbreak of anal fibropapillomatosis following rectal palpation in a herd of beef heifers was reported (Tweddle and White, 1977). Although many studies have described anogenital tumorigenesis in cattle, few attempts have been made to detect BPVs from anogenital tumors in cattle. The first detection of BPV1 from the penile papilloma was reported in 1981 (Saveria Campo et al., 1981). A recent report from New Zealand showed the detection of BPV2 from anal fibropapillomas of heifers (Munday et al., 2018). Although the studied samples did not derive from the neoplasms, BPV22 classified in the genus *Dyokappapapillomavirus* was identified from vaginal biopsy/swab samples of Holstein cows with vulvovaginitis (Bauermann et al., 2017). Another recent paper described the detection of a novel BPV from genital tract swabs of healthy dairy cows (Ling et al., 2019). BPVs are one of the well-studied PVs in animals however, significant association with bovine anogenital tumorigenesis is uncertain due to limited publications.

Based on these backgrounds, etiological roles of BPVs in bovine anogenital tumorigenesis needs to be clarified in order to develop prevention and treatment strategies for anogenital tumor diseases. Moreover, studies on anogenital-associated PVs in animals are essential from the viewpoint of comparative biology with anogenital-associated HPVs. Therefore, the present chapter intended to clarify pathogenetic roles and genomic features of anogenital-associated BPVs.

Materials and Methods

Sample collection

A total of five Holstein Friesian dairy cows kept in Chiba and Ibaraki Prefectures, Japan were included in the present study (Table 1-1). All the cows were in a healthy state at the moment of anogenital lesion excision. The macroscopic view of the lesions in cow numbers 1 to 3 are shown in Fig. 1-1A-C). Cow numbers 1, 2, and 3 had a history of artificial insemination (AI) before excision of the lesion. Cow number 4 had a history of one parity. A total of four lesions with an approximate size of 3 mm each were observed in this cow and excised on August 29th, September 6th, and December 2nd in 2019. Two vulval papilloma lesions adjacent to each other were observed on the 29th of August. Due to the limitation of the sample volume, one (B190830-1) was stored at -30 °C for DNA extraction and the other (B190830-2) was fixed in 10% buffered formalin for histological analysis. For that reason, histological analysis was unavailable for sample B190830-1. The macroscopic observation of case B190909 is shown in Fig. 1-2A. No macroscopic photos were available for B190830-1 and B190830-2. Cow number 5 had second parity prior to the day of the lesion excision. One vulval papilloma lesion sized approximately 10 mm was observed and excised on October 12th, 2019 (Fig. 1-2B). The sample number of this case was termed B191016. The respective sample IDs were numbered based on the arrival date to the diagnostic laboratory.

Histopathological analyses

Each of the excised papilloma sample was fixed in 10% buffered formalin and embedded in paraffin following hematoxylin and eosin (HE) staining for histological diagnosis. The respective sections were also subjected to immunohistochemistry to identify PV antigen. A cocktail of two monoclonal antibodies (clone BPV-1/1H8 + CAMVIR; Abcam, Cambridge, MA, U.S.A.), which was produced against the major capsid protein of BPV1 and HPV16 was applied. Horseradish perioxidase labeled detection kit (Envision +system) was used, and antigen-antibody complex was visualized by the chromogen treatment with 3,3'-diaminobenzidine (DAB). Each section was counterstained with Mayer's hematoxylin. BPV antigen-positive sample, which is unrelated to the present cases was used as a positive control. The primary antibodies were substituted with tris-buffered saline for the negative control.

DNA extraction and validation

Genomic DNA from frozen tissue samples (B160303, B160620, B160805, B190830-1, B190909, B191016, and B191204) were extracted using QIAamp DNA Mini Kit (Qiagen). For formalin-fixed paraffin-embedded (FFPE) tissue sample (B190830-2), genomic DNA was extracted

using QIAamp DNA FFPE Tissue Kit (Qiagen). Each DNA was extracted following the manufacturer's instructions. The quantity and quality of the DNA samples were determined using NanoDrop Lite (Thermo Fisher Scientific) and detection of bovine beta actin by conventional PCR. Conventional PCR was performed using KOD FX Neo DNA polymerase (Toyobo) and primers designed to amplify bovine beta actin named Bovine beta actin F/R (Table 1-2). As DNA fragmentation can be caused upon formalin fixation (Arreaza et al., 2016), a primer pair designed to detect a shorter length of bovine beta actin, named bovine beta actin F2/R2 was applied for DNA template extracted from FFPE sample, B190830-2 (Table 1-2).

PCR mixture was prepared in 200μL PCR tube each as follows: 10μL 2×PCR Buffer for KOD FX Neo, 4μL dNTPs (2mM), 0.4μL forward primer (10μM), 0.4μL reverse primer (10μM), and 0.4μL KOD FX Neo (1 U/μL). Template DNA was added between 40 to 60 ng per PCR reaction and distilled water was filled up to a total volume of 20μL. PCR reaction for Bovine beta actin F/R primer pair was conducted by two-step cycling condition referring to the manufacturer's instruction as follows: pre-denaturation for 2 min at 94 °C followed by 39 cycles of PCR amplification comprising denaturation at 98 °C for 10 sec, and extension at 68 °C for 15 sec. PCR for Bovine beta actin F2/R2 was performed by three-step cycling condition as follows: pre-denaturation at 94 °C for 2 min , denaturation at 98 °C for 10 sec, annealing at 63 °C for 30 sec, followed by extension at 68 °C for 30 sec. Distilled water instead of the template DNA was applied for negative controls. PCR products were electrophoresed on 2% agarose gels stained with GelRed (Biotium) and the bands were visualized by UV-exposure.

BPV detection

Detection of BPV genomic DNA was validated by conventional PCR by KOD FX Neo DNA polymerase, applying two pairs of consensus primers, subAup/subAdw and subBup/subBdw (Kawauchi et al., 2015; Maeda et al., 2007). These two primer pairs were previously designed based on the conserved nucleotide sequence of BPV L1 among subgroup A (e.g. BPV1, BPV2, BPV5) and subgroup B (e.g. BPV3, BPV4, BPV6) BPVs (Maeda et al., 2007). PCR conditions for subAup/subAdw and subBup/subBdw primer pairs were performed by three-step cycling protocol by modifying the annealing temperature noted in Table 1-2. Positive and negative controls were validated by applying synthesized DNA (0.5 ng/μL) and distilled water, respectively. PCR mixture was prepared as described. PCR results were confirmed by gel-electrophoresis and UV-exposure as mentioned.

BPV genotyping by sequencing

To determine the BPV genotype, direct sequencing was performed. SubAup/subAdw and subBup/subBdw-positive PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following the manufacturer's instructions. Purified PCR products were bidirectionally sequenced with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The sequencing reaction was prepared as follows: 2μL of BigDye 5×Buffer, 1μL of BigDye Terminator v3.1, 1μL of forward or reverse primer (10μM). Appropriate volume of PCR-purified DNA was applied based on the size of the PCR amplicon following manufacturer's recommendation. Distilled water was filled up to 10μL volume. BigDye reactions were performed as follows: 1 min incubation at 96 °C following 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C, and 4 min at 60 °C. Thereafter, 40μL of 75%-volume isopropanol were added and kept at room temperature for 15 min. Following the centrifugation for 20 min at the speed of 15,000 rpm, lysate was discarded and 70%-volume ethanol was applied. The products were centrifuged at 15,000 rpm for 5 min and the ethanol was discarded. Fifteen-microliter-volume of Hi-Di Formamide (Thermo Fischer Scientific) was added and vortexed for 5 min avoiding light. Hi-Di-added products were heat-shocked for 2 min at 95 °C and subsequently cooled on ice for 5 min. Products were replaced to 96 well sequencing plate and subjected to sequencing using 3130xl genetic analyzer (Applied Biosystems). Sequencing results were analyzed with Molecular Evolutionary Genetics Analysis Version 7 (MEGA 7) (Kumar et al., 2016) or MEGA X (Kumar et al., 2018a). Sequence identity was determined using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

Sequence characterization of full-genome (B190830-1, B191016) and L1 open reading frame (B160303, B160620, B160805, B190830-2)

To identify the complete genome of each BPV in cases B190830-1 and B191016, inverse PCR was performed. In brief, outer-ward facing primers based on the known sequence of subBup/subBdw-amplified products were designed for each case. For case B190830-1, PCR primers designed to amplify 7217 bp of BPV28 named BPVJPN NIAH3 53-74 bp/BPVJPN NIAH3 98-75 bp were applied (Table 1-4). For case B191016, a primer pair amplifying 6852 bp of BPV29 named BPV29 L1 7056 (F)/BPV29 L1 6625 (R) were used (Table 1-5). Inverse PCR reactions were performed with KOD FX Neo by modifying the annealing temperature (Tables 1-2 and 1-4) and extension period based on the three-step cycling protocol. Amplified products were electrophoresed on GelRed-stained 1% agarose-gel.

As PVs are classified based on the complete nucleotide sequence of the L1 major capsid protein, L1 sequence was characterized for cases B160303, B160620, B160805, and B190830-2.

Based on the BPV genotype determined by sequencing results of subAup/subAdw- and subBup/subBdw-amplified products, primers amplifying the L1 of BPV1 (B160805), BPV2 (B160303, B160620), and BPV28 (B190830-2) were designed. For BPV1 and BPV2 (BPV1/BPV2)-detected cases, BPV1&2 E5/BPV1&2 757 LCR primer pair was used in the PCR reaction (Table 1-3). Considering the DNA fragmentation due to formalin-fixation in case B190830-2, primers were designed to detect short fragments of BPV28 L1 (Table 1-4).

The success in PCR of each case was confirmed by gel-electrophoresis. Respective amplicons were column-purified and subjected to direct sequencing by applying sequencing primers listed in Tables 3 and 4 for BPV1/BPV2-detected cases (B160805, B160303, B160620) and BPV28-detected case (Table 1-4). Column-purification and sequencing were performed as described.

Sub-cloning and sequencing

Sub-cloning of the complete L1 sequence was performed for BPV1/BPV2-detected cases (B160303, B160620, B160805). A primer pair named BPV1&2 E5 (F)/ BPV1&2 757 LCR (R) were used to amplify the 4180 bp and 4171 bp products of BPV1 and BPV2, respectively which includes the complete L1 region (Table 1-3). The respective amplicons were cloned into pCR 2.1-TOPO vector (Invitrogen) following the plasmid purification using NucleoSpin Plasmid QuickPure (Macherey-Nagel).

Complete genome of BPV28 and BPV29 identified from cases B190830-1 and B191016, respectively, were cloned into pCR XL-2 TOPO vector (Invitrogen). For BPV28 (B190830-1), three pairs of primers named BPV28 667 (F) /3391 (R), BPV28 3237 (F)/6424 (R), and BPV28 6327 (F)/BAA5-1012 bp (R) (Table 1-4) were applied for PCR and each amplicon was cloned. For BPV29 (B191016), two PCR products amplified with primer pairs named BPV29 E1 1059 (F)/L1 6255 (R) and BPV29 L1 6043 (F)/E1 1106 (R) were cloned (Table 1-5).

Sequence of the respective clones were conducted using BigDye Terminator v3.1 Cycle Sequencing kit and 3130xl genetic analyzer (Applied Biosystems) with a modification of applying 0.25μL of BigDye Terminator v3.1 instead of 1μL. Fifty-nanogram of plasmid DNA was added and filled up to 10μL with distilled water in each BigDye reaction. In addition to sequencing primers used for direct sequencing, M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers were applied for each clone to confirm the success in sub-cloning.

RNA extraction, DNA elimination, and cDNA synthesis

Total RNA from frozen (B160303, B160620, B160805) and FFPE tissue (B190830-2, B191016) samples were extracted using RNeasy mini kit (Qiagen) and WaxFree™ Paraffin Sample RNA Extraction Kit (TrimGen), respectively following the manufacturer's recommended protocol. Genomic DNA was eliminated with recombinant DNase I (RNase-free) (Takara) and the success in DNase treatment was confirmed by conventional PCR against two bovine beta actin primer pairs (bovine beta actin F/R and F2/R2) as described. cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara) and each cDNA sample was stored at -30°C until use.

Reverse-transcriptase PCR (RT-PCR)

Reverse-transcriptase PCR (RT-PCR) was performed to evaluate the mRNA expression of BPV early and late genes in each case. KOD FX Neo DNA polymerase was used for RT-PCR and synthesized cDNA of the respective cases were applied as template. For BPV1/BPV2-detected cases (B160303, B160620, B160805), four primer pairs targeting the E2, E5, E6, and L1 named BPV1rnaE2-F/R (Zhu et al., 2019a); BPV1,2 E5-F/R; BPV1,2 E6-F/R; and subAup/subAdw (Maeda et al., 2007) were used (Tables 1-2 and 1-4). For BPV28-identified (B190830-2) and BPV29-identified (B191016) cases, four primer pairs detecting three early genes (E1, E2, E10) and one late gene (L1) of the respective BPVs were applied (Tables 1-4 and 1-5). Genomic DNA extracted from the respective samples was used as positive control in each reaction. The RT-PCR reactions were performed by modifying the annealing temperature suitable for each primer pair. RT-PCR results were confirmed by electrophoresis on 2% agarose gel and bidirectional sequencing following the same method as described.

Sequence analyses

A phylogenetic tree was built based on the L1 nucleotide sequence of 116 isolates of animal and human PVs in the Papillomavirus Episteme PaVE (Van Doorslaer et al., 2017), including BPVs identified in the present study and PVs previously identified in the anogenitals of animals. The tree was constructed with MEGA X by the neighbor-joining method (Saitou and Nei, 1987; Tamura et al., 2004) with 1000 bootstrap replicates. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site.

Sequence identities of the L1 nucleotide between BPV types including the reference and isolates identified in this research were determined by ClustalW tool (https://www.genome.jp/tools-bin/clustalw). Isolates B190830-2 and B191016 were included in the complete genome-based

ClustalW analysis. The reference BPVs included in these analyses were referred from PaVE (Van Doorslaer et al., 2017) and GenBank databases.

To determine the ORFs and non-coding region of the BPVs identified in cases B190830-2 and B191016, complete sequences were deposited in Open Reading Frame Finder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/).

Sequence data identified in this research were deposited in DDBJ/EMBL/GenBank database under accession numbers of LC426021 (B160303), LC426022 (B160620), LC426023 (B160805), LC519594 (B190830-1), LC521687 (B190830-2), and LC514113 (B191016) (Table 1-1).

Results

Histopathology

The macroscopic observations on cases B160303, B160620, and B160805 were composed of a bland population of spindle-shaped mesenchymal cells proliferating in streams and was covered by acanthotic epidermis. The thickened epidermis occasionally extended into the underlying dermis as broad rete pegs (Fig. 1-1D–I). Based on these histological findings, all of the three cases (B160303, B160620, B160805) were diagnosed as fibropapilloma. In case B160620, PV antigen was immunohistochemically positive in the nucleus of a few keratinocytes at the stratum granulosum of the hyperplastic epidermis (Fig. 1-3), while no positive signal was observed for cases B160303 and B160805.

In four HE-stained specimens (B190830-2, B190909, B191016, B191204), papillary projections of stratified squamous epithelium were observed and histologically diagnosed as papilloma (Fig. 1-2C-F). Nuclear inclusion bodies were not found in any these cases. Immunohistochemically, PV antigen was negative in the epithelial cells of all four cases.

BPV detection

Detection of BPV DNA was confirmed by PCR using subAup/subAdw and subBup/subBdw primer pairs following electrophoresis. In cow numbers 1 to 3 (B160303, B160620, B160805), all three cases were positive with subAup/subAdw while no band was detected for subBup/subBdw primer pair. In cow numbers 4 and 5 (B190830-1, B190830-2, B190909, B191016, B191204), no band was observed for the PCR product amplified with subAup/subAdw primer pair. In cow number 4, with the subBup/subBdw primer pair, a single positive band between 600 and 700 bp were observed for case B190830-1, while B190909 and B191204 were negative. Similarly, a single

band was observed with subBup/subBdw-amplified product of the DNA sample collected from cow number 5 (B191016).

The direct sequencing result of the subBup/subBdw-amplified product showed sequence homology of 99.81% (523/524 nucleotides, nt) to BPV28 (GenBank accession number: LC500686). Considering the DNA fragmentation upon formalin-fixation in case B190830-2, subAup/subAdw and subBup/subBdw primer pairs may have not been suitable for this sample. Taking the sequencing result of B190830-1, the same BPV genotype infection may have occurred in sample B190830-2. Therefore, a primer pair detecting 163 bp fragment of BPV28 L1 region, a primer pair named BPV28 L1 6642 (F)/BPV28 L1 6804 (R) (Table 1-2), was used to confirm the detection of BPV28 in sample B190830-2. Electrophoresis and direct sequencing confirmed that BPV28 was also detected in sample B190830-2.

In cow number 5, a single band sized between 600 and 700 bp was observed for subBup/subBdw-amplified product of sample B191016, while no band was observed with subAup/subAdw primer pair. The direct sequencing result of the B191016 subBup/subBdw-amplified product was closest to BPV11 (GenBank accession number: AB543507) but below 80% homology, suggesting an unclassified BPV.

Complete BPV L1 sequences of BPV1 and BPV2 identified from cow numbers 1 to 3

The complete L1 nucleotide sequence of BPV1 identified from cow number 3 (B160805) were 100% identical to the four BPV1 isolates registered in the GenBank database including the reference BPV1 (GenBank accession numbers: KY886226.1, J02045.1, X02346.1, U13843.1). BPV2 L1 sequence identified from cow numbers 1 and 2 (B160303 and B160620) presented 100% identity with one BPV2 isolate each, (GenBank accession numbers: MF045490.1 and X01768.1, respectively). The nucleotide sequence of cases B160303 and B160620 were identical except for the 1456th nucleotide of the L1 (“A” for B160303 and “T” for B160620). Due to this nucleotide substitution, the 486th amino acid substitution was also noted as “T” for B160303 and “S” for B160620.

Sequence characteristics of BPV28 identified in B190830-1 and B190830-2 from cow number 4

PCR and sequencing results of samples B190830-1 and B190830-2 confirmed the detection of partial BPV28 L1 sequence. BPV28 had been identified recently, originated from the cutaneous wart excised from the face of a cow (GenBank accession number: LC500686). As the lesion site observed in cow number 4 was distinct from this case (LC500686), the complete BPV28 sequence identified in the vaginal papilloma was determined to investigate the viral sequence variation between

the origin of the anatomical locations (face/vulva) and tissue sites (cutaneous/mucosal). The complete BPV28 genome identified by inverse PCR and sequencing of case B190830-1 was 7264 bp in size and harbored 99.78% nucleotide sequence homology (7253/7269 nt) to the reference sequence (GenBank accession number: LC500686). The sequence homology determined by each ORF was E1: 99.89% (1825/1827 nt); E2: 100% (1254/1254 nt); E4: 100% (459/459 nt); E10: 100% (129/129 nt); L1: 99.61% (1530/1536 nt); L2: 99.49% (1576/1584 nt); and LCR: 100% (477/477 nt). The amino acid sequences of E2, E4, E7, and E10 were 100% identical to the reference. E1 amino acid showed 99.84% (607/608) identity with a one-point mutation of R454K. L2 had 99.62% (525/527) amino acid sequence identity. In the L2, six nucleotide insertions of "GGG GGT" at nucleotide positions 4010 to 4015 resulting in the insertion of two glycines (G) at the 82nd amino acid was observed in case B190830-1. Remarkably, five nucleotide deletions of "CAG CT" was observed at positions 5829 to 5833 locating in the L1. Based on the results using NCBI ORF finder tool, this deletion resulted in a disruption of the reading frame, separating the L1 into two proteins (Fig. 1-4A). Based on the positions of reference BPV28, the two separated L1s were: 5654 to 5855 nt (492 bp, 163 amino acids) and 5746 to 6894 nt (1149 bp, 382 amino acids). The complete sequence results of the BPV28 clones of B190830-1 were 100% identical to the direct sequencing results.

Since the BPV28 L1 sequence result harbored a frameshift mutation in sample B190830-1, the full sequence of BPV28 L1 in case B190830-2 was also determined. The L1 of case B190830-2 was a single ORF (1536 bp, 511 amino acids), showing no "CAG CT" deletion at positions 5829 to 5833, unlike B190830-1 (Fig. 1-4A).

Full-genome characterization of the unclassified BPV (BPV29) in B191016 from cow number 5

As the sequencing result of subBup/subBdw-amplified product of case B191016 suggested the detection of an unclassified, novel BPV type, inverse PCR and bidirectional sequencing was further conducted to determine the unknown sequence. The success in inverse PCR was confirmed by electrophoresis. The direct sequencing results of the PCR-purified product revealed that the unclassified BPV was 7282 bp in the complete genome, with a GC content of 42.4% (3087 GC/7282 bp). The viral genome was composed of L1 (5613-7130 nt, 1518 bp), L2 (4012-5601 nt, 1590 bp), E1 (965-2800 nt, 1836 bp), E2 (2742-3998 nt, 1257 bp), E4 (3208-3732 nt, 525 bp), E7 (670-975 nt, 297 bp), E10 (343-471 nt, 129 bp), and LCR (7131-7282 nt, 1-342 nt, 494 bp) (Fig. 1-4B). Based on the L1 nucleotide homology determined by ClustalW analysis, the most identical BPV genotype was BPV6 (GenBank accession number: AB845589.1) showing 78% similarity (Table 1-6). Since this unknown BPV L1 sequence was dissimilar to BPV6 L1 for over 10%, the unclassified BPV should be

classified as a novel genotype and designated BPV29. This BPV29 is suggested to be classified in the genus, *Xipapillomavirus*, species, *Xipapillomavirus* 1. The L1 nucleotide-based phylogenetic analysis showed that the novel BPV29 was clustered within *Xipapillomavirus*-classified PV types (Fig. 1-8). The sequencing results of the BPV29 clones were 100% identical to those of the direct sequencing results.

Conserved motifs of BPV28 and BPV29 sequences

LCR is recognized as a regulator of viral transcription and replication by binding to its respective cellular binding sites. These conserved binding sites have been determined in various PVs, known as TATA box, activator protein 1 (AP-1), AP-2, nuclear binding factor 1 (NF-1), octamer binding protein 1 (Oct-1), and polyadenylation sites (polyA) (Desaintes and Demeret, 1996). In the LCR of BPV28 identified from case B190830-1, sequences of the conserved binding sites were observed. One polyA binding site (AATAAA, 6969 nt), three NF-1 binding motifs (TTGGC, 7002 nt, 7116 nt, 7147 nt), one AP-1 binding site (TKWNTMA, 7062 nt), four AP-2 binding sites (SCACMY, 6946 nt, 7051 nt, 7090 nt, 7233 nt), one Oct-1 binding motif (AANWGYAB, 7167 nt), and three TATA boxes (TATAA, 58 nt, 7121 nt, 7152 nt) were identified (Walter G Hubert, 2005) (Fig. 1-5A). In the LCR of various PVs, two binding sites, known as E1-binding site (E1BS) (Sedman et al., 1997) and E2BS are shown to be conserved (Rogers et al., 2011). In the LCR of BPV28, three E2 binding sites ($ACCN_6GGT$, 27 nt, 43 nt, 7213 nt) (Rogers et al., 2011) were present, while no E1BS motif ($ATNGTTN_3AACNAT$) was observed (Fig. 1-5A). As described, the conserved transcription factor-binding sequences of PVs were observed in the LCR of BPV28.

In the LCR of BPV29, two TATA boxes (124 nt and 291 nt), two AP-1binding motifs (33 nt and 226 nt), three AP-2 binding motifs (9 nt, 21 nt, 56 nt) were confirmed (Fig. 1-5B) (Walter G Hubert, 2005). Two NF-1 motifs (72 nt and 89 nt) and two Oct-1 motifs (129 nt and 179 nt) were also identified (Walter G Hubert, 2005). In the LCR of BPV29, three E2BS (185 nt, 261 nt, 276 nt) were present (Fig. 1-5B). Similar to BPV28 LCR, no E1BS was observed in BPV29.

The retinoblastoma protein (pRB)-binding domain (LXCXE) has been shown to contribute to host cell transformation and this LXCXE motif is conserved within the E7 of various PVs, including *Xipapillomavirus*-classified BPVs (Narechania et al., 2004). In BPV28 and BPV29, the LXCXE motif was observed as LHCEE and LDCDE, respectively at E7 amino acid positions 24 to 28. Another conserved motif noted in the PV E6 and E7 ORFs is the zinc-binding domain ($CXXC\text{-}X_{29}\text{-}CXXC$), which contributes to cellular transformation (Narechania et al., 2004; Roman and Munger, 2013). In

the E7 of both BPV28 and BPV29, one zinc-binding domain was observed at amino acid positions 47 to 83.

mRNA expressions of BPV1, BPV2, BPV28, and BPV29

The success in DNase-treatment using subAup/subAdw (for cases B160303, B160620, B160805) and bovine beta actin F2/R2 (for cases B190830-2, B191016) primer pairs were confirmed in all tested samples. mRNA (cDNA) expression of three early genes (E2, E5, E6) were confirmed in BPV1/BPV2-detected samples (B160303, B160620, B160805), but L1 was negative in all three cases (Fig. 1-6A-D). Similarly, mRNA expression of early genes (E1, E2, E10) were confirmed in both BPV28 (B190830-2) and BPV29 (B191016)-identified cases, while no L1 expression was observed (Fig. 1-7A and B). Sequencing results of RT-PCR-positive products were 100% identical to that of respective positive controls in all tested samples.

Discussion

In the present study, two classical *Deltapapillomavirus*- (BPV1, BPV2) and two *Xipapillomavirus*-classified (BPV28, BPV29) BPV genotypes were identified from bovine anogenital lesions. In BPVs, genotypes classified as the genus, *Deltapapillomavirus* and *Xipapillomavirus* have been associated with fibropapillomas and epitheliotropic lesions, respectively (Daudt et al., 2018). The histopathological findings of the respective cases were consistent to this PV classification/lesion phenotype characteristics.

