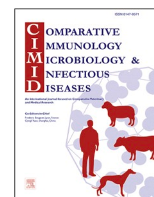
Comparative Immunology, Microbiology and Infectious Diseases 83 (2022) 101768

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid



Genome typing, histopathology, and evolution of BPV30, a novel *Xipapillomavirus* type isolated from Bovine papilloma in Costa Rica

Ramses Alfaro-Mora [a,1], Rosanna Zobba [b,c,1], Elisabetta Antuofermo [b,c], Giovanni Pietro Burrai [b,c], Roberto Solinas [b], Gaby Dolz [d], Marco Pittau [b,c], Alberto Alberti [b,c,*]

[a] *Universidad Latina de Costa Rica, Costa Rica*
[b] *Dipartimento di Medicina Veterinaria, Università degli studi di Sassari, Italy*
[c] *Mediterranean Center for Disease Control, University of Sassari, Italy*
[d] *School of Veterinary Medicine, Universidad Nacional de Costa Rica, Costa Rica*

A R T I C L E I N F O

Keywords:
Oncoviruses
Bovine papillomatosis
Viral infection
Cancer
Proliferative lesions
Central America

A B S T R A C T

Xipapillomavirus includes a group of viruses almost exclusively reported in both beef cattle and dairy breeding, in which they induce papillomatosis and occasionally malignant tumors. Bovine papillomaviruses (BPVs) infection impacts greatly on animal productions, and this is amplified by their cosmopolitan distribution. Cutaneous proliferative lesions in bovines can relate to leather depreciation and impaired milk production by giving rise to obstruction of the teat and hygiene limitations, often leading to hemorrhagic mastitis. This study reports the identification of a novel *Xipapillomavirus* type associated with udder papilloma in a Jersey cow in Costa Rica. Viral genome was fully sequenced and molecularly characterized. Histopathology and viral phylogeny and evolution are also presented and discussed by comparison with already described BPVs. Based on results, a novel *Xipapillomavirus* type, namely BPV30, is proposed. BPV30 is a typical *Xipapillomavirus* 2 most similar to BPV12, from which it separated roughly 18 million years ago. The absence of E6 and the presence of E10 in BPV30 confirm an E6 loss occurring along the clade leading to BPV12. The identification of this novel BPV is fundamental to the development of specific prophylactic tools, which represent the most effective weapon to fight viral circulation, to prevent infections, and eventually controlling associated proliferative lesions.

1. Introduction

Viruses included in the family *Papillomaviridae* are small, non-enveloped, icosahedral viruses with a circular, 7–8 kilobases (Kb) long, double-strand DNA genome [22,23]. Papillomaviruses (PVs) infect epithelia of vertebrates and are frequently reported in *Amniotes* [30]. However, the recent identification of PV in fish [21] could be indicative of a wider host-tropism, including amphibians, in which no PVs have been so far identified. PVs live as harmless viruses in the animal and human skin microbiota but can also cause proliferation of skin and mucosae and result in distinct clinical presentations, from self-limited papilloma/fibropapilloma to invasive cancer [33,35,36,39,8,34,40].

According to PAVE episteme (https://pave.niaid.nih.gov/) 448 viral types have been described in human, and this number overcomes the total number of viruses identified in animal hosts (N = 226). Consequently, it is feasible that the diversity of animal PVs is still largely unexplored [10].

Animal PV have been rescued from 93 host species. Most papillomaviruses have been rescued from mammalian hosts (209 PVs). On the other hand, only 9 PVs have been described in birds, 5 in reptiles and 3 in fishes. As a general rule, domestic species are usually more investigated than wildlife.

Currently, 29 bovine papillomaviruses (BPVs) have been reported and assigned to 4 genera except for 3 unclassified viruses (Table 1): viral genera show remarkably different richness in species, with *Xipapillomavirus* and *Dioxipapillomavirus* at the extremes in terms of number of species (respectively 15 and 1).

Also, BPV genera seems to be associated with different pathological manifestations (Table 1) with *Deltapapillomavirus* and *Xipapillomavirus* containing oncogenic species, and all BPV types inducing bovine papillomatosis [7, 25, 6, 3].

BPVs predominantly infect dairy cattle even if infection is

* Corresponding author at: Dipartimento di Medicina Veterinaria, Università degli studi di Sassari, Italy.
E-mail address: alberti@uniss.it (A. Alberti).

[1] Contributed equally

https://doi.org/10.1016/j.cimid.2022.101768

Available online 13 February 2022

0147-9571/© 2022 Elsevier Ltd. All rights reserved.

Table 1
Taxonomical status and pathological features of the 29 bovine papillomaviruses identified so far worldwide.

Genus/Species	Types	Lesions	Genebank ID
Deltapapillomavirus 4	BPV1, BPV2, BPV13 BPV14	Papillomas and fibropapillomas of skin, udders, and teats; urinary bladder cancer. Equine sarcoid	X02346, M20219, JQ798171, KP276343
Dyokappapapillomavirus	BPV16, BPV18, BPV22	Papillomas	KU519391, KU519393, KY705374
Dioxipapillomavirus	BPV7	Cutaneous papillomas	DQ217793
Epsilonpapillomavirus	BPV5, BPV8, BPV25	Cutaneous papillomas	MG252779, AF457465, DQ098913
Xipapillomavirus	BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV12, BPV15, BPV17, BPV20, BPV23, BPV24, BPV26, BPV28, BPV29	Papillomas, cancer in BPV4 positive animals eating bracken fern	KU519392, KU519395, KX098515, MG602223, MG281846, AF486184, X05817, AJ620208, AB331650, AB331651, AB543507, KM983393, JF834523, LC500686, LC514113
Unclassified	BPV19, BPV21, BPV27	Papillomas	KU519394, KU519396, MH512005

recurrently reported both in beef cattle and dairy breeding [2,3] and therefore BPVs infection impacts greatly on animal productions. Infected animals may show weight loss and impaired growth rate [3,24], breathing and feeding difficulties, and this may lead to the need for euthanasia [9]. Mastitis and consequent reduction in milk production may result from bacterial opportunistic infections [20,25,31,9]. The economic impact of infection is amplified by BPVs cosmopolitan distribution.

This study reports the identification of a novel BPV type associated with cutaneous udder papilloma in a Jersey cow in Costa Rica. Viral genome was fully sequenced and molecularly characterized. Histopathology and viral phylogeny and evolution are also presented and discussed by comparison with already described BPVs.

2. Material and methods

2.1. Case study and samples

A two-year-old Jersey cow exhibiting a cauliflower-like lesion on the udder was reported to the Veterinarian in charge of the dairy cattle herd in the county of San Carlos (Alajuela province, Costa Rica, see Supplementary material). A tissue sample representative of the lesion was collected and sent to the Universidad Nacional de Costa Rica, where it was divided into two portions of which one was fixed in 10% neutral buffered formalin and paraffin-embedded, while the second tissue portion was saved in ethanol and frozen at −20 C°. Sampling was performed by following the relevant guidelines and regulations according to Costa Rica laws.

2.2. Histopathology

Formalin-fixed tissue sample was dehydrated with increasing alcohol concentrations and paraffin-embedded. Section (3 μm) were stained with hematoxylin and eosin (H.E.) and slides were evaluated at light microscopy (Nikon Eclipse 80i). Tissues were imaged using Nikon Eclipse 80i and digital computer images were recorded with a Nikon Ds-fi1 camera.

Formalin-fixed tissue sample was dehydrated with increasing alcohol concentrations and paraffin-embedded. Section (3 μm) were obtained with a microtome (RM2245, Leica Biosystems), and stained with hematoxylin and eosin (H.E.) in an automatic multistainer (ST5020, Leica Biosystems). Slides were then evaluated at light microscopy (Nikon Eclipse 80i), as described previously [33]. Tissues were imaged using Nikon Eclipse 80i and digital computer images were recorded with a Nikon Ds-fi1 camera.

2.3. DNA extraction and FAP PCR

Total DNA was extracted from 25 mg tissue samples replicates using the DNeasy Blood and Tissue kit (Qiagen, Italy), following manufacturer recommendations.

To investigate the presence of PV DNA in samples FAP59/FAP64 PCR was performed, based on the use of degenerated FAP59/FAP64 primers [16] targeting about 480 bp of the L1 fragment conserved in diverse PV types. FAP59/FAP64 PCR was performed in 50 μl volume reactions each containing 150 ng DNA by concentrating primers and other reagents according to Qiagen recommendations for Taq polymerase. An initial denaturation step at 94 °C for 10 min, followed by 40 cycles of denaturation (1 min), annealing at 50 °C (1 min), and extension at 72 °C (1 min), were included in the PCR profile. A final extension at 72 °C for 2 min was used.

FAP59/FAP64 PCR products were run in a 1.5% agarose gel and detected with GelRed® Nucleic Acid Gel Stain (BIOTUM, Italy) by using the Bio-Rad GelDoc EZ imager (Bio-Rad, Italy). FAP59/FAP64 PCR products were purified in spin columns and sequenced on both strands by automated Sanger sequencing (BMR genomics, Italy). Local similarity between FAP59/FAP64 regions and sequences deposited in the GenBank was investigated by using The Basic Local Alignment Search Tool (BLAST).