Both BPV1 and BPV2 have been detected in cutaneous papillomas of dairy cattle (Dagalp et al., 2017). These two genotypes were also identified in the mucosal sites, such as urinary bladder cancers of cattle, but this etiology is suggested to be caused by immunosuppression via bracken fern grazing (Campo et al., 1992; Z. Yuan et al., 2007b). Related to BPV1-detection from the mucosal sites, a previous report demonstrated BPV1-detection from esophagus and some nodules of the upper-intestinal tract of a steer with severe-papillomatosis (Shimakura et al., 2018). BPV2 DNA was identified in fibropapilloma emerged on the anus of heifers (Munday et al., 2018), suggesting that both BPV1 and BPV2 have an potential to cause lesions on the mucosal sites. This study also demonstrated the association of BPV types, 1 and 2, with anognital fibropapilloma development in domestic cattle, indicating the mucosal tropism of both types.

In this study, two unique BPV types, 28 and 29, were identified in the vulval papillomas of cows, which demonstrates the detection of *Xipapillomavirus*-classified BPVs from the bovine genitals for the first time. Prior to this detection of BPV28, complete sequence of BPV28 identified

from cutaneous papilloma from the face of a steer was characterized as a novel genotype (GenBank accession number: LC500686). However, this case showed concurrent detection with BPV1 and BPV2, and histological analysis was not performed to due to the sample limitation. The present study characterized BPV28 sequence and histology for the first time, identified from bovine vulval papilloma.

In cow number 4, after the excision of samples B190830-1 and B190830-2, similar wart-like lesions were observed at the vulva. Although their histological observations were similar (samples B190909 and B191204), no PV DNA was detected. The etiology of BPV-negative papilloma following BPV-positive papilloma excision needs to be further studied. Interestingly, in cow number 4, one of the BPV28s identified in case B190830-1 had five nucleotide deletions in the L1, resulting in a frameshift mutation and two L1 ORFs (Fig. 1-4A), while the other BPV28 detected from the adjacent sample (B190830-2) did not have such a mutation. To minimize possible artifacts, the five-nucleotide deletion (5829-5833 nt) was confirmed by bidirectional sequencing by applying three sequencing primers, designated as primer 10 (F: 5162-5183 nt), primer 11 (F: 5680-5703 nt), and primer 11 (R: 6385- 6364 nt) (Table 1-4) for both clone and direct sequencing. To the author's knowledge, there are no such reports describing L1 frameshift mutations due to nucleotide deletions in naturally identified cases in animal PVs. In a previous study related to humans, HPV6a harboring a large deletion in L1 was identified in recurrent cases of laryngeal papilloma, showing similar histology to that of HPV6a wild-type-identified cases (Suzuki et al., 1995). Unfortunately, due to the sample limitation, we could not address the histological significance between BPV28-identified cases with L1 frameshift (B190830-1) or without mutation (B190830-2). Another previous study of HPV demonstrated that the N-terminal deletion of HPV16 L1 resulted in a mixed population of capsids of variable sizes (Varsani et al., 2006). As the L1 protein comprises the viral capsid along with L2, sequence deletion in L1 may alter morphological structures of the viral capsid. However, etiological and/or morphological variations due to the L1 frameshift mutation observed in sample B190830-1 cannot be clarified in the present study, and the underlying phenomenon needs to be further analyzed.

Another *Xipapillomavirus* 1-classified BPV type 29 identified in this study showed similar genome structures to the other *Xipapillomavirus* 1-classified BPVs, such as BPV3 and BPV4. The nucleotide sequence of BPV29 showed the highest homology not only to L1 but also to the complete genome sequence of BPV6 (GenBank accession number: AB485589) (Table 1-6), which is also classified as *Xipapillomavirus* 1. BPV6 infection has been associated with bovine teat papillomatosis and detected in Japan (Hatama et al., 2008; Maeda et al., 2007; Ogawa et al., 2018), but it is still uncertain how BPV29 has evolved.

The present study analyzed mRNA expressions of both early and late genes by RT-PCR. BPV transcription is regulated by E2 (Munday, 2014), and cellular transformation is known to be generated by oncogenes, such as E5 and E10 (Van Doorslaer and McBride, 2016). In general, viral replication of BPV occurs only in differentiating cells (Chen et al., 2009) and may vary between anatomical location, tissue, and/or cells (Shimakura et al., 2018). In all five tested samples (B160303, B160620, B160805, B190830-2, B191016), mRNA detection of the early regions was confirmed while L1 was negative. This suggests that the active virus was present, but none or very few complete (encapsulated) viruses were being produced. So theoretically, L1 protein should not be expressed within the lesions. Immunohistochemically, PV antigen was not detected in the analyzed specimens except for case B160620 (Table 1-1 and Fig. 1-3). Since the primary antibody applied for IHC was produced against BPV1 L1 antigen, the negative results for IHC were acceptable. In the specimen of case B160620, BPV L1 antigen was observed within the terminally differentiating keratinocytes in the stratum granulosum (Fig. 1-3). The positive signal was limited to very few keratinocytes so BPV L1 mRNA may have been below the detection limit of RT-PCR.

The presence of transcriptionally-active PVs could become one of the key factors in viral transmissions (Altamura et al., 2018). Unlike the papilloma outbreaks as reported previously (Maeda et al., 2007; Tweddle and White, 1977), no anogenital papilloma lesions were observed within the analyzed herds in this study. From the aspect of veterinary hygiene, it is important to keep in mind that breeding routine such as rectal palpation, ultrasonography, the use of controlled internal drug release device and artificial insemination could become a route of viral transmission.

Conclusion

The present study demonstrates the association of BPVs classified as *Deltapapillomavirus* (BPV1, BPV2) and *Xipapillomavirus* (BPV28, BPV29) with bovine anogenital lesions. This report will update the etiological knowledge of anogenital-associated BPVs including genomic characteristics of two novel BPVs, BPV28 and BPV29. BPVs have been studied for decades, but the discovery of BPVs including the two classical types, 1 and 2, from anogenital neoplasms have suggested their potential to cause anogenital tumors in cattle. Animal studies are valuable not only for the veterinary field, but also for human medicine, as animal models are one of the essential tools to study human diseases. Studies of anogenital-associated PVs in animals have a positive impact on diverse research fields.

Table 1-1 Summary data of clinical information for five dairy cows with anogenital lesions, histology, and BPV information.

Cow number	Sample ID	Collection date	Breed	Age	Sex	Sample storage	Location of the lesion	Histological diagnosis	PV IHC	BPV genotype	GenBank accession number
1	B160303	Feb. 29, 2016	Holstein Friesian	1	Female	Frozen and FFPE	Vulva	Fibropapilloma	-	BPV2	LC426021
2	B160620	Jun. 17, 2016	Holstein Friesian	2	Female	Frozen and FFPE	Vulva	Fibropapilloma	+	BPV2	LC426022
3	B160805	Aug. 2, 2016	Holstein Friesian	2	Female	Frozen and FFPE	Anus	Fibropapilloma	-	BPV1	LC426023
4	B190830-1	Aug. 29, 2019	Holstein Friesian	2	Female	Frozen	Vulva	Not available	Not available	BPV28 (frameshift in L1)	LC519594
	B190830-2					FFPE	Vulva	Papilloma	-	BPV28	LC521687
	B190909	Sep. 6, 2019				Frozen and FFPE	Vulva	Papilloma	-	Not detected	-
	B191204	Dec. 2, 2019				Frozen and FFPE	Vulva	Papilloma	-	Not detected	-
5	B191016	Oct. 12, 2019	Holstein Friesian	2	Female	Frozen and FFPE	Vulva	Papilloma	-	BPV29	LC514113

GenBank/EMBL/DDBJ accession numbers of the BPV-positive cases are noted for the respective sample IDs.
Abbreviations: BPV, bovine papillomavirus; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry

Table 1-2 Primer information used for detecting bovine beta actin and BPVs.

Experiment	Applied samnples	Primer name	Sequence (5' to 3')	Target	PV Position (nt)	Product size (bp)	Tm (°C)	Reference
DNA quality/quantity validation	B160303, B160620, B160805 B190830-1, B190909, B191204, B191016	Bovine beta actin (F) Bovine beta actin (R)	GTCAGTGGGCCGTCCTGCCTTG GGGACACCACAAGGGGCAGTCG	Bovine beta actin		411	68	This study
	B190830-2	Bovine beta actin (F2) Bovine beta actin (R2)	CTGCGGCATTCACGAAACTAC ATCTGCACCGTCCGCTA	Bovine beta actin		202	63	This study
BPV detection	All samples	subAup subAdw	CCAGAYTAYYTMAAAATGGC ATAAMKGCTAGCTTATATTC	BPV1, BPV2, BPV5, BPV7, BPV8, BPV13 (L1, degenerate)	Degenerate, L1	433-455	50	Maeda *et al.*, 2007; Kawauchi *et al.*, 2015
	All samples	subBup subBdw	TWYAATAGGCCCTTTTGGAT TTMCGCCTACGCTTTGGCGC	BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV12 (L1, degenerate)	Degenerate, L1	590-611	55	Maeda *et al.*, 2007; Kawauchi *et al.*, 2015
	B190830-2	BPV28 L1 6642 (F) BPV28 L1 6804 (R)	TCTCTTGCCACCAGATGTCC TGCTGCGTTTGCCTATCTGA	BPV28	6642-6661 6804-6785	163	55	This study

The nucleotide position is based on the sequence of the reference BPV sequence.
Abbreviations: bp, base pairs; BPV, bovine papillomavirus; F, forward; nt, nucleotide; R, reverse; Tm; annealing temperature

Table 1-3 Primers used in PCR, reverse-transcriptase PCR (RT-PCR), and sequencing for cases B160303, B160620, and B160805.

Experiment	Applied samnples	Primer name	Sequence (5' to 3')	Position (nt)	Product size (bp)	Reference
PCR and sub-cloning	B160303, B160620, B160805	BPV1&2 E5 (F)	CACTACCTCCTGGAATGAACATTTCC	3787-3812 (BPV1), 3788-3813 (BPV2)	4180 (BPV1), 4171 (BPV2)	Roperto *et al.*, 2008
		BPV1&2 757 LCR (R)	GATGGTGTGATTATTGTTAAC	21-1 (BPV1, BPV2)		Nasir *et al.*, 2007
Sequencing		BPV1_seq_5141	CTACATGTCAGGTACTCATTG	5140-5160 (BPV1), 5139-5159 (BPV2)		Shimakura *et al.*, 2018
		BPV1_seq_5641	TCTCCCTCCAACCCCTGTAAG	5640-5660 (BPV1), 5633-5653 (BPV2)		
		BPV1_seq_5721	CGGAGCGCCTGCTAACTATA	5720-5739 (BPV1), 5713-5732 (BPV2)		
		BPV1_seq_6141	ATGGCGCCTGCCCTCCTCTTG	6140-6160 (BPV1), 6133-6153 (BPV2)		
		BPV1_seq_7390 (R)	CTTACCCCGCATCTACCGCC	7389-7370 (BPV1), 7382-7362 (BPV2)		
		BPV1&2 LCR inv. (R)	ATGCTGGACAGGATGTGTACCG	7231-7210 (BPV1), 7223-7202 (BPV2)		Nasir *et al.*, 2007 (Reverse complement of BPVLCR F primer)
RT-PCR		BPV1rnaE2-F	GTGGTAGAGGTGGAGTTTGA	2953-2972 (BPV1), 2950-2969 (BPV2)	265	Zhu *et al.*, 2019
		BPV1rnaE2-R	CAGAAGAGGTGGATGAGACA	3217-3198 (BPV1), 3214-3195 (BPV2)		
		BPV1,2 E5-F	TTGTTCTTGGGACTAGTTGCTG	3899-3920(BPV1), 3903-3924(BPV2)	100	This study
		BPV1,2 E5-R	CTGTACAGGAGCACTCAAAATGA	3998-3976(BPV1), 4002-3980(BPV2)		
		BPV1,2 E6-F	GTGCAGRGAGCCTCTYACAGAA	147-168 (BPV1, BPV2)	234	This study
		BPV1,2 E6-R	AGTTTKCCCCCACAGTAGCA	380-361 (BPV1, BPV2)		

The nucleotide position is based on the sequence of the reference genotype of BPV1 and BPV2 (GenBank accession numbers: X02346 and M20219).
Abbreviations: bp, base pairs; BPV, bovine papillomavirus; F, forward; nt, nucleotide; R, reverse; seq, sequence; Tm; annealing temperature

Table 1-4 Primers used in PCR, reverse-transcriptase PCR (RT-PCR), and sequencing for cases B190830-1 and B190830-2.

Experiment	Applied sample	Primer name	Sequence (5' to 3')	Position (nt)	Product size (bp)	Reference
Inverse PCR		BPVJPN NIAH3 53-74 bp	GTAACCGCTGTTGATAGCACAC	6357-6378	7217	
		BPVJPN NIAH3 98-75 bp	CTGAGATTGTAAAGTTTGTTCCAC	6379-6402		
PCR for sub-cloning						
		BPV.p18 667(F)	CACTGTGAGTCTCCTCTGTCCA	667-688	2725	
		BPV.p18 3391(R)	GCTGTTGCTGTCTGGTTCCT	3391-3372		
		BPV.p18 3237(F)	ACATCCCAGAAACTCCAGGAAAC	3237-3259	3188	
		BPV.p18 6424(R)	GGCTCGGGATTGGTTTTATGAAC	6424-6402		
		BPV.P18 6372 (F)	GTTGATAGCACACGTGGAACAAAC	6372-6395	1705	
		reverse BAA5-1012 bp	TCGCTATCAGATTCACTACATTCTGC	808-783		
Sequencing		primer.1(F)	GTTGTTACTTGTACCGAATGCG	14-36		
		primer.1(R)	TGCAAAACACTGGCTTAGCGCACGC	713-689		
		primer.2(F)	TGCGCTAAGCCAGTGTTTTGC	692-712		
		primer.2(R)	TGTAACACGCTTGTGCTGTAGTCC	1210-1187		
		primer.3(F)	GGGAAAGATATTGGTGCAAACAAG	1155-1178		
		primer.3(R)	AGGCTAGGCGTGCATAATTGTAAG	1767-1744		
	B190830-1	primer.4(F)	GGGCTTACGATCATGATTATGTTG	1705-1728		
		primer.4(R)	GCAGGTTTTCCAGACTCATC	2419-2400		
		primer.5(F)	GATGCTACCAGAGCTACTTGGTC	2184-2206		
		primer.5(R)	CTCTGGTTTAGCCATGAAAGTCTC	2826-2803		
		primer.6(F)	CCTGGTCTTTGACAGAAACA	2777-2796		
		primer.6(R)	TCGTAGGCCTCGTTTATAAG	3503-3484		
		primer.7(F)	GGAAGATCTCGAACACCTGA	3436-3455		
		primer.7(R)	GTCGTGCAACATTTGCTCC	4043-4025		
		primer.8(F)	CCCACAGGGTACACACCATTAG	3965-3986		
		primer.8(R)	GGCTGGATTGTCAAAATCCC	4660-4641		
		primer.9(F)	AATAGACGACTGGTACAACAGG	4568-4589		
		primer.9(R)	CGTGTACTCTCATCCTCATCAG	5220-5199		
		primer.10(F)	GACATTGAAACCACTGTAACCC	5162-5183		
		primer.10(R)	GACTCTGTCATCATCATTTGCAC	5795-5773		
		primer.11(F)	GCAGAGGTCAGCCACTAGGAGTTG	5680-5703		
		primer.11(R)	GTTCCACGTGTGCTATCAACAG	6385-6364		
		primer.12(F)	CCAGTGACCAGCAAATCTATAATAGG	6268-6293		
		primer.12(R)	CCATCAGTGTGCAAGCAGTTATTATC	6957-6932		This study
		primer.13(F)	AACCTAGAACAGTACTCATTGGG	6747-6769		
		primer.13(R)	AAAAGACGCAAAGTTTGCC	170-152		
PCR and sequencing for L1		BPV28 L1 5272 (F)	TGACACCAACACAGCTACATTC	5272-5293	267	
		BPV28 L1 5538 (R)	TCACATCCCCCTTTTCGTCTT	5538-5518		
		BPV28 L1 5481 (F)	CGGCTACTAGCTGTTGGACA	5481-5500	349	
		BPV28 L1 5829 (R)	AAAGCTGCACCTGTTTTGGG	5829-5810		
		BPV28 L1 5543 (F)	CCCTAAAGTCTCTGGAAGCCA	5543-5563	435	
		BPV28 L1 5977 (R)	ATGTTCCCAAACCCTGTGTCA	5977-5957		
		BPV28 L1 5799 (F)	GTATGCATTGACCCAAAACAGG	5799-5820	477	
		BPV28 L1 6275 (R)	GTCACTGGAAACCAAAGACCC	6275-6255		
		BPV28 L1 5954 (F)	GTGTGACACAGGGTTTGGGAAC	5954-5975	473	
		BPV28 L1 6426 (R)	CGGGCTCGGGATTGGTTTTA	6426-6407		
		BPV28 L1 6372 (F)	AGCACACGTGGAACAAACTT	6372-6391	433	
	B190830-2	BPV28 L1 6804 (R)	TGCTGCGTTTGCCTATCTGA	6804-6785		
		BPV28 L1 6762 (F)	TCATTGGGGCGCAAATTTCTT	6762-6782	247	
		BPV28 L1 7008 (R)	ATGCCAATCCAATCCAGGTGT	7008-6988		
RT-PCR		BPV28 E1 1010 (F)	TAGAGCTGCGCCAAAAAGGA	1010-1029	124	
		BPV28 E1 1133 (R)	TCCCTGCGAAACTAAGCTCC	1133-1114		
		BPV28 E2 3133 (F)	CGACAACGAGCCCAAAAAGG	3133-3152	157	
		BPV28 E2 3289 (R)	TTGATCTCGAACTCGACCGC	3289-3270		
		BPV28 E10 108 (F)	ATGCCGCTTCGTTGGTTAT	108-126	129	
		BPV28 E10 236 (R)	TCAATCCCATCCATGTAAATCATCT	236-212		
		BPV28 L1 5799 (F)	GTATGCATTGACCCAAAACAGG	5799-5820	179	
		BPV28 L1 5977 (R)	ATGTTCCCAAACCCTGTGTCA	5977-5957		

The nucleotide position is based on the sequence of the reference genotype of BPV28 (GenBank accession number: LC500686).
Abbreviations: bp, base pairs; BPV, bovine papillomavirus; F, forward; nt, nucleotide; R, reverse; Tm; annealing temperature

Table 1-5 Primers used in PCR, reverse-transcriptase PCR (RT-PCR), and sequencing for case B191016.

Experiment	Applied sample	Primer name	Sequence (5' to 3')	Position (nt)	Product size (bp)	Reference
Inverse PCR		BPV29 L1 7056 (F)	TCTGCACCGAAAACGGTCAC	7056-7075	6852	
		BPV29 L1 6625 (R)	CCTCTAGTGCTGTCAACTGCA	6625-6605		
PCR for sub-cloning		BPV29 E1 1059 (F)	GCCTGTCTGATTTGTCTGACCTA	1059-1081	5197	
		BPV29 L1 6255 (R)	CAGATTTACTGGCTTGCAGCTTT	6255-6233		
		BPV29 L1 6043 (F)	ATACAGCAATGGACCCTAAGCAA	6043-6065	2346	
		BPV29 E1 1106 (R)	CTTGCTCGCATTCAGCATTATCA	1106-1084		
Sequencing		BPV29 seq. 403 (R)	GAGACAAGAAATTAAACCCAC	403-383		
		BPV29 seq. 440 (F)	CTGCTATAAATGAGCTGCATGG	440-461		
		BPV29 seq. 988 (R)	AATACCTTTTGCATTAGGAT	988-969		
		BPV29 seq. 1059 (F)	GCCTGTCTGATTTGTCTGACC	1059-1079		
		BPV29 seq. 1411 (R)	TACAAGCCCACCTCTATTGT	1411-1392		
		BPV29 seq. 1707 (F)	GCAGGGACACAGTTTGGAATC	1707-1727		
		BPV29 seq. 2005 (R)	GCCTGCCAGCCTCGCATAAT	2005-1986		
		BPV29 seq. 2408 (F)	GCAGTTTTAGATGATGCAAC	2408-2427		
		BPV29 seq. 2609 (R)	AGCACATAATTCTACTGTGC	2609-2590		
		BPV29 seq. 3082 (F)	GATCGCATGTGACTGTTATT	3082-3101		
		BPV29 seq. 3142 (R)	CATAGAGTGTATTCCATAGC	3142-3123		
		BPV29 seq. 3476 (F)	CACGCGGTCGCAGTCGTCTT	3476-3495		
		BPV29 seq. 3571 (R)	GATAGCGGCGCTCTCCGTCT	3571-3552		
	B191016	BPV29 seq. 4004 (F)	GCATTAAAATGGTTCGTGCAGC	4004-4025		This study
		BPV29 seq. 4195 (R)	CTTTGCCACTGCTGATTCCAAG	4195-4174		
		BPV29 seq. 4467 (F)	CACAATTGGTGTTGATGAAG	4467-4486		
		BPV29 seq. 4839 (R)	GTCAGTGATTTTAACCTGCTG	4839-4819		
		BPV29 seq. 5073 (F)	CTTGCGAATAGGTGGACATG	5073-5092		
		BPV29 seq. 5397 (R)	CAGGAATAATTTGACAGATCC	5397-5377		
		BPV29 seq. 5474 (F)	CTTACTCACCAGTGAACCCT	5474-5493		
		BPV29 seq. 5970 (F)	CCTGCCTTCAATAAATTTAG	5970-5989		
		BPV29 seq. 6039 (R)	CCCTGTCATCATCTGTTTGTGG	6039-6018		
		BPV29 seq. 6487 (F)	GGTCTCCAAGTGGCTCATTA	6487-6506		
		BPV29 seq. 7190 (R)	CATAAAGTAGAACAATAACC	7190-7171		
RT-PCR		BPV29 E1 1996 (F)	GCTGGCAGGCGAAGATTCTA	1996-2015	127	
		BPV29 E1 2122 (R)	TCCCATGGACATTTCACGCA	2122-2103		
		BPV29 E2 3662 (F)	TCCTGGAGAAGTGGGAACGA	3662-3681	157	
		BPV29 E2 3799 (R)	GTGAGAATGCGAAGTGCGTC	3818-3799		
		BPV29 E10 321 (F)	GTGGAGCTTCGGTTCCATCA	321-340	150	
		BPV29 E10 470 (R)	CAATCCCATCCATGCAGCTC	470-451		
		BPV29 L1 6186 (F)	CAGGACGGGGAAATGTGTGA	6186-6205	181	
		BPV29 L1 6366 (R)	ACATCTGTTCCCGTTTGGCA	6366-6347		

The nucleotide position is based on the sequence of the reference genotype of BPV29 (GenBank accession number: LC514113).
Abbreviations: bp, base pairs; BPV, bovine papillomavirus; F, forward; nt, nucleotide; R, reverse; seq, sequence; Tm; annealing temperature

Table 1-6 Nucleotide sequence homology scores based on the L1 and full-genome of the reference BPVs determined by CLUSTAL W.

	BPV1	BPV1 (B160805)	BPV2	BPV2 (B160303)	BPV2 (B160620)	BPV3	BPV4	BPV5	BPV6	BPV7	BPV8	BPV9	BPV10	BPV11	BPV12	BPV13	BPV14	BPV15	BPV16	BPV17	BPV18	BPV19	BPV20	BPV21	BPV22	BPV23	BPV24	BPV25	BPV26	BPV27	BPV28	BPV28 (B190830-1)	BPV28 (B190830-2)	BPV29
BPV1 (X02346)		-	87	-	-	58	57	60	57	57	60	56	56	56	58	87	68	56	56	57	56	57	58	57	55	58	57	60	58	56	57	57	-	57
BPV1 (B160805, LC426023)	100		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BPV2 (M20219)	84	84		-	-	57	56	59	57	57	60	57	57	57	58	91	68	56	56	56	56	57	58	58	55	57	57	59	57	56	57	57	-	56
BPV2 (B160303, LC426021)	84	84	99		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BPV2 (B160620, LC426022)	84	84	99	99		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BPV3 (AF486184)	56	56	57	56	56		73	57	72	58	57	71	73	71	66	58	57	70	59	63	58	58	65	58	57	78	72	58	72	57	73	73	-	74
BPV4 (X05817)	55	55	55	55	55	74		57	73	57	57	74	70	78	65	57	57	73	58	61	57	58	64	57	58	72	76	57	74	57	71	71	-	75
BPV5 (AF457465)	62	62	64	64	64	54	54		56	57	72	56	57	57	58	60	60	56	56	58	58	58	57	57	54	57	57	73	58	57	57	57	-	56
BPV6 (AJ620208)	55	55	58	58	58	73	72	55		56	56	71	70	72	65	56	56	71	58	62	57	57	64	58	57	73	70	57	69	56	70	70	-	78
BPV7 (DQ217793)	55	55	55	55	55	56	54	53	55		57	55	56	56	57	57	56	56	56	56	56	58	57	58	56	58	56	56	57	61	57	57	-	57
BPV8 (DQ098913)	61	61	60	60	60	56	55	72	56	54		57	57	57	58	60	60	57	56	58	58	57	58	57	55	58	58	80	57	57	58	58	-	57
BPV9 (AB331650)	55	55	56	56	56	75	74	56	72	53	54		70	74	65	57	57	73	58	61	56	58	64	57	58	72	71	57	71	55	70	70	-	73
BPV10 (AB331651)	56	56	55	56	56	72	71	58	71	55	55	72		70	66	57	57	69	58	61	58	59	66	59	56	73	70	57	72	56	73	73	-	71
BPV11 (AB543507)	56	56	56	56	56	74	78	53	73	55	55	75	71		65	56	56	74	59	62	57	58	64	57	58	72	74	57	76	56	70	70	-	74
BPV12 (JF834523)	56	56	57	57	57	69	68	57	68	55	55	67	67	67		58	58	63	58	63	59	59	64	58	58	66	65	58	65	57	66	66	-	66
BPV13 (JQ798171)	85	56	88	88	88	56	55	60	57	56	60	56	56	55	57		69	56	56	56	57	57	57	58	55	57	57	60	57	56	58	57	-	57
BPV14 (KP276343)	73	85	72	72	72	56	56	64	57	52	62	55	56	55	58	72		56	57	57	57	57	57	58	54	57	59	60	57	58	57	57	-	56
BPV15 (KM983393)	55	73	55	55	55	74	73	56	72	53	55	75	72	75	68	55	57		58	61	57	57	62	57	58	70	70	56	70	57	69	69	-	72
BPV16 (KU519391)	53	55	53	53	53	57	59	54	60	52	54	61	54	56	56	53	54	56		59	61	58	58	58	62	60	59	57	59	56	58	58	-	58
BPV17 (KU519392)	53	53	56	56	56	63	61	55	63	54	54	61	59	62	64	53	56	63	55		59	59	64	59	55	63	62	58	63	56	63	63	-	61
BPV18 (KU519393)	53	53	54	54	54	58	57	53	58	37	54	55	56	57	53	53	51	58	53	54		58	58	57	59	58	58	57	56	57	59	59	-	57
BPV19 (KU519394)	58	53	57	57	57	58	60	56	58	56	53	58	59	59	61	57	57	58	57	57	52		59	69	57	58	58	57	58	58	57	57	-	59
BPV20 (KU519395)	53	58	53	53	53	67	67	55	68	55	53	68	68	67	68	53	57	68	52	61	58	58		58	58	65	65	57	67	57	66	66	-	63
BPV21 (KU519396)	60	53	59	59	59	57	58	55	60	55	56	56	58	58	58	59	57	58	55	57	56	69	57		56	58	58	57	58	58	58	58	-	59
BPV22 (KY705374)	55	60	57	57	57	56	56	58	57	54	54	56	59	55	56	56	56	55	64	54	61	54	56	55		57	57	55	56	56	57	57	-	58
BPV23 (KX098515)	55	55	56	56	56	79	73	53	76	55	54	74	73	75	69	56	56	74	61	62	57	59	66	58	58		72	57	73	57	73	73	-	75
BPV24 (MG602223)	55	55	55	55	55	72	77	54	73	55	56	73	71	76	67	55	55	74	61	62	58	59	67	58	56	73		58	77	57	72	72	-	70
BPV25 (MG252779)	59	55	60	60	60	56	77	72	57	54	82	56	56	56	56	60	61	56	55	54	54	54	59	55	57	55	58		59	57	58	57	-	57
BPV26 (MG281846)	54	59	54	54	54	73	77	56	72	54	55	74	72	81	70	54	57	73	55	62	56	59	67	57	55	74	74	56		57	72	72	-	71
BPV27 (MH512005)	54	54	53	53	53	55	77	53	52	62	52	54	53	55	55	54	54	54	52	54	51	57	53	57	53	55	55	52	54		57	57	-	57
BPV28 (LC500686)	55	55	56	56	56	73	77	51	72	57	55	74	72	75	69	56	56	75	57	61	58	57	68	58	57	74	73	56	74	54		99	-	71
BPV28 (B190830-1, LC519594)	55	55	57	57	57	73	77	51	72	56	55	74	72	75	69	56	56	75	57	61	58	56	68	58	57	74	73	56	74	54	99		-	71
BPV28 (B190830-2, LC521687)	55	55	56	56	56	73	77	51	73	56	55	75	72	75	69	56	56	75	57	61	58	57	69	58	57	74	74	56	75	54	99	100		-
BPV29 (B191016, LC514113)	56	56	57	57	57	74	77	54	77	56	54	73	72	75	70	57	57	72	57	63	58	59	67	59	56	76	72	56	74	56	74	74	74	

The homological scores against the reference BPV genotypes determined by CLUSTAL W analysis are noted for BPV1, BPV2, BPV28, and BPV29-identified cases. The L1 and whole-genome based sequence homological scores are noted in blue and black, respectively. GenBank accession number and/or sample ID of each BPV sequence is noted in parentheses.
Abbreviation: BPV, bovine papillomavirus

Fig. 1-1

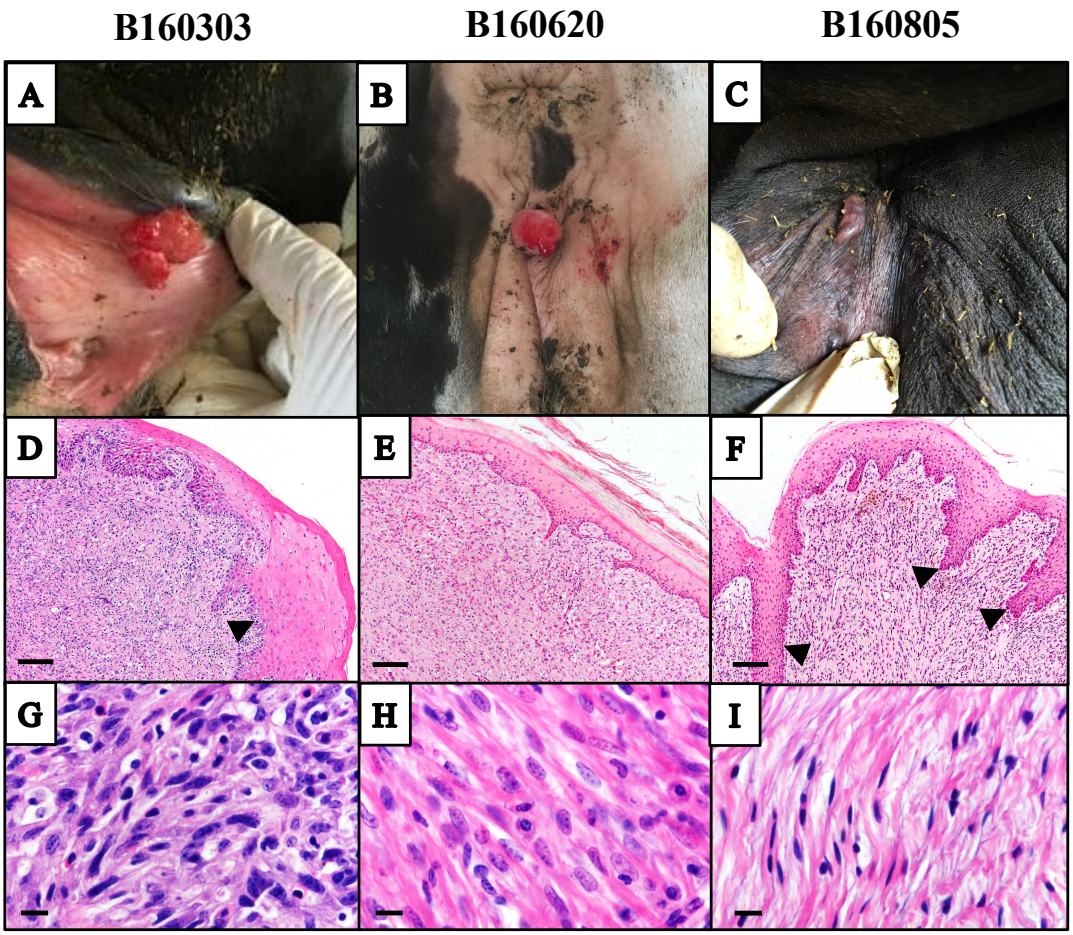


Fig.1-1

Macroscopic and histopathological features of two vulval and one anal fibropapilloma cases.

(A-C) Macroscopic findings of two vulval (A, B) and an anal (C) fibropapillomas.

(D-I) Histopathological features of exophytic nodular mass on the vulva/anus.

The lesion in all cases consists of a bland population of spindle-shaped mesenchymal cells proliferating in streams and is covered by acanthotic epidermis, which occasionally forms broad rete pegs (arrowheads).

(A, D, G) Case number B160303, vulval.

(B, E, H) Case number B160620, vulval.

(C, F, I) Case number B160805, anal.

Hematoxylin and eosin staining, bars, 100 μm (D–F) and 10 μm (G–I).

Fig. 1-2

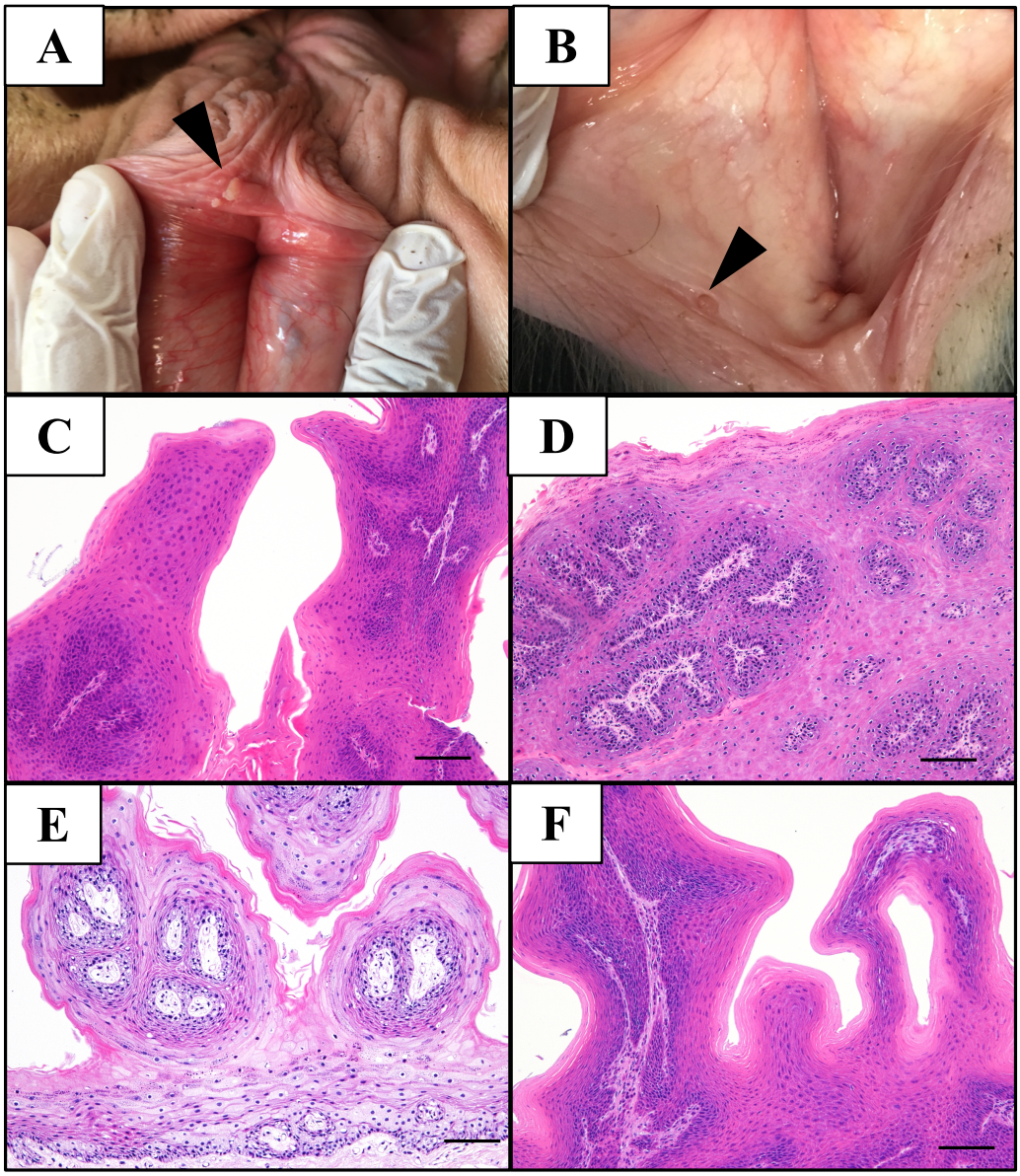


Fig. 1-2

Macroscopic and histological observations of vulval papilloma lesions in cow numbers 4 and 5.

(A)Vulval papilloma lesions (arrowhead) observed in case B190909.

(B)Vulval papilloma lesions (arrowhead) observed in case B191016.

Histologically, papillary projections of stratified squamous epithelium were observed in four cases.

(C) B190830-2. (D) B190909. (E) B191204. (F) B191016

Hematoxylin and eosin staining, bars, 100 μm (C–F).

Fig. 1-3

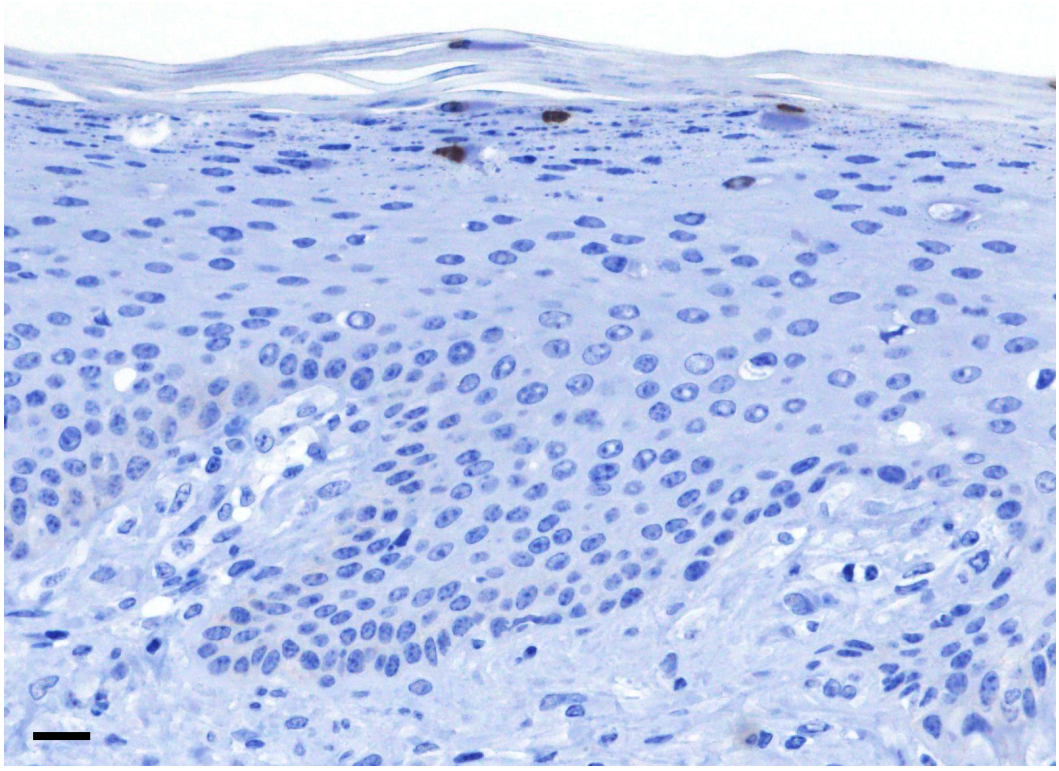


Fig. 1-3

Immunohistochemical detection of papillomaviral antigen in case B160620.

Immunoreactivity for papillomaviral antigen is observed in the nucleus of few keratinocytes within the stratum granulosum of the hyperplastic epidermis. Bar, 20 μm.

Fig. 1-4

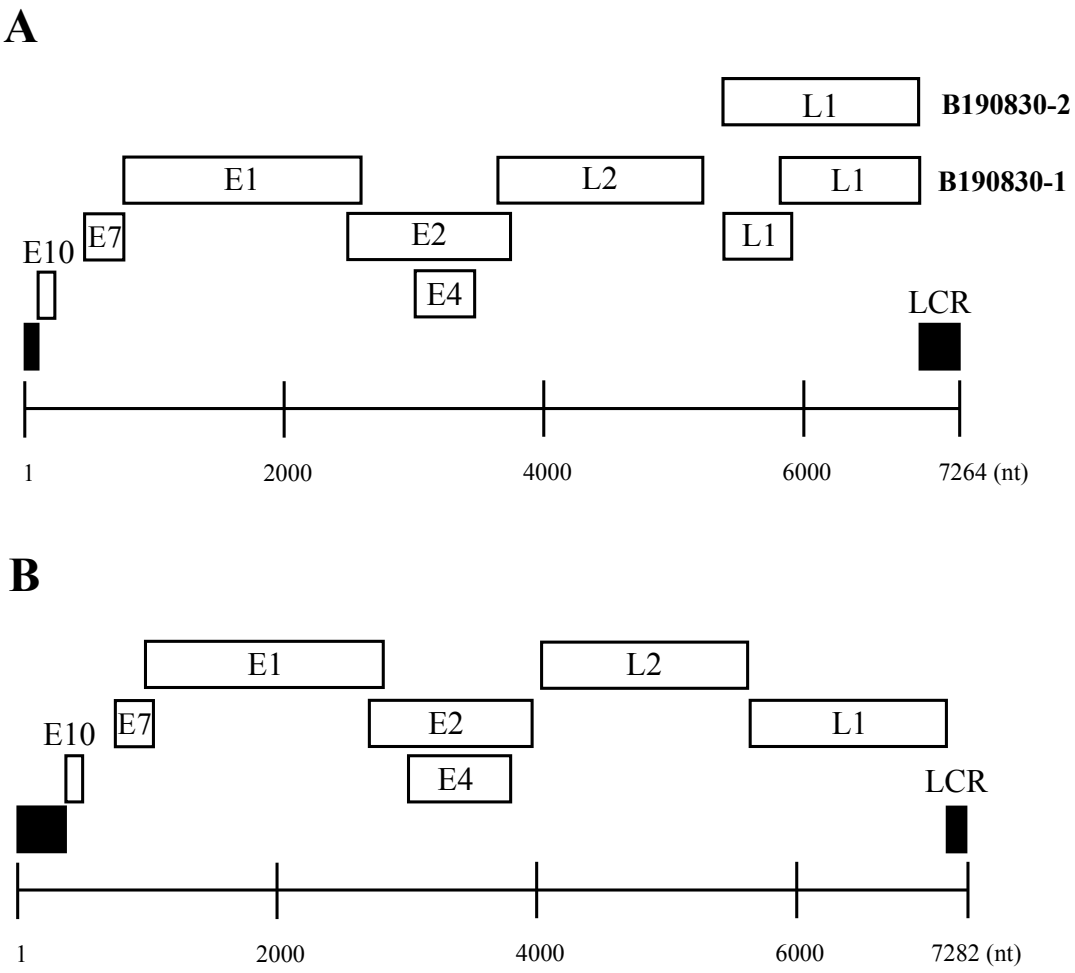


Fig. 1-4

Genomic structures of BPV28 reference, BPV28 with L1 frameshift mutation, and BPV29.

The circular genomes of BPV28 and BPV29 are shown in a linearized form.

(A) BPV28 with the L1 frameshift mutation identified in sample B190830-1 and the complete BPV28 structure of B190830-1 (cow number 4).

(B) The reference structure of BPV29 identified in sample B191016 (cow number 5).

Fig. 1-5

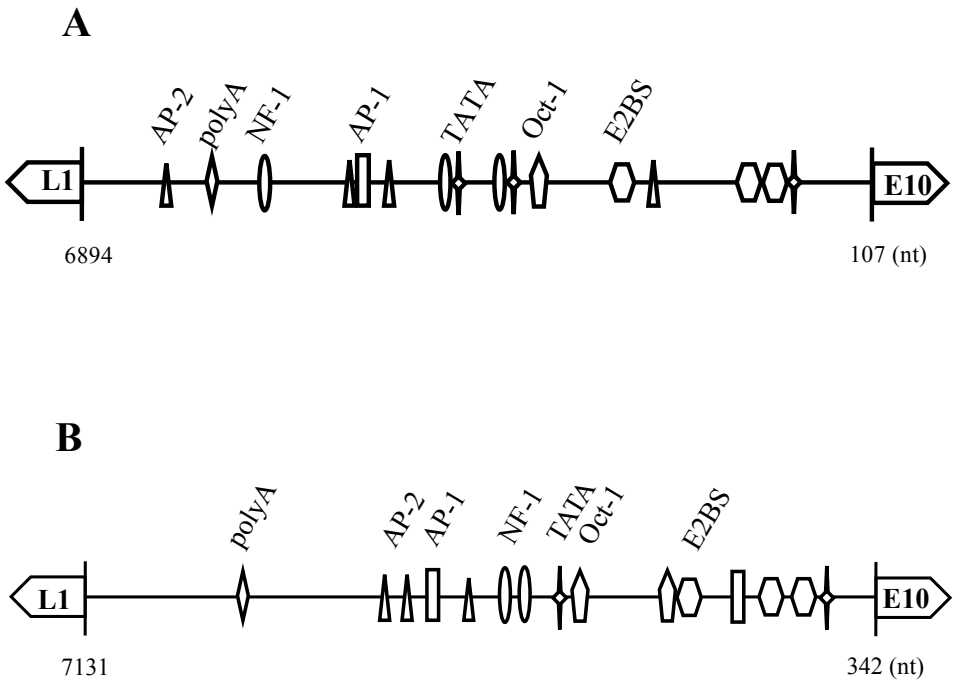


Fig. 1-5

Putative motifs in the LCR of BPV28 and BPV29.

Respective motifs are depicted in different symbols along with their names.

(A) BPV28 LCR of case B190830-1.

(B) BPV29 LCR of case B191016.

Abbreviations: AP-1, activator protein 1 binding site; E2BS, E2 binding site; LCR, long control region; NF-1, nuclear binding factor 1 binding site; nt, nucleotide; Oct-1, octamer protein 1 binding site; poly A, polyadenylation site; TATA, TATA box.

Fig. 1-6

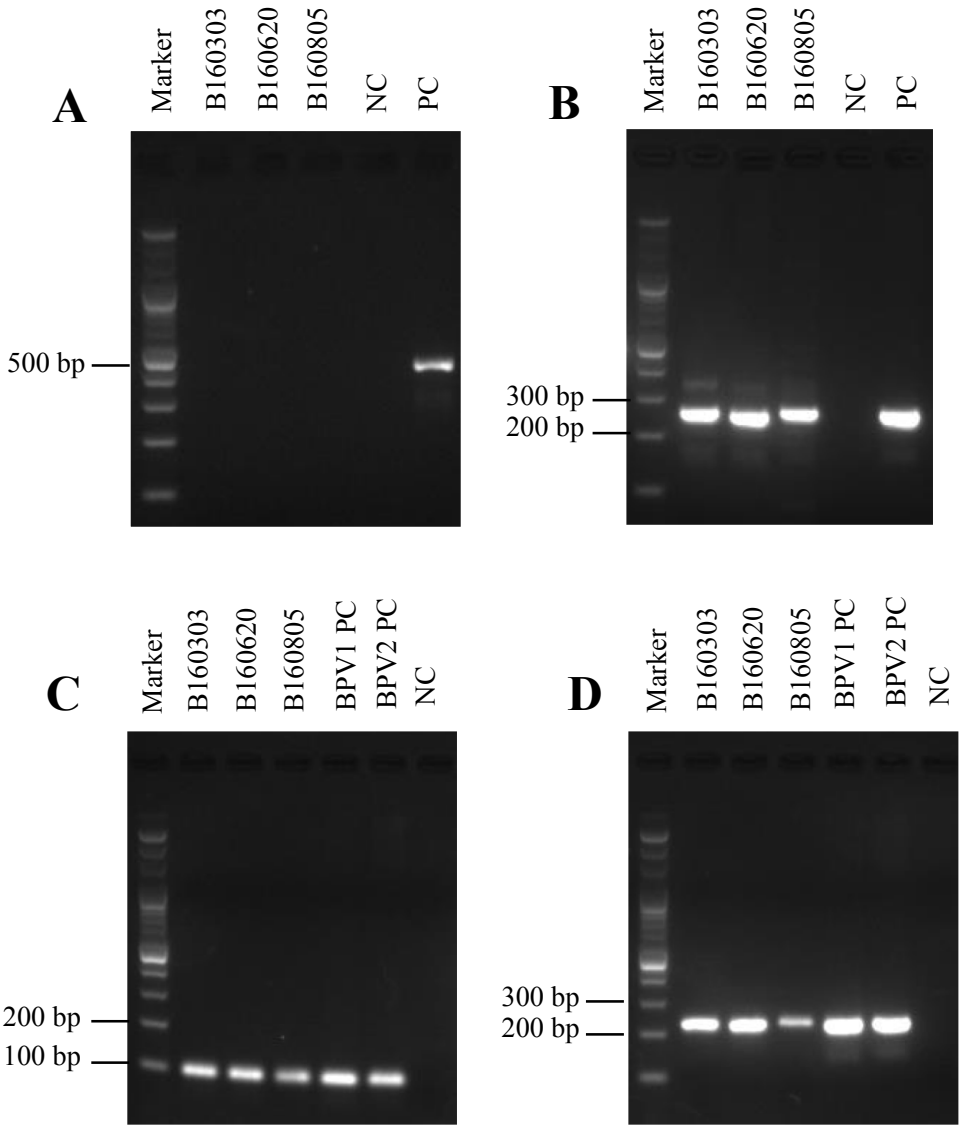


Fig. 1-6

Reverse-transcriptase PCR (RT-PCR) results of BPV1 and BPV2-identified cases (B160303, B160620, B160805).

Agarose gel electrophoresis of RT-PCR products are shown. mRNAs of three early regions (E2, E5, E6) were detected in all three cases, while L1 was not.

(A) mRNA L1 (B) mRNA E2 (C) mRNA E5 (D) mRNA E6

Abbreviations: NC, negative control; PC, positive control.