2.4. Full genome amplification, cloning and genome sequencing

The TempliPhi 100 Amplification Kit (GE Healthcare, Italy) was used following protocols previously described [28], as modified by Alberti et al. [1] in order to perform Rolling Circle Amplification (RCA). Briefly, 10 μl sample buffer aliquots were mixed with 5 μl total DNA extractions, heated at 95 °C for 3 min, and transferred on ice. Ten microlitres of TempliPhi reaction buffer, 0.4 μl of TempliPhi enzyme mix containing phi 29 DNA polymerase, random hexamers in 50% glycerol, and 0.4 μl of 10 mM dNTPs were premixed and added to each cooled sample. Final reaction was then incubated at 30 °C for 16 h. Phi 29 polymerase was eventually inactivated (65 °C for 10 min). RCA products aliquots (5 μl) were digested with restriction enzymes *Eco*RI, *Bam*HI, and *Sac*I (New England Biolabs, Italy) in separated digestions, and run in 0.8% agarose gel to visualize the presence of a restriction profile consistent with the length of a papillomavirus genome, or of multiple bands with sizes adding up to a typical papillomavirus genome length (about 7–8 Kb). Upon *Sac*I digestions of the RCA products (see Supplementary material), obtained from DNA derived from the mammary cauliflower lesion, 5 bands adding up to about 7 Kb were cut from agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Italy), following vendor recommendations. Three bands, approximately 2.8, 2.2, and 1.2 in size, were successfully cloned into pRSET B and sequenced by primers walking (see Supplementary material for primers). Sequencing gaps among the 3 bands were filled by standard amplification and sequencing (see Supplementary material).

2.5. Sequence analysis and genome characterization

The L1 taxonomy tool in PAVE episteme (https://pave.niaid.nih.gov/) was used to investigate similarity of the novel virus with all extant PV genome sequences. Also, nucleotide and amino acid similarities were investigated with MUSCLE [13].

Sequencing results were analyzed with MEGA version 7 (MEGA7) software [18]. Putative open reading frames (ORFs) were predicted with the ORF finder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). BPV30 full genome sequence was submitted to the animal PV reference group for PV name approval.

2.6. Phylogenetic analyses and time trees

The nucleotide sequences of BPV30 and 127 other PVs were imported in DAMBE version 4.2.7 [41] and aligned at the amino acid level with ClustalW. Then, nucleotide alignments were obtained by using the aligned amino acid sequences as an alignment guide. This approach was followed independently for the E1, E2, and L1 ORFs, and according to Gottschling and cowokers [17], a compiled alignment was obtained by joining the 3 orfs together.

PVs evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible (GTR) model [27]. GTR was identified as the best-suited evolutionary model for our data among 24 different nucleotide substitution models by using the: "find best DNA/protein Models" option of MEGA7 [18], and by selecting the model with the lowest BIC score (Bayesian Information Criterion), considered to describe the substitution pattern the best. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.2187)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 4.29% sites). All positions containing gaps and missing data were eliminated. There was a total of 3217 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [18].

Bootstrapping [14] with 1000 iterations was used to evaluate statistical support of internal branches of the tree. MEGA7 was also used to scale to time phylogenetic trees, and branch lengths were calibrated according to UuPV1 and PlpPV1 divergence time, calculated considering an overall evolutionary rate of the feline PV viral coding genomes of 1.95×10^{-8} (95% confidence interval 1.32×10^{-8} to 2.47×10^{-8}) nucleotide substitutions per site per year [29] and assuming a constant evolutionary rate for all PVs. Branch lengths calibrations were based on relative times obtained with MEGA.

2.7. Nucleotide sequence accession numbers

The nucleotide sequence of the complete BPV30 genome has been deposited in the GenBank sequence database under accession number OL672227.

3. Results

Histologically the lesion was characterized by multiple elongate papillary projections of thickened squamous epithelium supported by fine fibrovascular core, progressing from a hypertrophied stratum basale to a thickened stratum spinosum and granulosum and covered by orthokeratotic hyperkeratosis (Fig. 1A). Neoplastic polygonal cells, with distinct cell borders, had a moderate eosinophilic cytoplasm and round, centrally located, nuclei with finely stippled chromatin and with one, central, basophilic, ovoid nucleolus. Multifocally, the stratum granulosum showed increased irregular keratohyalin granules and swelling scattered squamous cells with abundant clear cytoplasm and pyknotic nucleus surrounded by a clear halo (koilocytes, Fig. 1B). The morphological diagnosis of the lesion was skin viral papilloma.