Fig. 1-7

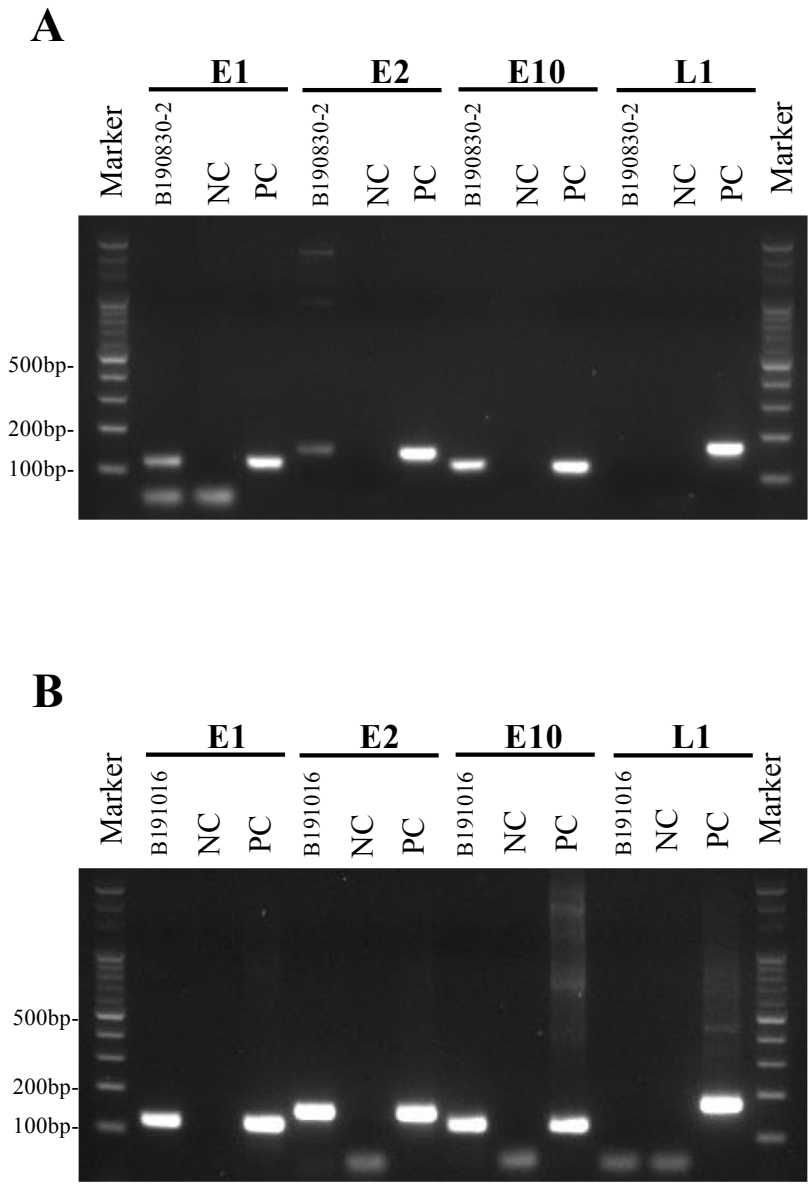


Fig. 1-7

Reverse-transcriptase PCR (RT-PCR) results of BPV28 and BPV29-identified cases (B190830-2 and B191016).

mRNAs of three early regions (E1, E2, E10) were detected in both two samples, while L1 was not.

(A) BPV28-positive case, B190830-2.

(B) BPV29-positive case, B191016.

Abbreviations: NC, negative control; PC, positive control.

Fig. 1-8

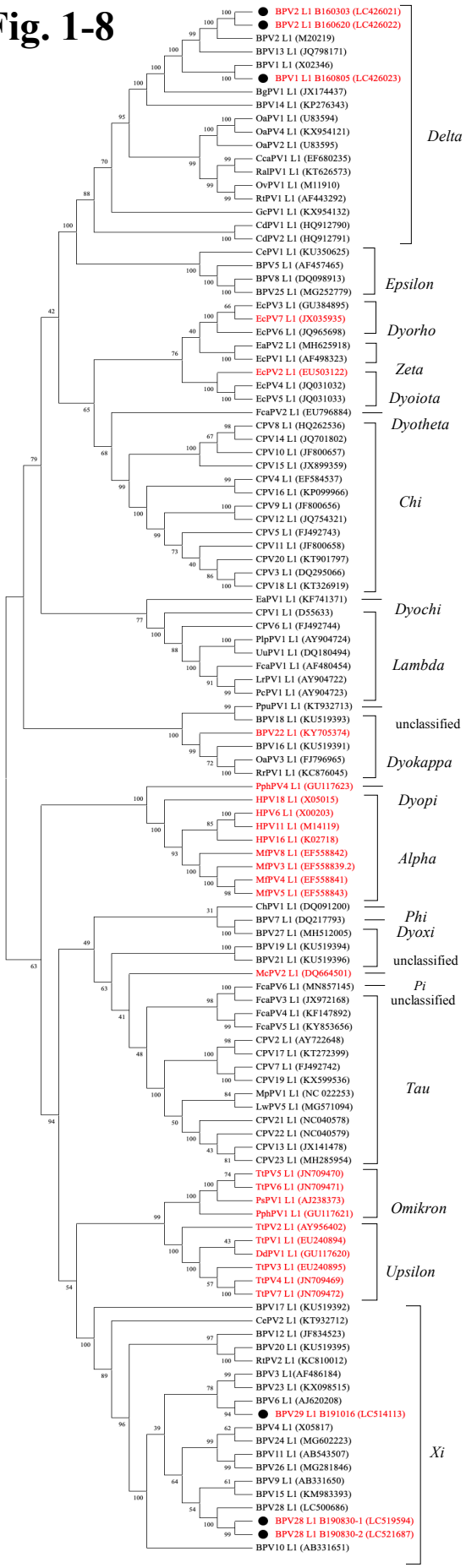


Fig. 1-8

Phylogenetic tree based on the L1 nucleotide sequence of animal and human papillomaviruses (PVs), including the isolates identified in the present study.

The full-L1 nucleotide sequence in total of 116 PV genotypes was included and the tree was constructed by the neighbor-joining method. in this analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Respective GenBank accession numbers are shown in parentheses. The BPV L1 sequences identified in the present study are noted by closed circles along with each sample ID. PVs identified in the anogenital tissue or lesions are colored in red. The genus of the corresponding PV is indicated in italics.

Abbreviations: BgPV, *Bos grunniens* PV; BPV, *Bos taurus* PV; CPV, *Canis familiaris* PV; CcaPV, *Capreolus capreolus* PV; CdPV, *Camelus dromedarius* PV, CePV, *Cervus elaphus* PV; ChPV, *Capra hircus* PV; DdPV, *Delphinus delphis* PV; EaPV, *Equus asinus* PV; EcPV, *Equus caballus* PV; FcaPV *Felis catus* PV; GcPV, *Giraffa camelopardalis* PV; HPV, Human PV; LrPV, *Lynx rufus* PV; LwPV, *Leptonychotes weddellii* PV; McPV, *Matomys coucha* PV; MfPV, *Macaca fascicularis* PV; MpPV, *Mustela putorius* PV; OaPV, *Ovis aries* PV; OvPV, *Odocoileus virginianus* PV; PcPV, *Puma concolor* PV; PlpPV, *Panthera elo persica* PV; PpuPV, *Pudu puda* PV; PsPV, *Phocoena spinipinnis* PV; RalPV, *Rupicapra rupicapra* PV; RtPV, *Rangifer tarandus* PV; TtPV, *Tursiops truncatus* PV; UuPV, *Uncia uncia* PV.

Chapter 2

Phylogeny-trait association analyses of Bovine papillomavirus types 1 and 2 identified in cattle and horses kept in Japan

Introduction

In chapter 1, BPV types, 1 and 2, (BPV1/2) were identified in mucosal (anogenital) fibropapillomas of domestic cows. BPV1/2, both classified in the genus, *Deltapapillomavirus* were shown to harbor tissue tropisms in the mucosal sites in addition to the cutaneous sites (Shimakura et al., 2018). Taken the general characteristics of PVs harboring strict tissue/species tropisms (DeVilliers et al., 2004), BPV1/2 are unique viruses as they also show cross-species infections to horses (Chambers et al., 2003a; Ragland et al., 1996). In the natural bovine host, BPV1/2 infections are associated with (fibro)papillomas showing subsequent spontaneous regression (Maxie, 2015). However, BPV1/2 infection to horses will cause benign but aggressive fibroblastic tumors, sarcoids (Ragland et al., 1996). Equine sarcoid is the most common skin tumor in horses (Knowles et al., 2016) developing on any cutaneous site of the body (Nasir and Campo, 2008) and may obstruct vital function (Nasir and Brandt, 2013). Surgical excision is one of the treatment approaches, however, hyperproliferation and/or reoccurrence is observed frequently (Chambers et al., 2003a). In addition to the genomic detection of BPV1/2, the association between BPV infection and sarcoid development was demonstrated by experimental BPV injection to horses (Hartl et al., 2011). As well as BPV1 and BPV2, BPV13 also classified in the species, *Deltapapillomavirus* 4 was detected from equine sarcoid lesions (Nasir and Brandt, 2013). To the author's knowledge, the cross-species infection of BPV1/2 to horses were first noted in (Ragland et al., 1966), however, the viral mechanism of the cross-species infections of BPV1/2 to horses are still not unraveled.

To elucidate this unique phenomenon of BPV1/2 infection to horses from the viewpoint of genomic analyses, previous studies compared BPV1/2 sequences between the species origin (cattle and horses). Earlier studies have shown sequence variants of BPV1, which were frequently detected from equine sarcoids and not or less-frequently from the bovine papilloma-derived BPV1 E2, LCR (Nasir et al., 2007; Trewby et al., 2014a), and E5 (Chambers et al., 2003b; Savini et al., 2015) nucleotides. In terms of E2 and LCR, sarcoid-associated sequence variants showed biological and functional significance *in vitro* using primary cells isolated from cattle and horses (Nasir et al., 2007). On the other hand, a recent study found that no equine-associated sequence variants of BPV1 were observed when compared with the bovine sequence (Koch et al., 2018), suggesting that BPV1 has been shared between two hosts. Although various studies have been seeking the cross-species infection based on the genomic and *in vitro* analyses, the underlying phenomenon of BPV1/2 cross-species infection is still undetermined.

As noted, genomic studies of BPV in equine sarcoids have been conducted worldwide. However, to the author's knowledge, it has been restricted to Europe (Chambers et al., 2003b; Nasir

et al., 2007; Savini et al., 2015; Szczerba-Turek et al., 2011; Trewby et al., 2014a; A.D. Wilson et al., 2013), Africa, Australia, and South America (Trewby et al., 2014a), and no reports have been received from Asian countries including Japan. To confirm the presence of sarcoid-associated BPV sequence variants, verification should be made, irrespective of national boundaries. Therefore, this study aimed to identify sequence characteristics of BPV1 and BPV2 in equine sarcoids and bovine papillomas collected in Japan. Moreover, this study aimed to clarify the phylogeny-trait association between BPV sequence and geographic origin or the host species.

Materials and Methods

Sample collection

Ten equine sarcoid and bovine papilloma samples each, provided by clinical veterinarians in Japan were analyzed in this study (Table 2-1). Two bovine papilloma cases (B160303 and B160805) from chapter 1 were included in the present analyses. The mean age of sarcoid-bearing horses and papilloma-bearing cattle was 15.3 ± 6.07 (mean ± standard deviation) and 1.8 ± 0.6 years, respectively at sample collection. Three equine sarcoid samples (sample numbers E171218-2, -3, -5) were FFPE tissue samples. The other seven sarcoid and ten cattle papilloma samples were fresh tissue samples (Table 2-1).

Histopathological analyses

Fresh equine/bovine-derived tissue samples were cut in half, one was stored at –30ºC for DNA extraction and the other was fixed in 10% buffered formalin for histological analysis. Formalin-fixed samples were embedded in paraffin and stained with HE for histological diagnosis. Histological examinations were unavailable for three cattle-derived cases, P9, P11, and P21 due to the limitation of sample volume. Histological examinations of samples E171218-2, -3, -5 were conducted in the Department of Applied Science, Nippon Veterinary and Life Science University and the other cases were performed in the Laboratory of Veterinary Pathology, the University of Tokyo.

DNA extraction and validation

DNA from frozen and FFPE samples were extracted with QIAamp DNA mini kit and QIAamp FFPE Tissue Kit (Qiagen), respectively following the manufacturer's instructions. The success in DNA extraction was validated by conventional PCR using KOD FX Neo DNA polymerase for beta actin. Primers named bovine β-actin (F/R), equine β-actin (F/R) and 192 equine β-actin (F/R)

for bovine papilloma, equine sarcoid, and FFPE-sarcoid DNA samples were applied, respectively (Table 2-2). For FFPE sarcoid tissue samples, a primer pair for detecting shorter length (192 bp) of equine β-actin was used considering the DNA fragmentation (Arreaza et al., 2016). PCR reactions were performed in a total of 20 µl reaction system described in Chapter 1. Template DNA was replaced with distilled water for negative controls. PCR conditions were set based on three-step cycle conditions specified in the manufacturer's protocol and by modifying the annealing temperature of the respective primers described in Table 2. Respective PCR products were electrophoresed on 2% agarose gels and the positive bands were visualized by UV-exposure. For further PCR experiments, primers designed to detect the shorter length (< 500 bp) of DNA in FFPE sarcoid tissue samples were used for the reason described above.

BPV detection

Detection of BPV genomic DNA was verified by conventional PCR and sequencing. Conventional PCR was performed in the conditions as described above. The primer pairs, BPV1&2 L1 subA modified (F/R) and 266 BPV-1,2 L1 (F/R) were used to detect BPV1/2 in seven frozen and three FFPE-sarcoid tissue samples, respectively (Table 2-2). BPV detection from ten bovine papilloma samples were conducted using two pairs of primers, subAup/subAdw and subBup/subBdw (Table 2-2) (Kawauchi et al., 2015). PCR-positive samples amplified with BPV1&2 L1 subA modified (F/R), 266 BPV-1,2 L1 (F/R), subAup/subAdw and subBup/subBdw primers were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Following the purification step, bidirectional sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit and 3130×l genetic analyzer sequencing machine (Applied Biosystem). BigDye reactions were performed using the same primers applied for PCR and by following the same protocol described in Chapter 1. Obtained sequences were analyzed using MEGA7 (Kumar et al., 2016) and MEGA X (Kumar et al., 2018a). Identical BPV genotype was determined with the BLAST from the NCBI.

Sequence characterization of bovine- and equine-derived BPV1/2

To further characterize the sequences of equine and bovine-derived BPVs, PCR and direct sequencing were conducted for four regions (E2, E5, L1, and LCR). Three regions, the E2, E5, and LCR have been previously studied to determine the sequence characteristics that account for cross-species infection (Chambers et al., 2003b; Nasir et al., 2007; Savini et al., 2015). Although previous studies describing the L1 sequence-trait association is limited, L1 sequence mutations have been shown to alter the vaccine efficacy (Godi et al., 2019). The L1 sequence was also included in this

analysis for future development of viral-like particle (VLP) vaccines for cattle and horses. For seven frozen equine sarcoid samples, two primer pairs designed to amplify the whole-genome of BPV1/2, BPV1&2 LCR inverse (F/R) and BPV1&2 856 LCR (F/R) were applied (Table 2-2). For FFPE sarcoid samples, primers designed to amplify small-sized (< 500 bp) genomes of BPV1 E2, E5, and LCR were applied (Table 2-3). Two bovine papilloma samples, B160705-4 and B160705-6 were both BPV1/BPV2-positive (Table 2-1). Accordingly, to amplify the respective genotypes including the early regions and LCR, type-specific primers named 6443/4938 BPV1 inverse (F/R) and 6204/4529 BPV2 inverse (F/R) were designed and used (Table 2-2). PCR mixture and conditions were the same as mentioned, except for the modification of extension period (30 seconds per 1 kbp) and the annealing temperature noted in Tables 2-2 and 2-3. Each PCR product was electrophoresed and the target bands were purified. Direct sequencing of FFPE sarcoid sample- and frozen equine/bovine tissue-derived products were performed with the respective primers used to amplify BPV1/2 DNA and extra sequencing primers (Tables 2-2 to 2-4) (Shimakura et al., 2018). Sequencing results were aligned using the ClustalW tool (Thompson et al., 1994) available in MEGA X software and sarcoid-associated sequence variants were determined. The sequence data identified in this study were subjected to the DDBJ/EMBL/GenBank database. Each accession number is listed in Table 2-1.

Phylogenetic tree construction and phylogeny-trait association analysis

Based on the sequence of BPV1/2 E2, E5, L1, and LCR, Bayesian phylogeny was estimated using Markov chain Monte Carlo (MCMC) method with MrBayes software (version 3.2.7a) (Ronquist et al., 2012). As the previous studies on BPV1 LCR sequence were available from the nucleotide (nt) positions 7266 to 7964 of the reference BPV1 (GenBank accession number: X02346.1) (Koch et al., 2018; Nasir et al., 2007; Trewby et al., 2014a), a Bayesian phylogenetic tree of BPV1 LCR was built based on this region . The MCMC analysis was run with general-time-reversible (GTR) model for 5,000,000 generations, with 200,000 generations of burn-in followed by sampling every 5,000 generations. Following the previous study, the substitution rate of each BPV1/2 gene was set as 1.95E-08 (Rector et al., 2007). Each tree was depicted with FigTree version 1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

The phylogeny-trait association (i.e. species and geographic origin) was determined using Bayesian Tip-association Significance (BaTS) software (Parker et al., 2008). The null distribution for three statistics, association index (AI), parsimony score (PS), and the monophyletic clade (MC) size were estimated with 1,000 replicates in the BaTS program by depositing the posterior tree output of the Bayesian analysis results. A p-value of <0.05 was considered statistically significant. In this

analysis, both reference sequences of BPV types 1 and 2 (GenBank accession numbers: X02346.1 and M20219.1) were excluded for the geographic trait analysis because the information on their geographic origins were unavailable. Sequence identity scores between each isolate was calculated using Clustal Omega tools (Sievers and Higgins, 2018). Among the reference sequences of equine sarcoid-derived BPV1, sequences that originated from the subspecies, *Equus (ferus) caballus* were applied in the analysis since donkey-derived sarcoid BPVs were not included in the present study.

Results

Histopathological observations of equine sarcoid and bovine papilloma

Histopathologically, all ten equine-derived samples were diagnosed as sarcoid, and cattle-derived samples were either fibropapilloma or papilloma (Table 2-1). HE sections and findings of two equine sarcoid cases, E170428-2 and E181025 and one bovine papilloma case, B180620-3 are shown in Figure 2-1. Equine sarcoids were composed of proliferation of fibrous tissue (Fig. 2-1A, B) and bovine papilloma showed papillary proliferation of squamous epithelium (Fig. 2-1C).

BPV genotyping of equine- and bovine-derived samples

Beta-actin of the all tested DNA samples were detected by PCR, confirming the success in DNA extraction. Table 2-1 summarizes the BPV genotype(s) identified in each sample by PCR and direct sequencing. Among the ten sarcoid samples, BPV1 and BPV2 were detected in seven and three sarcoid samples, respectively. In bovine-derived samples, two cases, B160705-4 and B160705-6 presented concurrent infection of several BPV genotypes (Table 2-1). Seven BPV1- and five BPV2-positives were confirmed in bovine papilloma-derived samples. These results confirmed that BPV1 and BPV2 were present in sarcoids of horses kept in Japan.

Neither equine-associated sequence mutations/variants in equine-derived BPV1/2

Direct sequencing results of the respective BPV1/2 viral nucleotides (E2, E5, L1, and LCR) aligned by ClustalW showed no sarcoid-associated nucleotide mutation /variant in any of the four regions when compared between equine- and bovine- derived sequences (Figs 2-4 and 2-5). Moreover, in BPV1, none of the sarcoid-associated variants noted in the previous reports (Chambers et al., 2003b; Nasir et al., 2007; Savini et al., 2015; Trewby et al., 2014a) were identified in the analyzed sequences (Fig. 2-4).

Bayesian phylogeny, phylogeny-trait association, and sequence identities of BPV1/2 isolates

Bayesian phylogenetic trees constructed upon the nucleotide sequences of BPV1 and BPV2, are shown in Figures 2-1 and 2-2, respectively. A Bayesian tree based on the BPV1 LCR (7266-7904 nt) described in the previous studies (Koch et al., 2018; Nasir et al., 2007; Trewby et al., 2014a; A.D. Wilson et al., 2013) and the database showed that overall, each BPV1 isolate harbored a close relationship with isolates of the neighboring/same geographic areas (Fig. 2-2D). Similarly, BPV1 E2 and L1 nucleotides of the analyzed isolates clustered within the respective geographic origins (Figs 2-2A, C), while no significant cluster was observed in the E5 (Fig. 2-2B). BPV2 neither showed significant clusters of geographic origins nor species in any of the four regions in the Bayesian phylogenetic tree (Fig. 2-3).

The statistic values of BPV1/2 phylogeny-trait association index calculated using BaTS are summarized in Table 2-5 (BPV1) and Table 2-6 (BPV2). The phylogeny-trait association is suggested to be stronger in low values for AI and PS and in high values for MC (Parker et al., 2008). In BPV1, AI and PS scores demonstrated significant correlation between phylogeny and geographic origin in E2, E5, and L1 (Table 2-5). In BPV1 LCR, both geographic origin and species were shown to be associated with the phylogeny (Table 2-5). The MC values of BPV1 showed that the clustering levels were significant in Japanese and Europe-derived isolates in all of the four regions (E2, E5, L1, LCR). On the contrary, only a few significant statistical scores were noted in BPV2, showing no clear phylogeny-trait association in either of the classifiers (Table 2-6).

The BPV1/2 sequence identity scores determined by Clustal Omega presented high values between the isolates. The scores in BPV1 were, E2: 99.6 ± 0.33% (mean ± standard deviation); E5: 98.7 ± 1.72%; L1: 99.4 ± 0.41%; LCR: 98.4 ± 0.95%. In BPV2, the scores were, E2: 99.5 ± 0.45%; E5: 99.7 ± 0.39%; L1: 99.4 ± 0.59%; LCR: 97.8 ± 2.29% (data not shown).

Discussion

This study confirmed BPV1/2 detection in equine sarcoid and bovine papilloma samples collected in Japan. The mean age of the sarcoid-affected horses at the time of sample collection (Table 2-1) was greater than that of the previous study (Knowles et al., 2016), but it has also been described that the risk of sarcoid development increases with age (Mohammed et al., 1992). Previous studies demonstrated that BPV1/2 harbor sarcoid-associated variants (Chambers et al., 2003b; Nasir et al., 2007; Savini et al., 2015) or geographically-specific features (Koch et al., 2018; Trewby et al., 2014a) between equine/bovine-derived sequence. The present study aimed to identify whether the BPV phylogeny and/or mutation share some relationship between the geographic origin and/or host species, as this attempt has not been previously made in Asian countries.

Sequence data of BPV1/2 (E2, E5, and LCR) described in the phylogenetic trees (Figs 2 and 3) harbored high sequence identities ranged from 97.8% to 99.7%. BPV1 isolates analyzed in this study did not retain any sarcoid-associated variants as reported previously (Chambers et al., 2003b; Nasir et al., 2007; Savini et al., 2015) (Fig. 4), while Bayesian phylogenetic trees of BPV1 E2, L1 and LCR showed clustering within the geographic origin. However, in BPV1 LCR, there were some exceptions in that some equine-derived isolate, for example, case E171218-5 (GenBank accession number: LC510382) branched within the European isolates (Fig. 2-2D). Conversely, one of the European isolates (GenBank accession number: KJ577566) showed a closer association with Japan-derived isolates than that of Europe (Fig. 2-2D). International transportation of horses may explain these exceptions.

The phylogeny-trait correlation analysis by BaTS showed the significant relationship in the geographic trait of BPV1 (E2, E5, L1, LCR) (Table 2-5). The region-based MC statistic of BPV1 was significant in Europe and Japan (Table 2-5), supporting the hypothesis of the geographically-specific association of BPV1 nucleotide mutations as described previously (Koch et al., 2018; Trewby et al., 2014a). The BPV1 LCR also harbored the phylogeny-trait association with species, but this result may be caused by the sampling bias that no bovine-derived BPV1 LCR sequence from South America, Australia, and Africa was available in the database (Fig. 2-2D). Further analysis is required to confirm the significant relevance between phylogeny-geographic trait association in BPV1 LCR by including more bovine-derived sequence data.

In BPV2, no significant phylogeny-trait relationship was observed (Fig. 2-3 and Table 2-6). These results could suggest that BPV2 data from both equine and bovine were less than those of BPV1. In the GenBank database, there were no complete-genome sequence of equine-derived BPV2 and only bovine-derived BPV2 from limited geographic regions (China and South America) were available. To verify the hypothesis of phylogeny-geographical trait association in BPV2, additional equine/bovine-derived sequence data deposited from worldwide are needed. To the author's knowledge, this is the first deposit of the BPV2 complete-genome sequence derived from equine sarcoids. It is important to deposit the complete or long PV sequence data and to specify the origin (e.g. country, host species, types of the lesion) to enrich the PV database.

Previous studies described the presence of equine sarcoid-associated variants in BPV1 E5 (Chambers et al., 2003b; Savini et al., 2015), known as the major oncoprotein taking a role in cellular transformation (Petti and DiMaio, 1994). The findings in this study neither supported the presence of sarcoid-associated BPV1 E5 sequence variants that has been previously noted (Chambers et al., 2003b; Savini et al., 2015) (Fig. 2-4). The BaTS analyses of BPV1/2 E5 demonstrated that no significant

association between two traits (geographic/species) and sequence were present (BPV1: Fig. 2B and Table 5; BPV2: Fig 3B and Table 6). When E5 nucleotides determined in this study were translated into 44 amino acids, neither BPV1 nor BPV2 E5 harbored mutations. A previous study also described that the mutations of E5 protein sequence were random and unlikely to be of biological significance (Koch et al., 2018). Based on these findings that E5 amino acids were 100% identical irrespective of the host species or the geographic origin, similar E5 protein may have potential to induce cellular transformation of both cattle and horses. In accordance with the results from BaTS analysis, at least in BPV1, it could be assumed that BPV1 is shared between local cattle and horses. This may imply that sarcoid development could be caused by BPV1 circulating in the nearby cattle, but equids have been considered a dead-end host (Nasir and Brandt, 2013).

Conclusion

The present study updates equine/bovine-derived BPV1/2 sequence database and their phylogeny-trait relationships. Molecular epidemiological analyses have shown that BPV1, and presumably BPV2, are shared between neighboring equids and bovids. Therefore, preventive strategies against BPV1/2 infection in horses need to be concerned especially for animals kept close with cattle. Moreover, these results suggest that a BPV1/2 vaccine could be applicable to both horses and cattle. To strengthen our geographically-specific hypothesis in both BPV1/2, additional universal sequences and similar epidemiological studies are needed as geographic sampling bias may alter the phylogeny-trait analysis. Furthermore, experimental studies to evaluate the biological significance of the sequence alterations are also needed to support our hypothesis. The present study will contribute to extend the knowledge of etiological roles, transmission, and infection of BPV1/2 in equids and bovids.

Table 2-1 Sample information, histological diagnosis, BPV genotype(s), and GenBank accession numbers of equine sarcoid and bovine papilloma lesions analyzed in this study.

Sample ID	Geographical area in Japan	Breed	Gender	Age (years)	Body site of the lesion	Histological diagnosis	BPV genotype	GenBank accession number
Equine								
E170428-1	Kanto	Haflinger	mare	17	external ear (left)	sarcoid	BPV2	LC510376
E170428-2	Hokkaido	Thoroughbred cross	gelding	8	foreskin	sarcoid (fibropapilloma)	BPV1	LC510377
E170801	Kanto	Arabian	mare	26	external ear (left)	sarcoid (fibropapilloma)	BPV1	LC510378
E171016	Kansai	Thoroughbred	gelding	24	sublingual	sarcoid (fibropapilloma)	BPV1	LC510379
E171218-2 (FFPE)	Katno	Thoroughbred	unknown	unknown	external ear (right)	sarcoid	BPV1	LC510380
E171218-3 (FFPE)	Katno	Thorouhbred	mare	11	elbow (right)	sarcoid	BPV1	LC510381
E171218-5 (FFPE)	Katno	Quarter horse	gelding	8	foreskin	sarcoid	BPV1	LC510382
E180925-2	Hokkaido	Thoroughbred	mare	17	eyelid (left)	sarcoid	BPV2	LC510383
E181025	Hokkaido	Thoroughbred	mare	12	fetlock (left, hindlimb)	sarcoid (pyogenic granuloma)	BPV2	LC510384
E190115	Kanto	Thoroughbred	gelding	15	chest (left)	sarcoid (pyogenic granuloma)	BPV1	LC510385
Bovine								
P9	Kyushu	Holstein Friesian	castrated male	1	shoulder	not determined	BPV2	LC510386
P11	Kyushu	Holstein Friesian	castrated male	2	neck	not determined	BPV2	LC510387
P21	Kyushu	Holstein Friesian	castrated male	2	face	not determined	BPV1	LC510388
B160303	Kanto	Holstein Friesian	female	2	vulva	fibropapilloma	BPV2	LC510389
B160701	Kanto	Holstein Friesian	female	2	teat	fibropapilloma	BPV1	LC510390
B160705-4	Hokkaido	Holstein Friesian	female	3	teat	fibroma	BPV1, 2, 6, 9, 10	LC510391 (BPV1), LC510392 (BPV2)
B160705-6	Hokkaido	Holstein Friesian	female	2	teat	fibropapilloma	BPV1, 2, 10	LC510393 (BPV1), LC510394 (BPV2)
B160805	Kanto	Holstein Friesian	female	2	anus	fibropapilloma	BPV1	LC510395
B180620-2	Tohoku	Japanese Black	castrated male	1	teat	papilloma	BPV1	LC510396
B180620-3	Tohoku	Japanese Shorthorn	castrated male	1	teat	papilloma	BPV1	LC510397

Abbreviation: BPV, bovine papillomavirus; FFPE, formalin-fixed paraffin-embedded

Table 2-2 Primer information applied to PCR.

Experiment	Applied samples	Primer name	Sequence (5′ to 3′)	Target and Position (nt)	Product size (bp)	Tm (°C)	Reference
DNA quality/quantity validation	Bovine papilloma samples	Bovine β-actin (F)	GTCAGTGGGCCGTCCTGCCTTG	Bovine β-actin	411	68	Chapter 1
		Bovine β-actin (R)	GGGACACCACAAGGGGCAGTCG				
	E170428-1, E170428-2, E170801, E171016, E180925-2, E181025, E190115	Equine β-actin (F)	ATGATGATATCGCCGCGCTC	Equine β-actin	459	52	This study
		Equine β-actin (R)	ATGTCCATCACGATGCCAGTG				
	E171218-2, E171218-3, E171218-5	192 Equine β-actin (F)	CGACATCCGTAAGGACCTGT	Equine β-actin	192	54	This study
		192 Equine β-actin (R)	GTGGACAATGAGGCCAGAAT				
BPV detection	Bovine papilloma samples	subAup	CCAGAYTAYYTMAAAATGGC	BPV1, BPV2, BPV5, BPV7, BPV8, BPV13 (L1, degenerate)	433-455	50	Maeda *et al.*, 2007; Kawauchi *et al.*, 2015
		subAdw	ATAAMKGCTAGCTTATATTC				
	Bovine papilloma samples	subBup	TWYAATAGGCCCTTTTGGAT	BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV12 (L1, degenerate)	590-611	55	Maeda *et al.*, 2007; Kawauchi *et al.*, 2015
		subBdw	TTMCGCCTACGCTTTGGCGC				
	E170428-1, E170428-2, E170801, E171016, E180925-2, E181025, E190115	BPV1/2 L1 subA modified (F)	CCAGACTACCTCAAAATGGCTGA	BPV1 (L1, 6289-6311) , BPV2 (L1, 6282-6304)	436(BPV1), 435(BPV2)	54	This study
		BPV1/2 L1 subA modified (R)	TAAAKGCTAGCTTATATTCTTCC	BPV1 (L1, 6702-6724), BPV2 (L1, 6695-6717)			
	E171218-2, E171218-3, E171218-5	266 BPV1,2 L1 (F)	GMYTAGATGCTAAGCAACAACAG	BPV1 (L1, 6038-6060), BPV2 (L1, 6031-6053)	266 (BPV1, BPV2)		This study
		266 BPV1,2 L1 (R)	TTTGAGGTAGTCTGGRTACARGC	BPV1 (L1, 6303-6281), BPV2 (L1, 6296-6274)			
Full-genome characterization of BPV1/2	E170428-1, E170428-2, E170801, E171016, E180925-2, E181025, E190115	BPV1&2 LCR inverse (F)	GTTAACAATAATCACACCATC	BPV1, BPV2 (LCR, 1-21)	7231 (BPV1), 7223 (BPV2)		Nasir et al., 2007 (Reverse complement of BPVLCRF/BPVLCRR primer)
		BPV1&2 LCR inverse (R)	ATGCTGGACAGGATGTGTACCG	BPV1 (LCR, 7231-7210), BPV2 (LCR, 7223-7202)			
	E170428-1, E170428-2, E170801, E171016, E180925-2, E181025, E190115	BPV1&2 856 LCR (F)	ATAGCAATGTGCTGTGTCAGTTG	BPV1 (LCR, 59-80), BPV2 (LCR, 60-81)	856 (BPV1, BPV2)	56	This study
		BPV1&2 856 LCR (R)	GGGTCTGTCAGCAGCTTTTTAT	BPV1(LCR, 7170-7192), BPV2(LCR, 7162-7184)			
Sequence characterization of BPV1 (L1, E2, E5, LCR)	P21, B160701, B160705-4, B160705-6, B160805, B180620-2, B180620-3	6443 BPV1 inv. (F)	ATGCCACCCTTAAAATACCCAGT	BPV1 (6443-6465)	6441 (BPV1)		This study
		4938 BPV1 inv. (R)	GCCACGTGATTTAGAGGCAATAC	BPV1 (4938-4916)			
Sequence characterization of BPV2 (L1, E2, E5, LCR)	P9, P11, B16303, B160705-4, B160705-6	6204 BPV2 inv. (F)	GGTGCTGCTGACTTTAAAACACT	BPV2 (6204-6226)	6285 (BPV2)		This study
		4529 BPV2 inv. (R)	CTTCATATGCTCCAGGACGAAGA	BPV2 (4551-4529)			

The nucleotide position is based on the sequence of the reference sequence of BPV1 and BPV2 (GenBank accession numbers: X02346 and M20219).
Abbreviations: bp, base pairs; F, forward; nt, nucleotide; R, reverse; Tm; annealing temperature

Table 2-3 Primers used in PCR and sequencing of formalin-fixed paraffin-embedded sarcoid tissue-derived DNA samples.

Primer pair	Sequence (5´ to 3´)	Target gene (PV genome, location of PV nucleotide)	Product size (bp)	Tm (°C)
2578 BPV1 (F)	TGATTGACGAGGAGGAGGATAGT	BPV1 (E1-E2, 2578-2600)	243	
2820 BPV1 (R)	CTGCTTGGCTCTCTCTTGACA	BPV1 (E2, 2820-2800)		
2771 BPV1 (F)	GACACTGCAGAGTACCACACTC	BPV1 (E2, 2771-2792)	213	
2983 BPV1 (R)	TTGCATTTCCATCAAACTCCACC	BPV1 (E2, 2983-2961)		
2729 BPV1 (F)	CACTGCTTTATGCTGCAAGGAAA	BPV1 (E2, 2729-2751)	258	
2986 BPV1 (R)	TGCTTGCATTTCCATCAAACTC	BPV1 (E2, 2986-2965)		
2931 BPV1 (F)	GTGCTTTAAGAAAGGCGCCAG	BPV1 (E2, 2931-2951)	167	
3097 BPV1 (R)	CGGCCATGGTGCAGTAGTAG	BPV1 (E2, 3097-3078)		
3055 BPV1 (F)	GCTGGGGCTGACGGAACT	BPV1 (E2, 3055-3072)	151	
3205 BPV1 (R)	ATGAGACACCAGCATACACTCTG	BPV1 (E2, 3205-3183)		
3102 BPV1 (F)	TGGACGCATTTACTATTCTCGCT	BPV1 (E2, 3102-3124)	278	
3379 BPV1 (R)	GAGGACCGTCCCGTACCC	BPV1 (E2, 3379-3362)		
3342 BPV1 (F)	CATCAGAGCAGGCCTCGGTT	BPV1 (E2, 3342-3361)	285	
3626 BPV1 (R)	GCACTTTACCTGGTTAGCAGTTC	BPV1 (E2, 3626-3604)		
3553 BPV1 (F)	ATGGATTCCACCTGTTAAAGGCA	BPV1 (E2, 3553-3575)	290	
3842 BPV1 (R)	TGATCAGAAGTCCAAGCTGGC	BPV1 (E2, 3842-3822)		
3796 BPV1 (F)	CTGGAATGAACATTTCCGGCTT	BPV1 (E2, 3796-3817)	242	
4037 BPV1 (R)	GCCAATAGCCAGTGATGTAAAGG	BPV1 (L2-E5, 4037-4015)		
5549 BPV1 (F)	TGCATCCCTCCTTGTTGAGG	BPV1 (L2, 5549-5568)	300	
5848 BPV1 (R)	GTGCAAATTGATTGGGATCAGGT	BPV1 (L1, 5848-5826)		
5787 BPV1 (F)	GGTCTCTGCAAATCAGTATAGGGT	BPV1 (L1, 5787-5810)	297	55
6083 BPV1 (R)	GGGGTACAGCCTAGCAACAG	BPV1 (L1, 6083-6064)		
6212 BPV1 (F)	GTGCAGCCAACTTCAAAGAAAT	BPV1 (L1, 6212-6233)	286	
6497 BPV1 (R)	ACTAGTGAGCCACTGGGAC	BPV1 (L1, 6497-6479)		
6390 BPV1 (F)	CTCGGAGAAAGAAGCCCCTAC	BPV1 (L1, 6390-6410)	275	
6664 BPV1 (R)	ACTCTGTTAGTGGGGTTCCA	BPV1 (L1, 6664-6645)		
6599 BPV1 (F)	GGGACAATACACGTGGTACTAAT	BPV1 (L1, 6599-6621)	291	
6889 BPV1 (R)	TTGCACATTTAGTTGCAGGAGA	BPV1 (L1, 6889-6868)		
6816 BPV1 (F)	TGTGCAGCCTCCTACCTCAT	BPV1 (L1, 6816-6835)	338	
7153 BPV1 (R)	GGTGCAGTTGACTTACCTTCT	BPV1 (LCR, 7153-7133)		
7032 BPV1 (F)	TGTGAGAAAACGAAGAATTAGCCA	BPV1 (LCR, 7032-7055)	302	
7333 BPV1 (R)	TCTTGCCAACAAAGCAATTATTCA	BPV1(LCR, 7333-7310)		
7289 BPV1 (F)	ATCCTGCCTTCTCAGCGAAA	BPV1 (LCR, 7289-7308)	290	
7578 BPV1 (R)	TCCTCCAAAAACCATAGAGACGC	BPV1 (LCR, 7578-7556)		
7516 BPV1 (F)	ACTGGTAAAAGTTTCCATTGCGT	BPV1 (LCR, 7516-7538)	223	
7737 BPV1 (R)	ACTTGTAGCTTGTCCTCACCAA	BPV1 (LCR, 7737-7716)		
7690 BPV1 (F)	ATTGAATGGGCGCATAATCAGC	BPV1 (LCR, 7690-7711)	216	
7905 BPV1 (R)	CCGGTTTCGGTGAGCTTAAAA	BPV1 (LCR, 7905-7885)		
7855 BPV1 (F)	GGCGGGAGCCAATCAAAATG	BPV1 (LCR, 7855-7874)	206	
115 BPV1 (R)	TTCTTGCAAAAGGTTTCAGGTCC	BPV1 (E6, 115-93)		

The nucleotide position is based on the sequence of the reference sequence of BPV1 and BPV2 (GenBank accession numbers: X02346 and M20219).
Abbreviations: bp, base pairs; F, forward; nt, nucleotide; R, reverse; Tm; annealing temperature

Table 2-4 Sequencing primers used to analyze fresh tissue-derived sarcoid and bovine papilloma-derived DNA samples.

Primer name	Sequence (5´ to 3´)	Target gene (location of PV nucleotide)	Reference
BPV1_seq_61	AAAAAGCTGCTGACAGACCC	BPV1 (61-80), BPV2 (62-81)	This study
BPV1_seq_191	TCAAAGACTTTCATGTTGTA	BPV1,2 (191-210)	Shimakura *et al.*, 2018
BPV1_seq_363 (R)	GCAGCATCTTATGCAAAGCC	BPV1,2 (363-344)	This study
BPV1_seq_621	GCCGTCCTACTAGGAAGCGAG	BPV1,2 (621-641)	Shimakura *et al.*, 2018
BPV1_seq_1210	CTAACCGTGTTCTTACGCCC	BPV1,2 (1210-1229)	This study
BPV1_seq_1621	AAGAGTGTTTGATGCTGCAG	BPV1 (1621-1640)	Shimakura *et al.*, 2018
BPV2_seq_1618	AAGAGTGCCTACTGATGCAG	BPV2 (1618-1637)	This study
BPV1_seq_2141	TATTGGCCCTCCAAACACAG	BPV1 (2141-2160)	
BPV1_seq_2641	CAAGAAACACAAATGCAGTTG	BPV1 (2641-2661), BPV2 (2638-2658)	Shimakura *et al.*, 2018
BPV1_seq_3141	CAGATTTAGTACAACAGGGC	BPV1 (3141-3160), BPV2 (3138-3157)	
BPV1_seq_3641	TGAAAAAGAACCATAGACATC	BPV1 (3640-3660)	
BPV1_seq_4169	AAGCTGCAGCAATAAAAATG	BPV1 (4169-4188)	This study
BPV1_seq_4641	ACTCGTCCACGGAGACCCTC	BPV1 (4640-4659)	Shimakura *et al.*, 2018
BPV2_seq_4639	ACTCTTCCACTGAAACTCTA	BPV2 (4639-4658)	
BPV1_seq_5374	AGTGCAACACGGCCTACAGG	BPV1 (5374-5393)	This study
BPV2_seq_5139	CTGCATGTCAGGTACTCCTTA	BPV2 (5139-5159)	
BPV1_seq_5535	CAGTAACTACACCTTGCATCCCT	BPV1 (5535-5557)	
BPV1_seq_5641	TCTCCCTCCAACCCCTGTAAG	BPV1 (5640-5660), BPV2 (5633-5653)	Shimakura et al., 2018
BPV1_seq_5721	CGGAGCGCCTGCTAACTATA	BPV1 (5720-5739)	
BPV2_seq_6133	ATGGTGCGTGTCCTCCTTTAG	BPV2 (6133-6153)	
BPV2_seq_6632	GCAGATGGAAACGCATTGTC	BPV2 (6632-6651)	This study
BPV1_seq_6808	GAAATAGGTGTGCAGCCTCCT	BPV1 (6808-6828)	
BPV1_seq_7113	ATGTTCTGTAAATGTAAAAC	BPV1 (7113-7132)	

The nucleotide position is based on the sequence of the reference sequence of BPV1 and BPV2 (GenBank accession numbers: X02346 and M20219).
Abbreviations: R, reverse; seq, sequence

Table 2-5 Statistical results of the Bayesian Tip-association Significance testing (BaTS) for BPV1 E2, E5, L1, and LCR.

Analysis	Statistic	Observed mean (95% CI)	Expected mean (95% CI)	Significance
BPV1				
E2 (Species)	AI	1.13 (0.51-1.75)	1.38 (1.11-1.63)	0.068
	PS	9.68 (8.00-11.00)	10.92 (9.08-12.27)	0.172
	MC (Equine)	2.29 (1.00-4.00)	2.38 (2.02-2.92)	0.96
	MC (Bovine)	2.67 (2.00-4.00)	2.54 (2.12-3.21)	0.993
E2 (Region)	AI	0.002 (0.00-0.00)	1.50 (1.24-1.74)	**0**
	PS	2.03 (2.00-2.00)	11.80 (10.09-13.11)	**0**
	MC (Japan)	9.85 (10.00-10.00)	2.42 (2.00-3.17)	**0.001**
	MC (China)	1.98 (2.00-2.00)	1.01 (1.00-1.06)	**0.001**
	MC (Europe)	13.99 (14.00-14.00)	2.15 (1.17-2.64)	**0.001**
E5 (Species)	AI	1.87 (1.05-2.71)	2.19 (1.81-2.49)	0.069
	PS	14.91 (12.00-17.00)	15.73 (13.89-16.99)	0.194
	MC (Equine)	6.62 (6.00-9.00)	3.85 (3.07-5.98)	0.051
	MC (Bovine)	2.47 (1.00-4.00)	2.38 (2.03-3.26)	0.972
E5 (Region)	AI	1.12 (0.5-1.74)	2.43 (2.20-2.73)	**0**
	PS	9.50 (8.00-10.00)	17.15 (15.64-18.24)	**0**
	MC (Japan)	5.15 (2.00-10.00)	1.98 (1.68-2.46)	**0.006**
	MC (Europe)	7.46 (6.00-12.00)	3.73 (3.01-5.63)	**0.001**
	MC (Africa)	1.99 (2.00-2.00)	1.01 (1.00-1.05)	**0.001**
	MC (China)	1.02 (1.00-1.00)	1.01 (1.00-1.05)	1
L1 (Species)	AI	1.33 (0.75-1.91)	1.52 (1.09-1.96)	0.239
	PS	9.86 (8.00-11.00)	10.90 (9.01-12.30)	0.176
	MC (Equine)	2.31 (2.00-4.00)	2.46 (2.02-3.01)	0.963
	MC (Bovine)	2.64 (2.00-4.00)	2.60 (2.15-3.30)	0.992
L1 (Region)	AI	0.03 (0.00-0.08)	1.64 (1.24-2.05)	**0**
	PS	2.02 (2.00-2.00)	11.78 (9.98-13.17)	**0**
	MC (Japan)	9.99 (10.00-10.00)	2.48 (2.02-3.23)	**0.001**
	MC (China)	2.00 (2.00-2.00)	1.02 (1.00-1.06)	**0.001**
	MC (Europe)	13.96 (14.00-14.00)	2.22 (1.74-2.80)	**0.001**
LCR (Species)	AI	1.78 (1.13-2.42)	2.35 (1.84-2.87)	**0.034**
	PS	12.88 (11.00-14.00)	16.00 (14.49-17.41)	**0.012**
	MC (Equine)	10.11 (10.00-11.00)	4.78 (3.61-6.64)	**0.017**
	MC (Bovine)	2.50 (1.00-4.00)	2.14 (1.64-3.02)	0.647
LCR (Region)	AI	1.45 (1.08-1.83)	3.60 (3.11-4.05)	**0**
	PS	10.52 (10.00-11.00)	25.34 (23.29-26.9)	**0**
	MC (Japan)	5.22 (2.00-10.00)	1.90 (1.46-2.99)	**0.002**
	MC (China)	1.43 (1.00-2.00)	1.01 (1.00-1.05)	1
	MC (Europe)	17.66 (16.00-20.00)	3.08 (2.42-3.73)	**0.001**
	MC (Africa)	2.00 (2.00-2.00)	1.25 (1.03-1.67)	**0.018**
	MC (South America)	1.00 (1.00-1.00)	1.01 (1.00-1.05)	1
	MC (Australia)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1

Statistic output based on the trait characteristics either of species and geographic origin for the respective BPV1 regions are shown.
P value below 0.05 was considered statistically significant, noted in bold numbers.
Abbreviations: AI, association index; CI, confidence interval; MC, monophyletic clade; PS, parsimony score

Table 2-6 Statistical results of the Bayesian Tip-association Significance testing (BaTS) for BPV2 E2, E5, L1, and LCR.

Analysis	Statistic	Observed mean (95% CI)	Expected mean (95% CI)	Significance
BPV2				
E2 (Species)	AI	0.25 (0.00-0.59)	0.42 (0.24-0.65)	0.078
	PS	1.89 (1.00-2.00)	2.63 (1.89-3.00)	0.252
	MC (Equine)	1.09 (1.00-2.00)	1.11 (1.00-1.32)	1.000
	MC (Bovine)	7.71 (7.00-10.00)	3.73 (2.43-7.70)	0.111
E2 (Region)	AI	0.45 (0.19-0.77)	0.67 (0.42-0.86)	0.085
	PS	3.78 (3.00-4.00)	4.38 (3.66-5.00)	0.414
	MC (Japan)	2.27 (1.00-4.00)	2.24 (1.49-3.58)	0.587
	MC (China)	1.03 (1.00-1.00)	1.12 (1.00-1.32)	1.000
	MC (South America)	2 (2.00-2.00)	1.04 (1.00-1.12)	**0.001**
E5 (Species)	AI	0.32 (0.00-0.63)	0.46 (0.31-0.61)	0.192
	PS	1.94 (1.00-2.00)	2.63 (1.94-2.94)	0.232
	MC (Equine)	1.05 (1.00-2.00)	1.12 (1.01-1.17)	1.000
	MC (Bovine)	4.37 (2.00-8.00)	3.64 (3.11-4.51)	0.193
E5 (Region)	AI	0.49 (0.12-0.91)	0.71 (0.53-0.85)	**0.012**
	PS	3.81 (3.00-5.00)	4.37 (3.76-4.83)	0.397
	MC (Japan)	2.27 (1.00-4.00)	2.32 (1.90-2.85)	0.667
	MC (China)	1.18 (1.00-2.00)	1.13 (1.00-1.18)	1.000
	MC (South America)	1.92 (1.00-2.00)	1.04 (1.00-1.06)	**0.001**
L1 (Species)	AI	0.04 (0.00-0.15)	0.49 (0.19-0.73)	**0.000**
	PS	1.79 (1.00-2.00)	2.67 (2.00-3.00)	0.221
	MC (Equine)	1.16 (1.00-2.00)	1.12 (1.00-1.99)	1.000
	MC (Bovine)	6.29 (5.00-11.00)	3.93 (2.29-5.63)	0.287
L1 (Region)	AI	0.68 (0.44-0.87)	0.76 (0.45-1.07)	0.403
	PS	4.00 (4.00-4.00)	4.36 (3.55-5.00)	0.461
	MC (Japan)	1.86 (1.00-3.00)	2.56 (1.31-4.37)	0.722
	MC (China)	1.00 (1.00-1.00)	1.14 (1.00-1.99)	1.000
	MC (South America)	2.00 (2.00-2.00)	1.04 (1.00-1.20)	**0.001**
LCR (Species)	AI	0.34 (0.25-0.53)	0.51 (0.14-0.90)	0.208
	PS	1.99 (2.00-2.00)	2.64 (2.00-3.00)	0.236
	MC (Equine)	1.00 (1.00-1.00)	1.12 (1.00-1.99)	1.000
	MC (Bovine)	7.49 (7.00-10.00)	3.64 (2.17-7.30)	0.093
LCR (Region)	AI	0.69 (0.52-0.77)	0.82 (0.41-1.21)	0.244
	PS	4.00 (4.00-4.00)	4.38 (3.05-5.00)	0.428
	MC (Japan)	2.40 (2.00-4.00)	2.39 (1.14-4.03)	0.764
	MC (China)	1.01 (1.00-1.00)	1.13 (1.00-1.99)	1.000
	MC (South America)	2.00 (2.00-2.00)	1.05 (1.00-1.95)	**0.001**

Statistic output based on the trait characteristics either of species and geographic origin for the respective BPV2 regions are shown. P value below 0.05 was considered statistically significant, noted in bold numbers.
Abbreviations: AI, association index; CI, confidence interval; MC, monophyletic clade; PS, parsimony score

Fig. 2-1

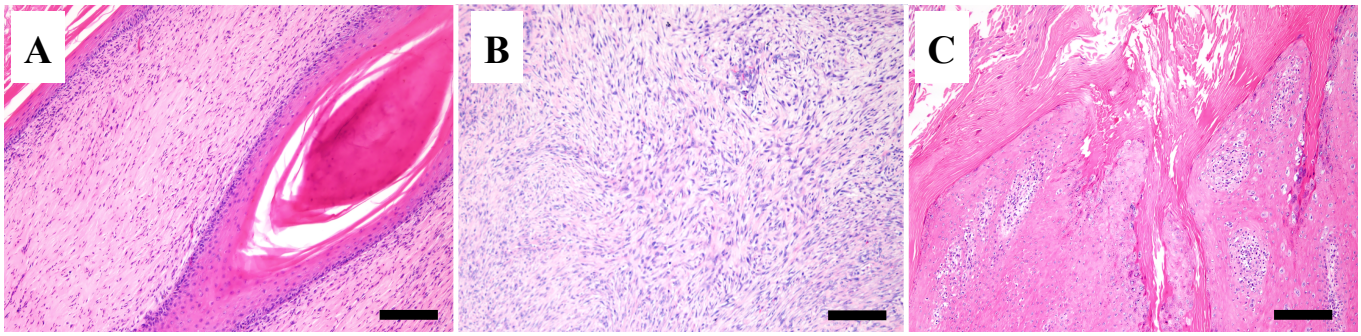


Fig. 2-1

Histopathological observations of equine sarcoid and bovine papilloma.