DNA was extracted from the lesion and a single clear band was obtained by FAP59/FAP64 PCR (see Supplementary material). Upon amplicon sequencing and BLASTN analyses, FAP59/FAP64 sequence was most similar to sequence MT674603 (99%) and JF834523 (84%), respectively representing a L1 fragment detected in a case of bovine papillomatosis in Brazil, and the homologous BPV12 L1 region. *Sac* I RCA product digestion (see Supplementary material) resulted in 5 bands consistent with full-length PV DNA (7–8 kilobases). Bands were numbered from 1 to 5, in descending order by size (B1: 2.8 Kb, B2: 2.2 Kb, B3: 1.2 Kb, B4: 1 Kb, B5: 0.2 Kb).

B1, B2, and B3 were successfully cloned into pRSET-B and sequenced by primer walking. Gaps among bands were amplified and sequenced (see Supplementary material for primers). The full-length PV genome sequence was assembled and, according to criteria established for PV taxonomy [4] resulted in the detection of a novel, unclassified PV genome, tentatively designated BPV30 (Bovine Papillomavirus type 30). Based on L1 comparison, BPV30 was most similar to BPV12 (*Xipapillomavirus* 2), with 75.58% identity.

BPV30 has a covalently linked circular genome adding up to 7194 bp. The complete nucleotide sequence of BPV30 (GenBank accession number OL672227) has a GC content of 40% and includes the canonical non-coding region (NCR) and the classical major PV ORFs E1, E2, E7, E5, L2, and L1. An E6 open reading frame is missing, and the E10 (nt 116–259, 49 aa) and E4 (133 aa) ORFs are present (Fig. 2A), similarly to other shared characteristics within *Xipapillomavirus* members. E4 [12] is mostly contained within the E2 ORF (nt 3424–3812), with the primary E4 gene-product being translated from a E1^E4 spliced mRNA

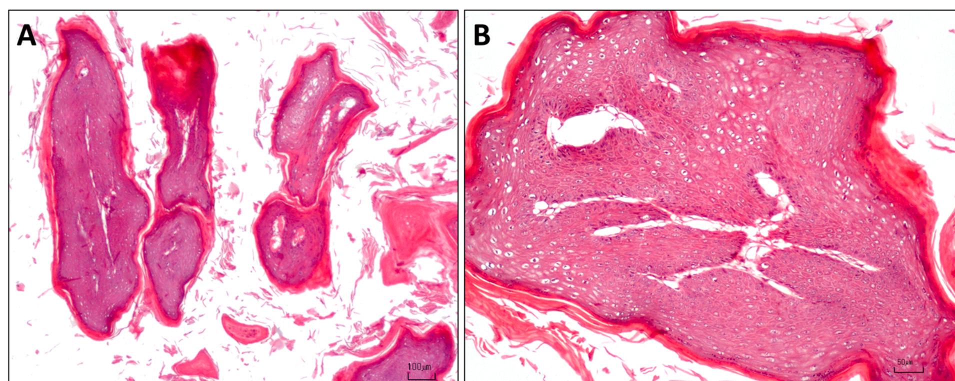


Fig. 1. Skin udder: bovine viral papilloma. (A) Exophytic multiple epidermal folds covered by orthokeratotic hyperkeratosis supported by fine fibrovascular stalks. H.E., Bar 100 μm. (B) Thickened stratum spinosum showing epithelial cells with abundant finely granular cytoplasm with eccentric vesiculate and scattered pyknotic nuclei surrounded by a clear halo (koilocyte) and increased keratohyalin granules in the stratum granulosum. H.E., Bar 50 μm.