(A) Equine sarcoid (E170428-2, BPV1-positive) composed of papillary proliferation of stratified squamous epithelium and fibrous tissue.

(B) Equine sarcoid (E181025, BPV2-positive) composed of proliferation of fibrous tissue with mild infiltration of inflammatory cells.

(C) Bovine papilloma (B180620-3, BPV1-positive), composed of papillary proliferation of squamous epithelium.

Hematoxylin and eosin staining, bars, 200 μm.

Fig. 2-2

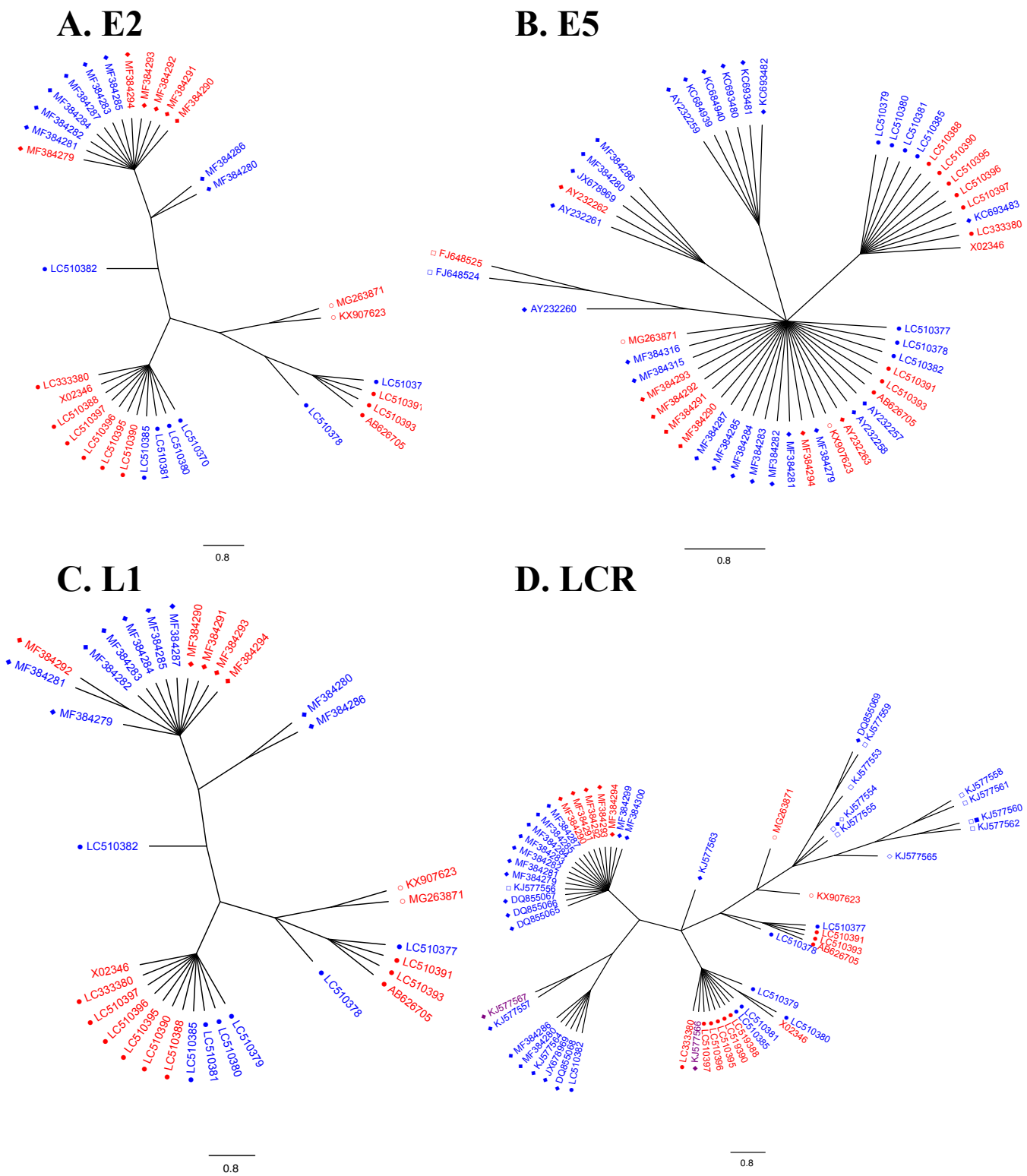


Fig. 2-2

Bayesian phylogenetic trees of BPV1 E2, E5, L1 and LCR nucleotides.

Bayesian phylogenetic trees were built by Markov chain Monte Carlo (MCMC) method. GenBank accession numbers of the respective isolates are shown. Equine- and bovine-derived sequences are colored in blue and red, respectively, and sequences identified in both species are colored in purple. The scale bar indicates percent variation per unit length. (A) BPV1 E2 (2608-3840 nt).(B) BPV1 E5 (3879-4013 nt). (C) BPV1 L1 (5609-7096 nt). (D) BPV1 LCR partial (7266-7904 nt).

The geographic origin of each isolate is noted using symbols, as follows:

Closed circle, Japan; Open circle, China; Closed rhombus, Europe; Open rhombus, South America; Closed square, Australia; Open square, Africa

Fig. 2-3

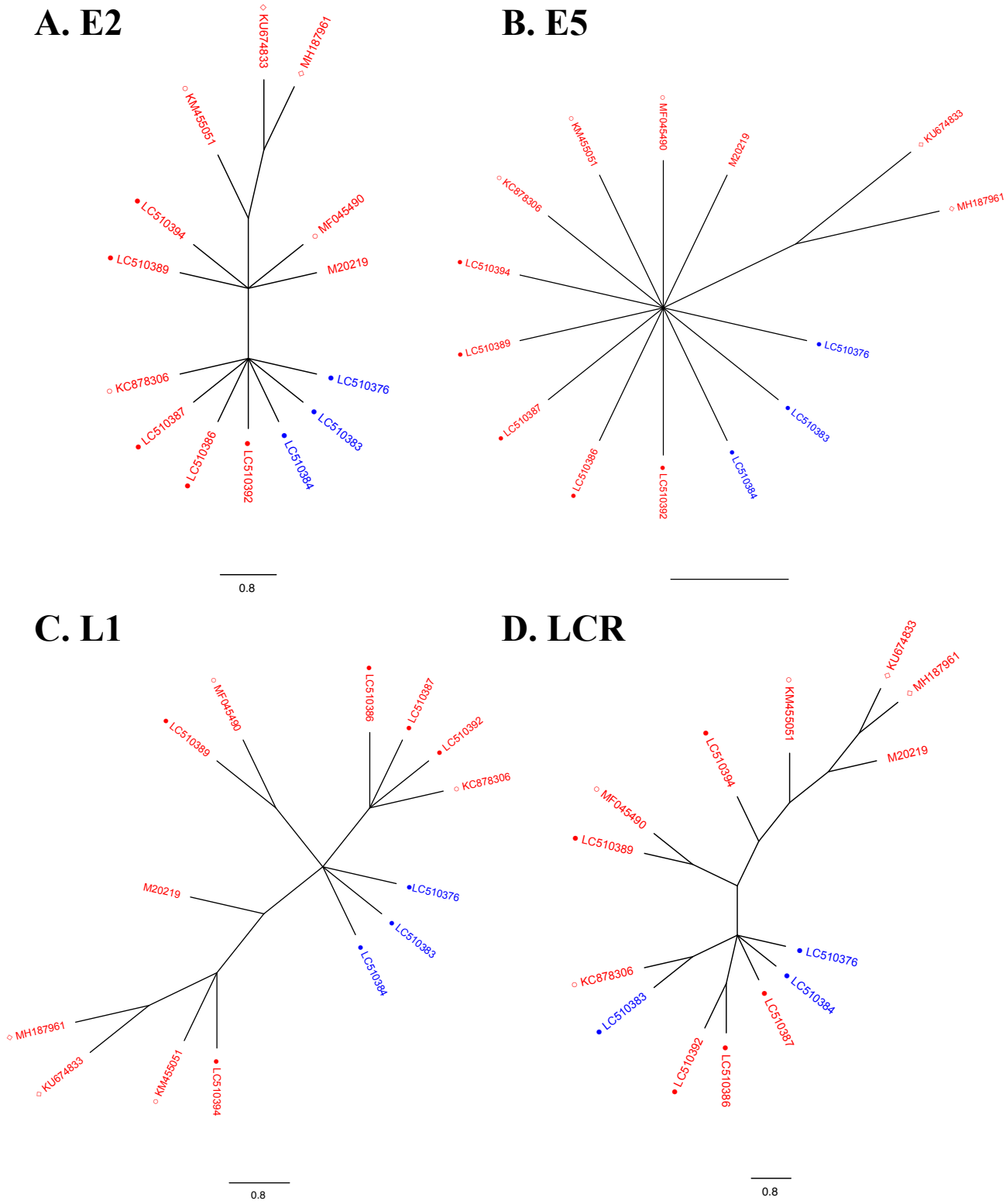


Fig. 2-3

Bayesian phylogenetic trees of BPV2 E2, E5, L1 and LCR nucleotides.

Bayesian phylogenetic trees were built by Markov chain Monte Carlo (MCMC) method. GenBank accession numbers of the respective isolates are shown. Equine- and bovine-derived sequences are colored in blue and red, respectively. The scale bar indicates percent variation per unit length. (A) BPV2 E2 (2608-3840 nt).(B) BPV2 E5 (3879-4013 nt). (C) BPV2 L1 (5609-7096 nt). (D) BPV2 LCR.

The geographic origin of each isolate is noted using symbols, as follows:

Closed circle, Japan; Open circle, China; Open rhombus, South America.

Fig. 2-4

BPV-1	E2														
	2764	2766	2917	3100	3255	3309	3382	3493	3544	3553	3547	3574	3648	3663	3723
BPV1 E170428-2 Equine Hokkaido (LC510377)	G	C	T	G	C	G	T	A	C	A	C	G	G	C	T
BPV1 E170801 Equine Kanto (LC510378)	G	C	T	G	A	G	T	A	C	A	C	G	A	C	A
BPV1 E171016 Equine Kansai (LC510379)	G	C	T	G	C	C	T	G	C	G	C	G	G	G	A
BPV1 E171218-2 Equine Kanto (LC510380)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 E171218-3 Equine Kanto (LC510381)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 E171218-5 Equine Kanto (LC510382)	G	C	T	T	C	C	C	A	C	G	C	T	G	C	T
BPV1 E190115 Equine Kanto (LC510383)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 B160701 Bovine Kanto (LC510390)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 B160805 Bovine Kanto (LC510395)	G	C	T	G	C	G	T	A	C	A	C	G	G	C	T
BPV1 B160705-4 Bovine Hokkaido (LC510391)	G	C	T	G	C	G	T	A	C	A	C	G	G	C	T
BPV1 B160705-6 Bovine Hokkaido (LC510393)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 B180620-2 Bovine Tohoku (LC510396)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 B180620-3 Bovine Tohoku (LC510397)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 P21 Bovine Kyushu (LC510388)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A
BPV1 reference (X02346)	G	C	T	G	C	C	T	G	C	G	C	G	G	C	A

BPV-1	E5															
	3886	3916	3919	3920	3922	3936	3937	3938	3942	3949	3951	3954	3955	3988	4003	4012
BPV1 E170428-2 Equine Hokkaido (LC510377)	T	T	T	G	A	T	G	T	T	A	T	T	G	C	G	A
BPV1 E170801 Equine Kanto (LC510378)	T	T	T	G	A	T	G	T	T	A	T	T	G	C	G	A
BPV1 E171016 Equine Kansai (LC510379)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 E171218-2 Equine Kanto (LC510380)	T	T	T	G	A	T	A	T	T	A	T	T	G	T	G	A
BPV1 E171218-3 Equine Kanto (LC510381)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 E171218-5 Equine Kanto (LC510382)	T	T	T	G	A	T	G	T	T	A	T	T	G	C	G	A
BPV1 E190115 Equine Kanto (LC510383)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 B160701 Bovine Kanto (LC510390)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 B160805 Bovine Kanto (LC510395)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 B160705-4 Bovine Hokkaido (LC510391)	T	T	T	G	A	T	G	T	T	A	T	T	G	C	G	A
BPV1 B160705-6 Bovine Hokkaido (LC510393)	T	T	T	G	A	T	G	T	T	A	T	T	G	C	G	A
BPV1 B180620-2 Bovine Tohoku (LC510396)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 B180620-3 Bovine Tohoku (LC510397)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 P21 Bovine Kyushu (LC510388)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A
BPV1 reference (X02346)	T	T	T	G	A	T	A	T	T	A	T	T	G	C	G	A

BPV-1	LCR																
	7146	7241	7305	7332	7362	7396	7406	7517	7538	7540	7585	7595	7598	7616	7617	7642	7644
BPV1 E170428-2 Equine Hokkaido (LC510377)	-	A	C	G	G	A	G	C	C	G	-	G	A	C	T	A	A
BPV1 E170801 Equine Kanto (LC510378)	-	A	C	G	G	A	G	C	C	G	-	G	A	C	T	A	A
BPV1 E171016 Equine Kansai (LC510379)	C	A	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 E171218-2 Equine Kanto (LC510380)	C	G	G	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 E171218-3 Equine Kanto (LC510381)	C	G	C	A	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 E171218-5 Equine Kanto (LC510382)	-	G	G	G	T	A	T	C	C	A	-	T	C	T	G	A	A
BPV1 E190115 Equine Kanto (LC510383)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 B160701 Bovine Kanto (LC510390)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 B160805 Bovine Kanto (LC510395)	-	G	C	G	G	A	G	C	C	G	-	G	A	C	T	A	A
BPV1 B160705-4 Bovine Hokkaido (LC510391)	-	G	C	G	G	A	G	C	C	G	-	G	A	C	T	A	A
BPV1 B160705-6 Bovine Hokkaido (LC510393)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 B180620-2 Bovine Tohoku (LC510396)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 B180620-3 Bovine Tohoku (LC510397)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 P21 Bovine Kyushu (LC510388)	C	G	C	G	G	A	G	T	C	G	-	G	A	C	T	A	G
BPV1 reference (X02346)	-	G	G	G	G	A	G	T	C	G	G	G	A	C	T	A	G

BPV-1	LCR (continued)																				
	7681	7684	7690	7712	7723	7736	7750	7751	7754	7785	7795	7816	7819	7833	7842	7879	7883	7913	7914	7936	81
BPV1 E170428-2 Equine Hokkaido (LC510377)	C	T	G	T	A	C	T	C	C	C	C	A	A	A	A	A	T	G	T	-	C
BPV1 E170801 Equine Kanto (LC510378)	C	T	G	T	A	G	T	C	C	C	A	A	G	A	A	A	T	G	T	-	C
BPV1 E171016 Equine Kansai (LC510379)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	A	C
BPV1 E171218-2 Equine Kanto (LC510380)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 E171218-3 Equine Kanto (LC510381)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 E171218-5 Equine Kanto (LC510382)	T	T	G	T	A	G	T	C	T	C	C	A	G	A	T	T	T	G	T	-	T
BPV1 E190115 Equine Kanto (LC510383)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 B160701 Bovine Kanto (LC510390)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 B160805 Bovine Kanto (LC510395)	C	T	G	T	A	C	T	C	C	C	C	A	A	A	A	A	T	G	T	-	C
BPV1 B160705-4 Bovine Hokkaido (LC510391)	C	T	G	T	A	C	T	C	C	C	C	A	A	A	A	A	T	G	T	-	C
BPV1 B160705-6 Bovine Hokkaido (LC510393)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 B180620-2 Bovine Tohoku (LC510396)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 B180620-3 Bovine Tohoku (LC510397)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	G	G	-	C
BPV1 P21 Bovine Kyushu (LC510388)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C
BPV1 reference (X02346)	T	T	A	T	G	G	T	C	C	C	C	A	G	A	A	A	T	A	G	-	C

BPV-1	L1																
	5699	5742	5766	5771	6016	6134	6136	6138	6144	6404	6410	6650	6659	6719	6799	6858	7008
BPV1 E170428-2 Equine Hokkaido (LC510377)	G	A	A	C	A	T	G	A	C	G	C	A	A	C	C	G	C
BPV1 E170801 Equine Kanto (LC510378)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 E171016 Equine Kansai (LC510379)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 E171218-2 Equine Kanto (LC510380)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 E171218-3 Equine Kanto (LC510381)	A	A	C	C	G	C	A	T	C	A	G	C	A	T	C	C	A
BPV1 E171218-5 Equine Kanto (LC510382)	G	A	C	C	G	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 E190115 Equine Kanto (LC510383)	G	A	C	C	A	T	G	A	C	G	C	A	A	C	C	G	A
BPV1 B160701 Bovine Kanto (LC510390)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 B160805 Bovine Kanto (LC510395)	G	G	C	C	A	T	G	A	C	G	C	A	A	C	C	G	A
BPV1 B160705-4 Bovine Hokkaido (LC510391)	G	G	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 B160705-6 Bovine Hokkaido (LC510393)	G	A	A	C	A	T	G	A	C	G	C	A	A	C	C	G	C
BPV1 B180620-2 Bovine Tohoku (LC510396)	G	G	C	C	A	T	G	A	C	G	C	A	A	C	C	G	A
BPV1 B180620-3 Bovine Tohoku (LC510397)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 P21 Bovine Kyushu (LC510388)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A
BPV1 reference (X02346)	G	A	C	C	A	T	G	A	C	G	C	C	C	C	G	C	A

Fig. 2-4

The varied sequence and its location of BPV1 E2, E5, L1 and LCR between equine/bovine-derived sequences identified in this study and from the previous studies.
If at least one sequence variation was observed between equine/bovine-derived isolate, its nucleotide sequence and the position is shown. BPV1 sequences which were previously reported in any of the papers (Chambers *et al.*, 2003; Koch *et al.*, 2018; Nasir *et al.*, 2007; Savini *et al.*, 2015) as sarcoid-associated variant is shown in red. Each nucleotide location is based on the reference sequence of BPV1 (GenBank accession number: X02346.1). Respective GenBank accession numbers are noted in parentheses.

Fig. 2-5

BPV-2	E2																									E5
	2609	2668	2669	2882	2916	3116	3240	3242	3243	3244	3245	3280	3281	3282	3334	3335	3391	3451	3507	3508	3579	3612	3681	3689	3690	3726	4005
BPV2 E170428-1 Equine Kanto (LC510376)	A	A	G	C	T	C	C	G	G	G	T	-	-	-	G	T	T	T	C	T	A	A	C	G	G	A	C
BPV2 E180925-2 Equine Hokkaido (LC510383)	A	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	T	T	C	G	A	A	C	G	G	A	C
BPV2 E181025 Equine Hokkaido (LC510384)	A	A	G	C	T	T	C	G	G	G	T	A	A	G	G	T	T	T	A	T	A	A	C	G	G	A	C
BPV2 B160303 Bovine Kanto (LC510389)	A	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	C	T	C	T	A	A	C	G	G	A	C
BPV2 B160705-4 Bovine Hokkaido (LC510392)	A	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	T	T	C	T	A	A	C	G	G	A	C
BPV2 B160705-6 Bovine Hokkaido (LC510394)	A	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	C	T	C	T	A	A	A	G	G	A	T
BPV2 P9 Bovine Kyushu (LC510386)	A	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	T	T	C	T	A	A	C	G	G	A	C
BPV2 P11 Bovine Kyushu (LC510387)	T	A	G	C	T	C	C	G	G	G	T	A	A	G	G	T	T	T	C	T	A	A	C	G	G	A	C
BPV2 reference (M20219)	A	G	C	G	A	C	T	-	-	-	C	A	A	G	T	A	C	C	C	T	G	T	C	C	C	T	C

BPV-2	LCR																																	
	7097	7120	7132	7150	7177	7257	7333	7348	7349	7370	7386	7389	7392	7397	7407	7418	7447	7449	7489	7498	7591	7633	7685	7725	7793	7803	7814	7836	7852	7894	50	52	87
BPV2 E170428-1 Equine Kanto (LC510376)	T	A	A	A	T	G	G	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	C	-	-	C	A	A	C
BPV2 E180925-2 Equine Hokkaido (LC510383)	T	A	C	A	T	G	A	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	C	-	-	C	C	A	-
BPV2 E181025 Equine Hokkaido (LC510384)	T	A	A	A	T	G	G	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	C	-	-	C	A	A	C
BPV2 B160303 Bovine Kanto (LC510389)	T	A	A	A	C	G	G	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	A	-	-	C	A	A	C
BPV2 B160705-4 Bovine Hokkaido (LC510392)	C	A	A	A	T	G	G	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	C	-	-	C	A	A	C
BPV2 B160705-6 Bovine Hokkaido (LC510394)	T	A	A	-	T	G	G	G	C	-	T	T	A	G	G	A	G	A	A	C	A	C	A	A	A	C	A	-	-	C	A	T	C
BPV2 P9 Bovine Kyushu (LC510386)	C	A	A	A	T	G	G	G	C	-	T	C	A	G	G	A	G	A	T	C	G	C	T	A	G	A	C	-	-	C	A	A	C
BPV2 P11 Bovine Kyushu (LC510387)	T	A	A	A	T	G	G	G	C	-	T	C	A	G	G	A	G	A	T	A	G	C	T	A	G	A	C	-	-	C	A	A	C
BPV2 reference (M20219)	T	G	A	-	T	C	G	C	T	A	C	T	T	T	C	G	A	C	T	C	G	-	T	T	A	C	A	A	C	-	C	A	-

BPV-2	L1																			
	5624	5699	5835	5885	5909	6126	6127	6133	6134	6167	6246	6251	6446	6567	6756	6845	6920	6925	7051	7056
BPV2 E170428-1 Equine Kanto (LC510376)	G	T	T	G	G	A	T	A	T	A	C	T	G	G	G	C	C	G	G	T
BPV2 E180925-2 Equine Hokkaido (LC510383)	G	T	T	G	G	A	T	A	T	A	C	T	G	G	G	C	C	G	G	T
BPV2 E181025 Equine Hokkaido (LC510384)	G	T	T	G	G	A	T	A	T	A	C	G	G	G	G	C	C	G	G	T
BPV2 B160303 Bovine Kanto (LC510389)	G	T	T	G	G	A	T	A	T	G	T	T	G	G	G	C	C	G	G	T
BPV2 B160705-4 Bovine Hokkaido (LC510392)	G	C	T	G	G	C	C	T	G	A	C	T	G	G	G	C	C	A	A	T
BPV2 B160705-6 Bovine Hokkaido (LC510394)	G	T	T	G	G	A	T	A	T	A	C	T	G	G	G	C	C	G	G	A
BPV2 P9 Bovine Kyushu (LC510386)	G	T	T	G	G	A	T	A	T	A	T	T	G	G	G	C	C	G	G	T
BPV2 P11 Bovine Kyushu (LC510387)	G	T	T	G	G	A	T	A	T	A	T	T	G	G	G	C	C	G	G	T
BPV2 reference (M20219)	A	C	T	T	A	C	T	A	T	A	C	T	A	A	C	T	G	A	G	T

Fig. 2-5

The varied sequence and its location of BPV2 E2, E5, L1 and LCR between equine/bovine-derived sequences identified in this study.

If at least one sequence variation was observed between equine/bovine-derived isolate, its nucleotide sequence and the position is shown. Each nucleotide location is based on the reference sequence of BPV2 (GenBank accession number: M20219.1). Respective GenBank accession numbers are noted in parentheses.

Chapter 3

Detection and sequence characterization of *Felis catus* papillomavirus types 3 and 4 associated with squamous cell carcinoma of cats in Japan

Introduction

In chapter 2, molecular epidemiological analyses of BPV types 1 and 2 demonstrated that PV sequence homologies and nucleotide substitutions could be affected by the geographical distributions. This phenomenon has also been described in HPV studies, and it has been considered that sequence variations observed between the geographical regions have potential to alter the HPV vaccine efficacy (Godi et al., 2019). From the aspect of PV vaccine development for animals, it is essential to create a cost-effective universal vaccine. In farm animals, economic efficiency becomes one of the issues in vaccine delivery. Hence, companion animals such as dogs, cats, and horses could become candidate animals for vaccine development. In terms of creating an animal PV vaccine, proper PV types should be inoculated to make practical vaccines suitable for each animal species. Moreover, a vaccine needs to be preventative against life-threatening malignant diseases than the benign ones.

Domestic cats have been kept worldwide as companion animals from ancient periods. To date, according to a survey demonstrated by Japan Pet Food Association, numbers of household cats have been increasing that the cat population became larger than dogs in Japan (”https://petfood.or.jp/data/” accessed on 27/11/2020). The increase in household cats are also noted in the United States and the United Kingdom, while a decline has been observed in Australia (Baldock et al., 2003). The mean life-span of domestic cats are becoming longer, which means that the cat could be affected by certain types of diseases such as cancers. A previous retrospective study noted the significant association between malignant tumor development and ages that malignant tumors were seen in older cats (median age 12 years) compared with benign tumors (Ho et al., 2018). Malignant cutaneous neoplasms such as BISCs, BCCs, and SCCs have been described to be associated with FcaPV infections (Munday et al., 2017b). Among the FcaPV-associated diseases, SCC has been one of the most diagnosed cutaneous neoplasms in cats.

In domestic cats, SCC accounts for 11-15% of cutaneous neoplastic diseases (Ho et al., 2018; Miller et al., 2012), and shares the most in oral neoplastic diseases (Stebbins et al., 1989). The significant cause of feline cutaneous SCC is undermined, but the previous studies suggest that UV-exposure could become one of the causative factors (Altamura et al., 2016; Bertone et al., 2003; Munday and Kiupel, 2010). Recent studies have suggested the etiological roles of FcaPVs in the cutaneous SCC development, as FcaPVs have been detected from cutaneous SCCs worldwide (Geisseler et al., 2016; Munday et al., 2011; O’Neill et al., 2011). Among the six types of FcaPVs, FcaPV2 has been identified the most from SCCs (Geisseler et al., 2016; Munday et al., 2011; O’Neill et al., 2011). However, it is uncertain that which FcaPV type(s) have associations with SCCs of cats kept in Asian countries including Japan. Based on the findings noted in Chapter 2 that PV sequences

could vary between geographic origins, we could hypothesize that FcaPV type-distributions may also become geographically divergent. In Asian countries including Japan, there is limited information about FcaPVs. In this study, FcaPV detection was conducted by conventional PCR. Both consensus and type-specific FcaPV primers were applied, because primers may affect the specificity and sensitivity for DNA amplification (Munday et al., 2011). This chapter has aimed to clarify the association of FcaPV with SCCs in cats kept in Japan, and to characterize the viral sequence which may alter viral pathogenicities.

Materials and Methods

Sample collection and histological diagnosis

Twenty-one feline SCC biopsy samples, collected in Japan between 2013 and 2015 were included in this study. SCC samples were fixed in 10% buffered-formalin and routinely embedded in paraffin and stained with HE for histopathological examination. Histopathological diagnoses of SCC were made on the consensus of two veterinary pathologists (certified by the Japanese College of Veterinary Pathologists) at the Department of Veterinary Pathology, the University of Tokyo. Clinical and histopathological findings of feline SCC cases are summarized in Table 3-1. The mean age at the diagnosis was 12.3 ± 2.3 (mean ± standard deviation) years with no sex predilection. In the present study, seven out of 21 cases were oral, nine were cutaneous, and the rest of the SCC case lesions were observed in other locations.

Immunohistochemistry (IHC)

IHC was performed to detect the PV antigen and p16 protein, with mouse anti-PV (clone BPV-1/1H8 +CAMVIR, Abcam, Cambridge, U.K.) and mouse anti-CDKN2A/p16INK4a (Becton Dickinson Company, Franklin Lakes, New Jersey, U.S.A.) monoclonal antibodies, respectively. Horseradish perioxidase labeled detection kit (Envision +system) was applied, and antigen-antibody complex was visualized by the chromogen treatment with 3,3'-diaminobenzidine (DAB). Sections were counterstained with Mayer' hematoxylin. A section of cutaneous bovine papilloma, positive for BPV2, was used as the positive control for anti-PV antibody. For the negative control, the primary antibodies were substituted with tris-buffered saline. Positive staining of more than 10% of the neoplastic tissue was considered to be p16-positive.

DNA extraction, validation, and papillomavirus detection

Genomic DNA from 21 FFPE tissue samples were extracted using Qiagen DNA FFPE Tissue kit (Qiagen). The success in DNA extraction of all samples were confirmed by PCR using feline beta-actin primer pair, ACTB (Kessler et al., 2009) (Table 3-2). DNA polymerase, KOD FX Neo (Toyobo) was applied in PCR reactions by following the manufacturer's instructions. PV detection was performed using two consensus primer pairs, MY09/11 (Manos et al., 1989) and CP4/5 (Tieben et al., 1994), which have been used to detect the L1 and E1 of various PVs. Additionally, three type-specific primer pairs, targeting the L1 of FcaPV2, FcaPV3, and FcaPV4 were applied (Table 3-2). PCR products were electrophoresed on 2% agarose gels, and the bands were visualized by UV-exposure.

Full-genome characterization of FcaPV4

The complete sequence of FcaPV4 genome was determined by PCR, sub-cloning, and sequencing. PCR primers were designed based on the reference sequence of FcaPV4 (GenBank accession number: KF147892). Considering the DNA fragmentation caused by formalin-fixation, each primer pair targets below 700 bp of FcaPV4 DNA (Table 3). The success in PCR was confirmed by gel-electrophoresis.

Sub-cloning, sequencing and sequence analyses

PCR-positive bands were cut followed by purification using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Purified products were subjected to bidirectional sequencing and sub-cloning. For sub-cloning, purified PCR products were cloned into pCR 2.1-TOPO vector or pCR_Blunt II-TOPO (Invitrogen) and processed to sequencing. Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing kit and 3130xl genetic analyzer (Applied Biosystems) by applying the respective primers used for PCR. Sequencing results were analyzed with MEGA 7 (Kumar et al., 2016) and MEGA X (Kumar et al., 2018b) software. The identical PV types were defined using the BLAST from the NCBI.

Phylogenetic tree construction and protein structural analysis

Based on the L1 nucleotide sequence isolates including 62 references of HPVs and animal PVs (Van Doorslaer et al., 2017), phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA X. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

In order to examine whether the amino acid substitutions, including the deletion affects the structure of L1 protein, L1 amino acid sequences of two FcaPV4-positive cases (13-136 and 14-1110) were deposited to SWISS-MODEL (https://swissmodel.expasy.org/interactive).

Reverse-transcriptase PCR (RT-PCR)

Reverse-transcriptase PCR (RT-PCR) was performed to evaluate the mRNA expression of E6 and E7 of the FcaPV4-positive samples. RNA extraction, DNA elimination, and cDNA synthesis were conducted as previously described (Chang et al., 2020b). In brief, total RNA from FFPE tissue blocks were extracted using RNeasy FFPE Kit (Qiagen) following the DNA elimination with RNAse-Free DNase Set (Qiagen). cDNA was synthesized by primer-specific reverse transcription using Transcriptor First Strand cDNA Synthesis Kit (Roche) and two primer pairs targeting FcaPV4 E6 and E7 (FcaPV4 inside E6 F/R and E7 F/R, Table 3-3). The expression of E6/E7 cDNA was confirmed by electrophoresis and bidirectional sequencing.

Results

Histopathological findings and immunohistochemistry

The histopathological examination of HE-stained feline SCC sections revealed the invasive growth of squamous epithelial tissue of the skin or the mucosa (Fig. 3-1). Severe nuclear atypia and abundant mitotic figures were observed. Nuclear inclusion bodies, indicative of papilloma virus infection, were not observed in the examined sections. Immunohistochemical detection for p16 showed diffuse positive staining in the neoplastic tissue of case numbers 13-136, 14-1110 (Fig. 3-2), and 5741-2013 (Table 3-1).

Positive staining of PV antigen in the nucleus of epithelial cells in the section of bovine fibropapilloma was observed, while no nuclear-positive staining was identified in the feline SCC samples (data not shown). Some of the paraffin blocks were not available for IHC due to insufficient amount of paraffin blocks (Table 3-1).

FcaPV detection

The PCR results of each sample is summarized in Table 3-1. PCR and electrophoresis revealed that two cases (13-0153 and 14-1110) were positive with CP4/5 primer pair, while no positive samples were observed by MY09/11 consensus primer. Sequencing revealed that cases 13-136 and 14-1110 were FcaPV3 and FcaPV4, respectively. The PCR-positive results of these two cases were also confirmed by applying type-specific primers of FcaPV3 and FcaPV4. One additional FcaPV4-

positive case was identified with FcaPV4 L1 F/R primer pair (Table 3-1). No FcaPV2-positive cases were detected within the analyzed samples.

The nucleotide sequence identity of FcaPV3- and FcaPV4-detected cases by type-specific primers were determined. The BLAST analysis showed that samples 13-0153 was 99.8 % (397/398 nt) identical to the reference FcaPV3 (GenBank accession number: JX972168). The FcaPV4-positive sample, case 14-1110 was 99.1 % (428/432 nt) identical to the reference sequence (GenBank accession number: KF147892). The FcaPV4-positive sample which was identified only by the type-specific primer (case 13-136), showed 95.4% (412/432 nt) sequence similarity to the reference FcaPV4 sequence.

PV genome evolutions are described to be slow and highly-conserved that the evolutionary rate was estimated to be 1.95×10^{-8} nucleotide substitutions per site per year in *Felidae* (Rector et al., 2007). Therefore, the full-genome characterization of both FcaPV4-identified isolates (13-136 and 14-1110) should be worth doing to enrich the FcaPV sequence database, and to discover sequence characteristics associated with the pathogenicity of FcaPV4.

Full-genome characterization and phylogeny of two FcaPV4 isolates

The complete genome-based FcaPV4 sequence identities of 13-136 and 14-1110 against the reference sequence was 96.7 % and 99.5 %, respectively (Table 3-4). The L1 nucleotide sequence of case 13-136 showed 95.7 % homology to that of the reference FcaPV4. Referring to the PV classification criteria of subtype (2-10% dissimilar from one another) (DeVilliers et al., 2004), isolate 13-136 was suggested to be classified as a novel FcaPV4 subtype. The nucleotide sequence of each PV genome region was identical to the reference sequence between 95.0 % to 98.1 % in case 13-136. The E2 showed the highest sequence identity and the L2 showed the lowest (Table 3-4). Another isolate, case 14-1110 showed higher identity than case 13-136, harboring over 98.2 % identities with the reference sequence. Among the analyzed genes, L2 showed the lowest identity also in case 14-1110 (Table 3-4).

The amino acid substitutions observed in cases 13-136 and 14-1110 were determined by comparing with the reference FcaPV4 sequence (Table 3-5). Overall, case 13-136 harbored frequent amino acid substitutions than 14-1110. In case 13-136, the numbers of amino acid substitutions observed in the two early genes, the E1 and E2 were four and nine, respectively (Table 3-5). In the E2, one deletion of glutamine (Q) at position 220 was noted. Case 13-136 showed some amino acid substitutions in both E6 and E7, case 14-1110 was 100% identical to the reference amino acid sequence (Tables 3-4 and 3-5). In the capsid proteins (L1, L2), similar amino acid substitutions were noted in

both cases. In the L1, the deletion of 335th tryptophan (W) and the substitution of 502th alanine (A) to threonine (T) were common amino acid alterations (Table 3-5). In the L2, in total of five similar amino acid substitutions were observed in the two FcaPV4-identified cases (Table 3-5).

Phylogenetic and L1 structural analyses

The phylogenetic analysis demonstrated that case 14-1110 L1 showed a closer relationship to the reference FcaPV4, while 13-136 branched between FcaPV4 and FcaPV5, both belonging to the genus, *Taupapillomavirus* (Fig. 3-3).

Based on the results of L1 structural analysis using the SWISS-MODEL, no significant alteration due to the presence/deletion of the 335th tryptophan in the L1 major capsid protein was noted in two FcaPV4 cases (13-136, 14-1110) (Fig. 3-4).

Reverse-transcriptase PCR (RT-PCR)

The results of RT-PCR confirmed the expression of FcaPV4 E6/E7 cDNA (mRNA) in both FcaPV4-identified cases (13-136 and 14-1110). The success in genomic DNA elimination was validated with the same RT-PCR primers by applying the DNase-treated RNA samples as template (Fig. 3-5). Sequence of the respective RT-PCR-positive products were identical to FcaPV4 E6 and E7 sequence.

Discussion

In the present study, association of FcaPV with feline SCC was determined by molecular and pathological analyses. Among the analyzed feline SCC cases, nine out of 21 cases were cutaneous and eight were oral SCCs. Three FcaPV-detected cases (13-136, 13-0153, 14-1110) were cutaneous SCCs (Table 3-1), which was similar to the previous findings that FcaPV-associated SCCs are common in cutaneous SCCs than oral SCCs (Munday and French, 2015). Although FcaPV has been detected from asymptomatic cats (Thomson et al., 2015), significant association between FcaPV infection and lesion development is still uncertain. In humans, co-infection of human immunodeficiency virus (HIV) is described to be associated with HPV-associated anogenital cancer development (Haga et al., 2017). Similarly, the immunodeficient status caused by FIV infection may become one of the risk factors for FcaPV-associated lesion development in cats (Egberink et al., 1992). The immune status of each cat included in this study was unknown, but defining the host immune status may become useful information to predict the prognosis, such as the potential to progress into metastatic SCCs from viral plaques/BISCs.

In the present study, immunohistochemical detection of p16 protein and PV antigen was performed. In cats, a previous study demonstrated that positive staining for p16 is one of the observations indicative of PV infection in SCCs (Munday et al., 2011). Based on the observations noted in the previous studies that PVs could be detected in dermatologically-healthy cats (Thomson et al., 2018, 2016), immunohistochemical detection of p16 within the SCC specimens are preferable to support the etiological roles of FcaPVs in the lesion development. As expected, both of the FcaPV4-positive cases (13-136, 14-1110) were immunopositive for p16 (Table 3-1 and Fig. 3-2). One sample, 5742-2013 was also p16-positive while neither FcaPV types, 2, 3, and 4 was detected by PCR. This result suggests that factors unrelated to PVs and/or involvement of other PV types may be present. No positive signals for PV antigen were observed within the all analyzed SCC specimens, including the FcaPV4-positive cases. This result was not surprising, because during the malignant progression, the L1 capsid antigen is suggested to be unexpressed (Azzimonti et al., 1999), and the primary antibodies used in this study (monoclonal BPV-1/1H8) was raised against the L1 major capsid protein.

This study confirmed the detection of two FcaPV types, 3 and 4, in feline SCCs by conventional PCR. One FcaPV3 and FcaPV4 each, was identified by CP4/5 consensus primer, but additional FcaPV4-positive result was confirmed by using FcaPV4 type-specific primer (Table 3-1). This result may be caused by the several mutations observed in case 13-136 have altered the sensitivity and/or specificity of the consensus primer. Application of PV-type specific primers are considered to be beneficial for detecting the target PV type, while it may have difficulties in identifying the unknown PV genotypes (Haga et al., 2013). In addition to FcaPV type-specific primers, designing the FcaPV consensus primers may be preferable for detecting the unknown genotype.

From the previous reports made from other geographical regions, FcaPV2 has been detected frequently among the known FcaPV types (Mazzei et al., 2017; Munday et al., 2011; O'Neill et al., 2011). In contrast, FcaPV2 was not detected in this study. This result are consistent to that of the human studies that the distribution of high-risk HPVs in differed depending on the geographical region (Crosbie et al., 2013). Although the sample size of this study is limited, the geographical difference may explain the discrepancies of the dominant FcaPV types in feline SCCs.

The complete nucleotide sequences of two FcaPV4 isolates were determined in this study and one case (13-136) turned out to be a subtype of FcaPV4. To the author's knowledge, the complete sequence of FcaPV4 available in the GenBank database is limited to one sequence (GenBank accession number: KF147892) from New Zealand. The present study updates the FcaPV4 sequence database, making the sequence directory more practical for further studies including *in silico* analyses. Among the two FcaPV4 isolates (13-136, 14-1110), 13-136 harbored more amino acid substitutions

than 14-1110 when compared with the reference FcaPV4 sequence (Tables 3-4 and 3-5). In the E2, 220th glutamine (Q) was deleted due to the nucleotide deletion of "CAG" at position 3158. E2 gene has been considered to regulate viral replication, transcription, and oncogene (E6 and E7) expression (Mcbrides et al., 1991). Thus, the amino acid substitutions including the 220th glutamine deletion in case 13-136 may alter its E2 function. In case 13-136, amino acid substitutions of two oncogenes (E6 and E7) were observed, while case 14-1110 was 100% identical to the reference sequence (Tables 3-4 and 3-5). mRNA expressions of FcaPV4 E6/E7 were observed in both cases, demonstrating that amino acid substitutions of E2, E6, and E7 noted in case 13-136 did not alter qualitative mRNA expression (Fig. 3-5). However, further considerations to verify the alteration in E6/E7 mRNA expression levels are needed.

The nucleotide and amino acid sequences were nearly 95% and 97% identical, respectively to the reference capsid genes (L1 and L2) in case 13-136, which was lower than the other analyzed ORFs (Table 3-4). In the capsid genes, some similar amino acid substitutions of 13-136 and 14-1110 were identified. In the L1, the deletion of 335th tryptophan (W) was observed in both 13-136 and 14-1110 (Table 3-5). However, no significant alteration of the L1 protein structure was observed by SWISS-MODEL analysis (Fig. 3-4).

Conclusion

In the present chapter, detection of FcaPV types 3 and 4, but not FcaPV2 was described in cutaneous SCC samples of cats kept in Japan. At least in FcaPV4-identified cases, p16 expression were confirmed demonstrating the etiological association of FcaPV4 with cutaneous feline SCC. Complete genome-based sequence analysis revealed that one case harbored frequent amino acid substitutions, and turned out to be a novel subtype of FcaPV4. The alteration of viral pathogenesis and/or expression levels due to the sequence substitutions needs to be further examined. The findings of this study suggest that inoculating FcaPV3 and FcaPV4 in addition to FcaPV2 needs to be considered in terms of developing a universal prophylactic vaccine for cats. The present study will extend the knowledge of FcaPV sequence diversities and its etiological roles in feline cutaneous SCCs.

Table 3-1 Descriptions of feline SCC samples and the results of PCR, sequencing, and p16 immunohistochemistry.

Sample ID	Breed	Age (years)	Sex	Anatomical site	PCR results					PV genotype (GenBank accession number)	p16 IHC
					CP4/5	MY09/11	FcaPV2-L1	FcaPV3-L1	FcaPV4-L1		
13-060	Mixed-breed	13	FX	Skin	-	-	-	-	-	ND	−
13-0107	Mixed-breed	12	FX	Oral (submandible)	-	-	-	-	-	ND	NA
13-136	Mixed-breed	14	FX	Skin	-	-	-	-	+	FcaPV4 (LC333412)	+
13-0153	Mixed-breed	12	MX	Skin (eyelid, left)	+	-	-	+	-	FcaPV3 (LC333418)	NA
13-0297	Mixed-breed	12	MX	Oral (lower gingia, right)	-	-	-	-	-	ND	−
13-0317	American shorthair	15	MX	Oral (tongue)	-	-	-	-	-	ND	−
13-848	Mixed-breed	10	FX	Auditory canal (left)	-	-	-	-	-	ND	−
13-882	Mixed-breed	16	FX	Oral (tongue)	-	-	-	-	-	ND	NA
13-944	Scottish fold	10	MX	Oral (tongue)	-	-	-	-	-	ND	−
14-778	Mixed-breed	13	M	Skin (upper jaw, right)	-	-	-	-	-	ND	−
14-1018	Mixed-breed	16	FX	Auditory canal (left)	-	-	-	-	-	ND	−
14-1110	American shorthair	10	MX	Skin (forelimb, digit, right)	+	-	-	-	+	FcaPV4 (LC33413)	+
15-291	Mixed-breed	10	FX	Oral (upper gingiva)	-	-	-	-	-	ND	−
15-358	Mixed-breed	13	FX	Skin (auditory canal, left)	-	-	-	-	-	ND	−
15-498	Mixed-breed	9	MX	Skin (external ear, left)	-	-	-	-	-	ND	−
15-528	Mixed-breed	16	MX	Oral (sublingual, right)	-	-	-	-	-	ND	−
15-577	Mixed-breed	15	FX	Oral (upper gingiva,right)	-	-	-	-	-	ND	−
15-615	Mixed-breed	10	MX	Esophagus	-	-	-	-	-	ND	−
15-637	Mixed-breed	11	FX	Skin (submandible)	-	-	-	-	-	ND	−
5741-2013	Mixed-breed	12	MX	Anus	-	-	-	-	-	ND	+
6997-2015	Mixed-breed	9	M	Oral (buccal mucosa)	-	-	-	-	-	ND	NA

Abbreviations: FX, spayed female; FcaPV, *Felis catus* papillloamavirus; M, male; MX, castrated male; NA, not available; ND, not detected; +, positive; -, negative.

Table 3-2 Primer used in PCR detecting feline beta-actin and papillomaviruses.

Primer Pair	Sequence (5´ to 3´)	Target gene (PV genome, location of PV nucleotide)	Product size (bp)	Tm (°C)	Reference
Feline ACTB (F)	CAACCGTGAGAAGATGACTCAGA	Feline β-actin	410	54	Kessler *et al.*, 2009
Feline ACTB (R)	CCCAGAGTCCATGACAATAACA				
MY09	GCMCAGGGWCATAAYAATGG	PV L1 (Consensus)	approximately 450	55	Manos *et al.*, 1989
MY11	CGTCCMARRGGAWACTGATC				
CP4	ATGGTACARTGGGCATWTGA	PV E1 (Consensus)	approximately 450	49	Tieben *et al.*, 1994
CP5	GAGGYTGCAACCAAAAMTGRCT				
FcaPV2 L1 (F)	CGCAAGGACAGAATAATGGTATTTGCT	FcaPV2 (L1, 7130-7156)	419	58	This study
FcaPV2 L1 (R)	AAGACGATCCGAGATGTCAACAT	FcaPV2 (L1, 7548-7525)			
FcaPV3 L1 (F)	TCTGGTAATCAGTATAGGGTGTTCAGAGT	FcaPV3 (L1, 5868-5876)	473	59	This study
FcaPV3 L1 (R)	ATTTCTAAAGGCACCCCTGATTTGTCTT	FcaPV3 (L1, 6340-6312)			
FcaPV4 L1 (F)	CTTTGGTAACCAGCGATTCC	FcaPV4 (L1, 6571-6590)	476	52	This study
FcaPV4 L1 (R)	CAATCTATCCTTCAAGTCCACTAC	FcaPV4 (L1, 7046-7022)			

The nucleotide position of FcaPV type-specific primers are referred to the reference nucleotide positions of FcaPV2, FcaPV3, and FcaPV4 (GenBank accession numbers: EU796884, JX972168, KF147892).
Abbreviations: ACTB, beta-actin; bp, base pairs; F, forward; R, reverse; Tm, annealing temperature

Table 3-3 PCR and sequencing primers for full-genome characterization of FcaPV4, and RT-PCR primers applied in this study.

Experiment	Primer name	Sequence (5' to 3')	Location (nt)	Product size (bp)	Tm (°C)
Full-genome characterization	FcaPV4 7820 (F)	CGACTGCGCAAGCAACATTA	204-223	423	
	FcaPV4 8242 (R)	CTGTTGCGCCCTGATTTGAG	607-626		
	FcaPV4 580 (F)	TAAGACTGGTTGTCAGCGCC	580-599	420	
	FcaPV4 999 (R)	GTCCTGCACCTTGCTTTTCG	980-999		
	FcaPV4 900 (F)	GACGCTACTGAACCAGCAGA	900-919	462	
	FcaPV4 1361 (R)	GAGCTCTCCAACAGCTCCTC	1342-1361		
	FcaPV4 1334 (F)	GTGTCAGGGAGGAGCTGTTG	1334-1353	498	
	FcaPV4 1831 (R)	TGTGTGCACAGTCCTTGACAT	1811-1831		
	FcaPV4 1774 (F)	GCAGCTGCTTGGCTAAAATGT	1774-1794	489	
	FcaPV4 2262 (R)	GTGCTTGCAGTCCACAGACA	2243-2262		
	FcaPV4 2126 (F)	GCCATTTCTGGTTGATGCCC	2126-2145	476	
	FcaPV4 2601 (R)	TCCCAAAACGAGACGTGGTC	2582-2601		
	FcaPV4 2578 (F)	TACTGACCACGTCTCGTTTTGG	2578-2599	473	
	FcaPV4 3050 (R)	CTGTGGTAGCATATTGCAGAG	3030-3050		
	FcaPV4 3010 (F)	TGTTAAGTTTGCAGCTGATGCTC	3010-3032	484	
	FcaPV4 3493 (R)	TCCCAGAGGGATGTAAGGAGA	3473-3493		
	FcaPV4 3366 (F)	CCCCCGATCTCTCCTTCG	3366-3383	460	
	FcaPV4 3825 (R)	TCAAGACCTCCAGGAACACA	3806-3825		
	FcaPV4 3772 (F)	TTTAAGGACTGTTCGCCTTCCT	3772-3793	471	
	FcaPV4 4242 (R)	TCAGCAGGCGTTTTAGCCTC	4223-4242		56
	FcaPV4 4223 (F)	GAGGCTAAAACGCCTGCTGAT	4223-4243	471	
	FcaPV4 4693 (R)	GGGGTTATTGTATTGCTGCCG	4673-4693		
	FcaPV4 4608 (F)	CCGATGACACAGCCATCTTGG	4608-4628	491	
	FcaPV4 5098 (R)	CAGTGCCCCTATATCCTCAAACC	5076-5098		
	FcaPV4 4920 (F)	ATAATAGACGCCTCAGGCAGG	4920-4940	474	
	FcaPV4 5393 (R)	ACTGCACTCCAGAAAAGTCCTC	5372-5393		
	FcaPV4 5065 (F)	CCCTACTCCTGGGTTTGAGGATA	5065-5087	660	
	FcaPV4 5724 (R)	GTTTTGCAGGAGGCAGGAACA	5704-5724		
	FcaPV4 5600 (F)	ACCTTTGACCTTCATCCAGGC	5600-5620	378	
	FcaPV4 5977 (R)	CGCAGTTTCCACACAAGTCT	5959-5978		
	FcaPV4 6348 (F)	CAGAGTATATGCAAATGGCCAG	6348-6369	445	
	FcaPV4 6792 (R)	CAAATTCCTCAGTATGTCTGAGAT	6769-6792		
	FcaPV4 6597 (F)	TTCAACAGGCCTTTCTGGCTGCAG	6597-6620	518	
	FcaPV4 7114 (R)	CTTGATTGGCCCAAGCCTGTTTG	7092-7114		
	FcaPV4 6966 (F)	CCAGACAAGGTGCCTCCTAAA	6966-6986	499	
	FcaPV4 7464 (R)	AGCAGAGCCAGGGATTAAGTG	7444-7464		
	FcaPV4 7145 (F)	GACTTCAAGCGCAAGGGCTA	7145-7164	472	
	FcaPV4 7616 (R)	CTGCTGCGATTTAGTTAGTTTCAC	7593-7616		
	FcaPV4 7557 (F)	TCTAACCTGTACCACTTACGGT	7557-7578	340	
	FcaPV4 7896 (R)	AGTTTTCCAAACTTTCCCCAGT	259-280		
RT-PCR	FcaPV4 inside E6 (F)	TAAGATCGCTTTCGATTTCAAGG	108-130	158	56
	FcaPV4 inside E6 (R)	CCCCAGTAAATTTTAAGACCCCA	243-265		
	FcaPV4 inside E7 (F)	ATTTGGATGAGCTGGTTTTGCC	451-472	139	53
	FcaPV4 inside E7 (R)	ACCAGTCTTAGGGTACTGTGAC	568-589		

Nucleotide location refers to the reference FcaPV4 (GenBank accession number: KF147892).

Abbreviations: bp, base pairs; F, forward; nt, nucleotide; R, reverse; RT-PCR, reverse-transcriptase PCR; Tm, annealing temperature

Table 3-4 Nucleotide an amino acid sequence identities (%) of two FcaPV4 isolates (13-136 and 14-1110) identified in this study.

Case number	Sequence	Complete genome	E1	E2	E4	E6	E7	L1	L2	LCR
13-136	Nucleotide	96.67% (7367/7620)	97.87% (1788/1827)	98.05% (1309/1335)	97.26% (567/583)	96.14% (398/414)	97.06% (297/306)	95.70% (1470/1536)	95.01% (1465/1542)	97.67% (419/429)
	Amino acid	-	99.34% (604/608)	97.97% (435/444)	94.82% (183/193)	95.62% (131/137)	96.03% (97/101)	97.26% (497/511)	97.08% (498/513)	-
14-1110	Nucleotide	99.49% (7577/7616)	99.95% (1826/1827)	99.9% (1333/1335)	99.8% (581/582)	99.76% (413/414)	100% (306/306)	99.61% (1530/1536)	98.18% (1514/1542)	99.77% (428/429)
	Amino acid	-	99.84% (607/608)	100% (445/445)	99.48% (193/194)	100% (137/137)	100% (101/101)	99.41% (508/511)	98.83% (507/513)	-

The identities against the reference FcaPV4 (GenBank accession number: KF147892) were determined by EMBOSS Needle Program. The identical nucleotide numbers are noted in parentheses.
Abbreviations: LCR, long control region.

Table 3-5 Amino acid substitutions observed in cases 13-136 and 14-1110.

Nucleotide number (nt)	Nucleotide substitution			Amino acid substitution	
	Reference	13-136	14-1110	13-136	14-1110
E6 (1-414 nt)					
55	G	T	G	A19S	
58	C	T	C	R20C	
158	G	A	G	R53K	
162	G	A	G	R60K	
277	A	G	A	N93A	
319	G	C	G	V107L	
E7 (411-716 nt)					
436	A	G	A	K9R	
561	G	A	G	V51I	
670	A	G	A	Y87C	
684	A	C	A	N92H	
E1 (688-2514 nt)					
1381	T	T	C	Y232H	
1741	T	T	A	Y352N	
2317	T	T	G	L544V	
2396	G	G	A	R570H	
2460	G	C	G		N591K
E2 (2501-3835 nt)					
2634	A	T	A	Y45F	
3136	G	C	G	Q212H	
3158	C	-	C		
3159	A	-	A	Q220-	
3160	G	-	G		
3179	G	A	G	V227I	
3193	G	C	G	Q231H	
3260	G	A	G	V254S	
3261	T	G	T		
3359	G	T	G	A287S	
3575	T	C	T	S359P	
3764	G	C	G	E422Q	
E4 (3009-3590 nt)					
3136	G	C	G	S43T	
3158	C	-	C		
3159	A	-	A	S51-	
3160	G	-	G		
3193	G	C	G	S62T	
3261	T	G	T	S85V	
3274	C	G	C	A89G	
3337	C	T	C	P110L	
3361	G	G	A		R118H
3463	C	G	C	P152R	
3469	A	G	A	D154G	
3559	G	A	G	R184K	
3580	G	C	G	R191P	
L2 (4121-5662 nt)					
4143	T	C	T	I8T	
4221	T	A	T	F34Y	
4455	G	C	C	G112A	
4459	A	T	T	E113D	
4469	A	G	G	I117V	
4840	C	G	C	D240E	
4915	G	C	G	E265D	
5125	G	T	G	E335D	
5145	G	G	A		S342N
5186	A	G	A	T356A	
5229	C	G	C	T370S	
5241	C	G	C	T374S	
5388	T	C	T	V423A	
5519	A	G	A	S467A	
5534	G	A	A	G472S	
5544	G	A	A	G475D	
L1 (5652-7187 nt)					
5661	A	G	G	N36D	
6033	C	T	C	L128F	
6203	A	T	A	E184D	
6493	G	A	G	S281N	
6496	A	T	A	Y282F	
6504	T	C	T	S285P	
6635	K	G	G	?328Q	
6652	G	-	-		
6653	G	-	-	W335-	
6655	T	-	-		
6866	C	T	C	P395S	
6881	G	C	G	E410D	
6900	A	G	A	I417V	
7155	G	A	A	A502T	
7165	C	G	C	T505S	
7177	A	G	A	K509R	
LCR (7188-7616 nt)					
7395	C	T	C		
7427	T	-	T		
7453	C	T	C		
7479	C	T	C		
7482	G	A	G		
7488	G	A	G		
7505	G	-	G		
7524	A	G	A		
7549	T	C	T		
7610	G	C	C		

Each nucleotide and amino acid position is referred to the reference FcaPV4 (GenBank accession number: KF147892). The deleted sequence is indicated by hyphen.
Abbreviations: LCR, long control region; nt, nucleotide

Fig. 3-1

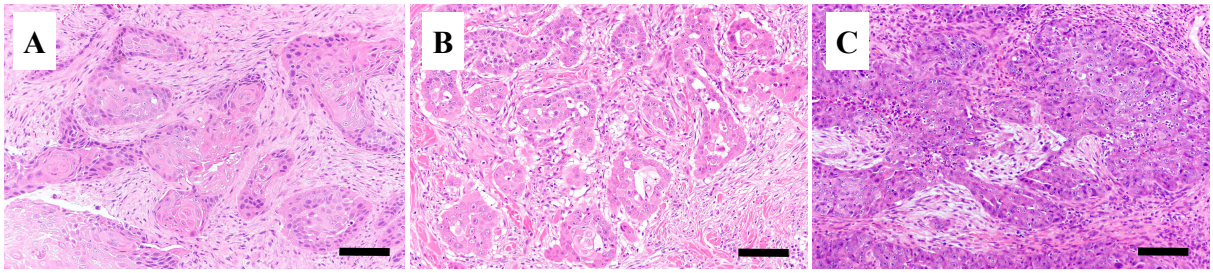


Fig. 3-1

Representative histopathological findings of feline squamous cell carcinoma.

Atypical squamous cells invade the fibrous tissue.

(A) Case number 14-1110, FcaPV4-positive.

(B) Case number 13-0153, FcaPV3-positive.

(C) Case number 14-0778, FcaPV-negative.

Hematoxylin and eosin staining, bars, 100 μm.

Fig. 3-2

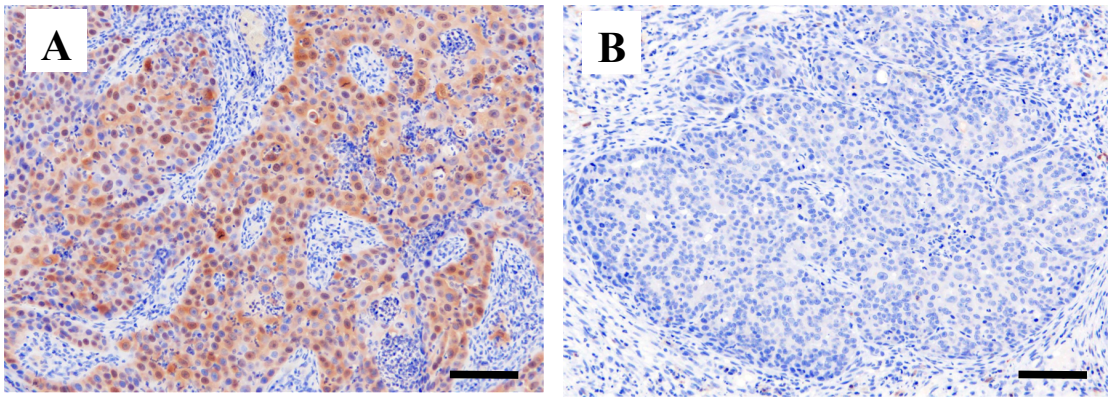


Fig. 3-2

Immunoshistochemical analysis against p16 protein in feline squamous cell carcinoma sections.

(A) Intense positive p16 staining in the nucleus of the basal cell membrane cells. (FcaPV4-positive, case number 14-1110).

(B) Negative for p16. (FcaPV-negative, case number 14-0778)

Bars, 100 μm.

Fig. 3-3

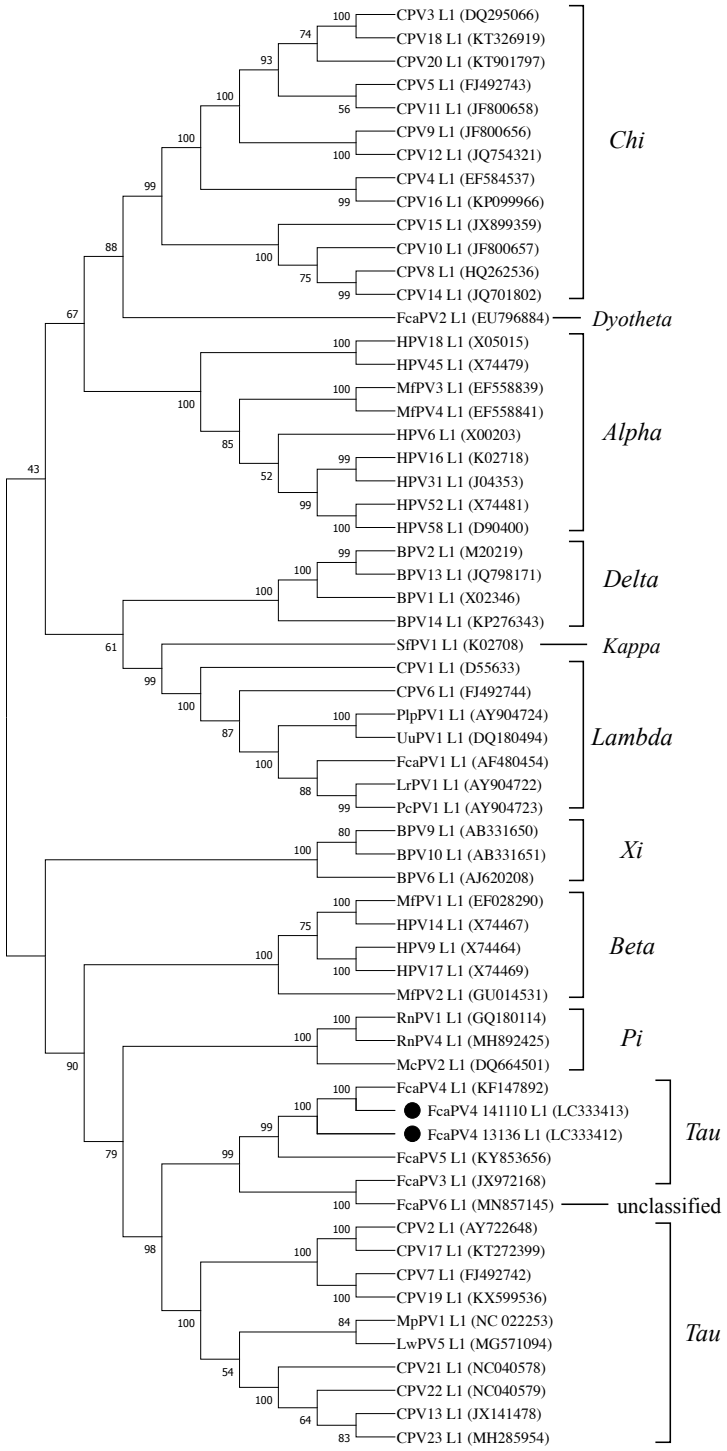
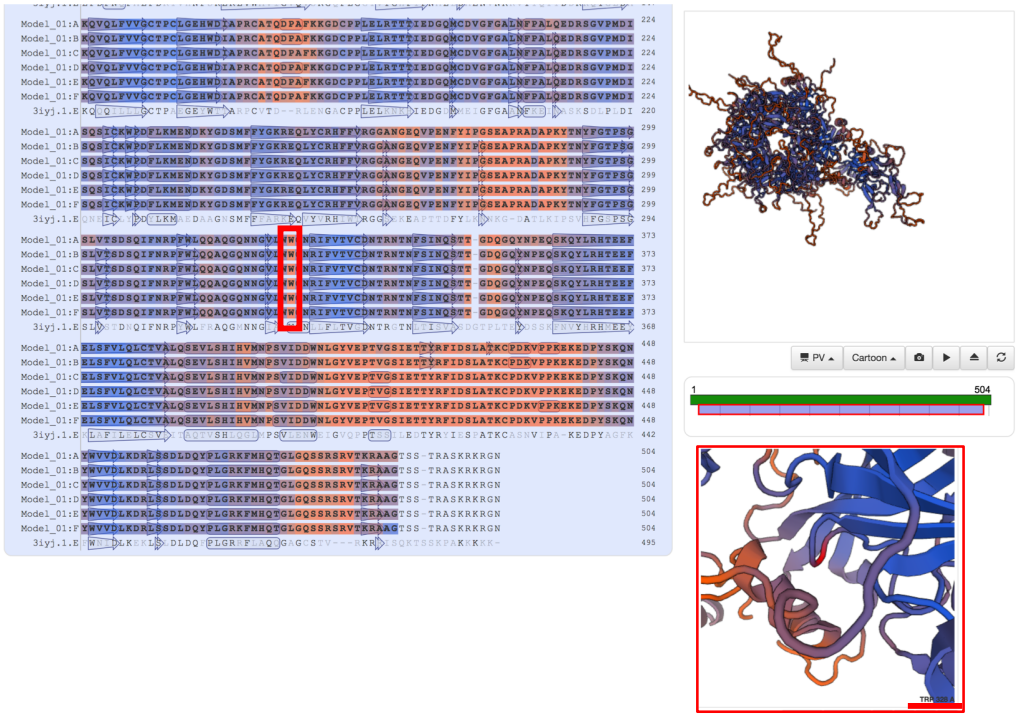


Fig. 3-3 Phylogenetic tree based on the L1 nucleotide sequence including two FcaPV4 isolates identified in this study and the reference PV genotypes.

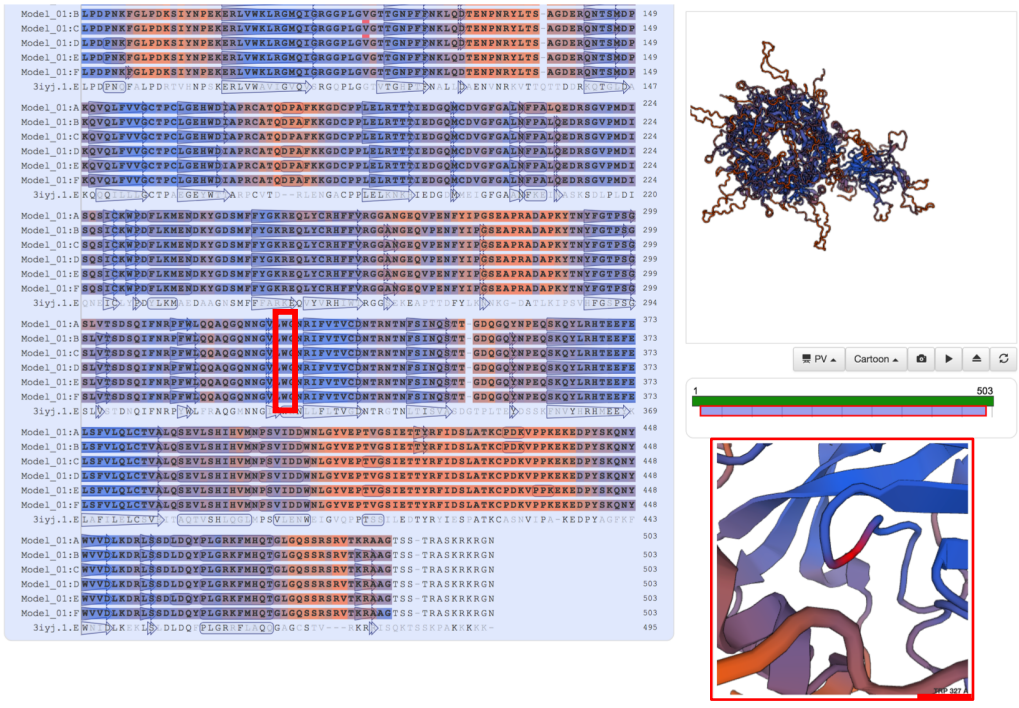
A total of 62 PV L1 nucleotide sequences were analyzed by Neighbor-Joining method with bootstrap replicates of 1000. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Each PV genus is noted by a bracket in italics. The respective GenBank accession numbers are shown in parentheses. Two FcaPV4 cases identified in this study are noted by closed circles. Abbreviations: BPV, *Bos taurus* PV; CPV, *Canis familiaris* PV; FcaPV; *Felis catus* PV; HPV, Human PV; LrPV, *Lynx rufus* PV; LwPV, *Leptonychotes weddellii* PV; McPV, *Mastomys coucha* PV; MfPV, *Macaca fascicularis* PV; MpPV, *Mustela putorius* PV; PcPV, *Puma concolor* PV; PlpPV, *Panthera leo persica* PV; RnPV, *Rattus norvegicus* PV; SfPV, *Sylvilagus floridanus* PV; UuPV, *Uncia uncia* PV.

Fig. 3-4

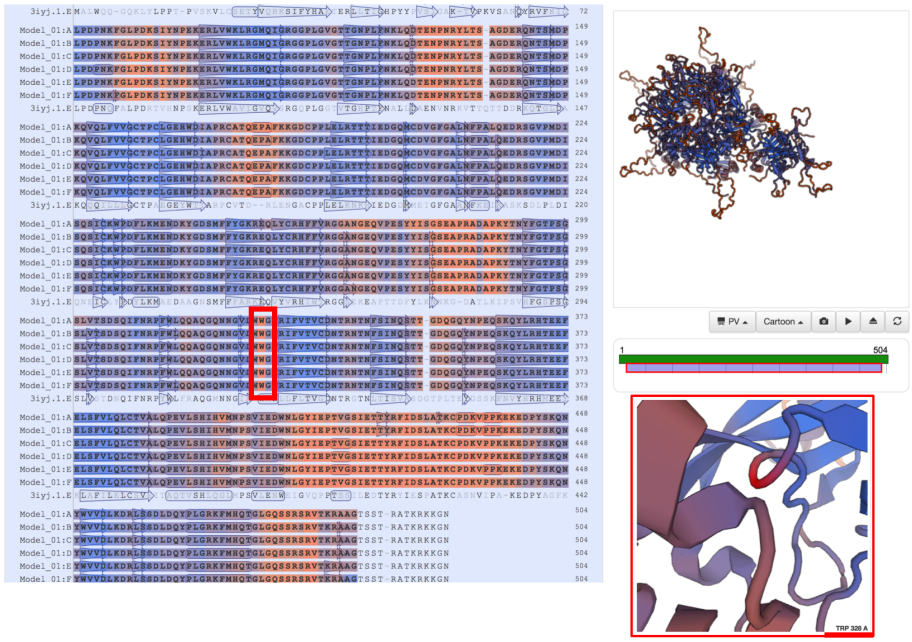
A



B



C



D

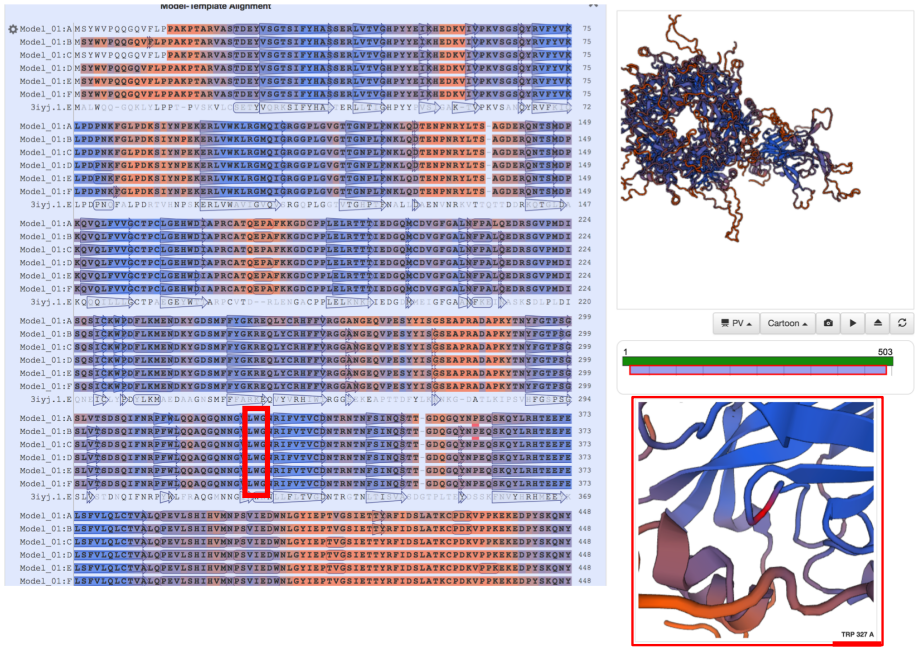


Fig. 3-4

FcaPV4 L1 major capsid protein structural analysis performed by SWISS-MODEL in cases 13-136 and 14-1110.

The L1 structure was modeled between the presence/deletion of 335th tryptophan (W).

No significant alterations of the L1 protein structure were observed in cases 13-136 and 14-1110.

(A) 13-136 L1 with 335th W. (B) 13-136 L1 without 335th W. (C) 14-1110 L1 with 335th W. (D) 14-1110 L1 without 335th W.

Fig. 3-5

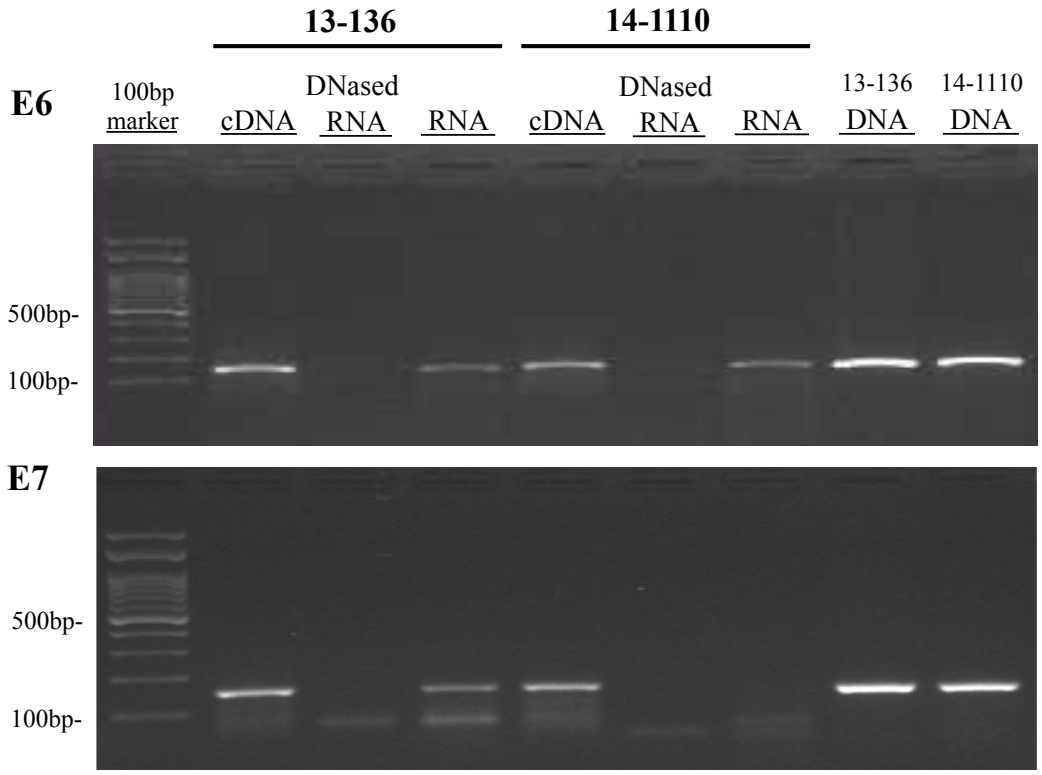


Fig. 3-5

FcaPV4 E6 and E7 mRNA expression by reverse-transcriptase PCR (RT-PCR).

Gel electrophoresis results confirmed the cDNA (mRNA) expression of FcaPV4 E6 (top) and E7 (bottom) by RT-PCR in case 13-136 and 14-1110.

A single band around the expected size of 158 bp (E6) and 139 bp (E7) were confirmed by RT-PCR in both cases.

Abbreviations: bp, base pair; DNased, DNase-treated.

General Conclusion

From the viewpoint of "One health", studies on infectious diseases harbor positive impact on both humans and animals. Over decades, animal PV studies have provided valuable knowledge of biological, and genomic features in the papillomaviral research field. Molecular epidemiological studies on pathogens provide fundamental resources for developing strategies on prevention and treatment against the infective agents. Especially for PVs, sequence characteristics become essential information as viral sequence phylogeny has been shown to associate with species/tissue/lesion-tropisms.

In Chapter 1, two classical BPV types, 1 and 2, were described to have potential to cause anogenital neoplasms in cattle. Moreover, detection of two novel types, BPV28 and BPV29, extends the knowledge on anogenital-associated BPV types. Findings on Chapter 1 provides valuable information on etiological and genomic features of anogenital-associated PVs in animals and contribute to developing preventive measures against BPV-associated anogenital lesion development.

Chapter 2 described that BPV1/2 sequence identities were associated with geographical trait and no sarcoid-associated variant was present, suggesting that BPV1/2 harbored by cattle have potential to infect nearby horses. Therefore, preventive measures against viral infection in horses needs to be considered especially for animals kept in close distance with cattle. Moreover, this study suggested that a prophylactic BPV1/2-vaccine could become applicable for both cattle and horses.

In Chapter 3, association of FcaPV types 3 and 4 in feline SCCs were described. Similar results to that of the BPV1/2 phylogeny-trait studies evaluated in Chapter 2, geographical discrepancies of FcaPV type-distribution in feline SCCs were noted. From the aspect of FcaPV-vaccine development for SCC prevention, in addition to FcaPV2, inoculating types 3 and 4 needs to be concerned.

Future contributions and significance

The present study described genomic and associated diseases of various PVs identified in domestic animals. These findings expand the knowledge on the molecular epidemiology of animal PVs and become meaningful to enrich sequence diversities and etiological roles of PVs. By strengthening the relevance between PV infection and disease development in the worldwide areas, the demand in significant prevention and treatment methods of PV-associated diseases would become accelerated in the veterinary field. Overall, molecular epidemiology of PVs related to domestic animals demonstrated by this study provides fundamental knowledge for further applied research, contributing to the establishment of PV-associated disease prevention and treatment strategies.

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