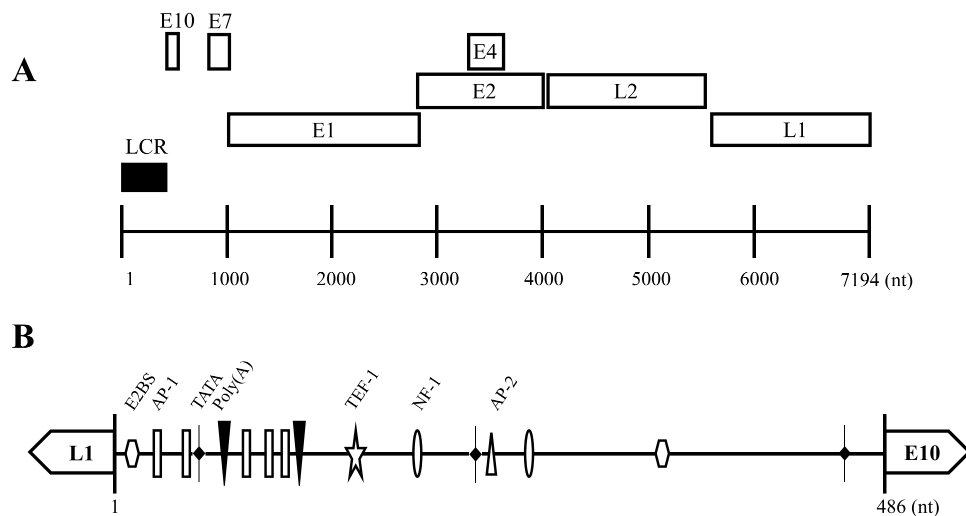


Fig. 2. : BPV30 genome organisation (A) and distribution of nt and aa features in LCR (B).

including the E1 initiation codon and adjacent sequences (ATGGCCTCTAAAG, nt 1087–1099).

Whole BPV30 genome and each ORF-coding regions were compared with the 29 reference BPV genotypes by CLUSTAL W analysis. ORF-coding regions (E4, E10, E1, E2 L2, L1, E7) were analyzed at both nucleotide and amino acid levels. For all genome regions and ORFs, BPV12 showed the highest score both at nucleotide and amino acid levels (Table 2). BPV30 LCR (485 bp) contains typical regulatory elements for virus replication and gene transcription and it is located between the L1 stop codon and the start codon of E10 (Fig. 2B). Two E2 binding sites (E2BS, ACCN6GGT) were identified in the BPV30 LCR at nt positions 7 and 327 (and an additional one at nt 7134), but no E1 binding site (E1BS) (ATTGTTN3AACAAT) was found. Three TATA box (TATAA) are located at nt 54, 261, and 438. BPV30 LCR contains 5 AP-1 binding motif (TKWNTMA) at nt positions 23, 52, 74, 94, and 100. Additionally, a TKSNTMA AP-1 binding motif consensus sequence (CTGAGTCAG) is located some 100 nt upstream the L1 ORF AP1 (TKWNTMA). One AP-2 binding motif (SCACMY) was identified at nt position 268. Two NF-1 motifs (TTGGC) were observed at nt 223 and 292, and a TEF1 binding site (YRCATDBYDB) at nt 141. Three polyadenylation signals (AATAAA) were predicted at nt position 62,102, and 6052.

Consistent with the *Xipapillomavirus* genome arrangement, a E10 ORF is located between the LCR and the E7 ORF, encoding a small protein (41 aa) with a highly hydrophobic aminoacid composition (75.5% hydrophobic residues). The BPV30 putative E7 protein was found to have one predicted zinc-binding domain (CXXC-X29 -CXXC) at aa positions 44–80 as well as a retinoblastoma protein binding site (LXCXE, aa positions 24–28). E1 ORF encodes the largest BPV30 protein (597 aa) containing a putative ATP-binding site (GPPNTGKS, aa 427–434) within the carboxy-terminal region of the ATP-dependent helicase. Additionally, four cyclin interaction motifs (RXL), essential to E1 association with cyclins and efficient phosphorylation, are predicted to be present at aa positions 68–70, 81–83, 95–97, 145–147, and 240–242.

E2 protein consists of a N-terminal transactivation domain (TAD) of about 200 residues (aa 1–197), a C-terminal DNA binding/dimerization domain of about 100 residues (aa 338–416), and a connecting “hinge” region of nearly 70 amino acids. BPV30 E2 protein lacks a leucine zipper domain (LX6LX6LX6L), that is present in some PVs E2.

BPV30 E4 ORF is predicted to encode a proline-rich (16 proline residues out of 133 amino acids) peptide containing a cyclin A interaction motif (RXL) in the C-terminal region.

The late region, composed of the two late genes encoding the major (L1) and minor (L2) capsid proteins contains a string of lysine and arginine residues, mainly at their carboxy termini both in the L1 and L2.

Phylogeny was successfully inferred based on a concatenated E1/E2/L1 nucleotide sequence alignment of BPV30 and 127 PV-types, representative of the different PV extant genera (Fig. 3). According to the most recent PVs classifications [19,32,4], maximum likelihood phylogenetic trees clustered the PVs in their respective genera. Main genera associations, consistent with what previously observed by Gottschling (2011) and later studies [10,19,33], were updated with novel PV species recently discovered after 2011. In particular, 6 main genera associations can be identified (Fig. 3): 1) Eta, Teta, Dyoepsylon, Dyozeta, and Psi genera clustering in a markedly distinct branch including turtles, birds, and bats PVs; 2) the *Artiodactyla*–infecting Delta, Epsilon and Xi viruses, grouping together with the PVs infecting *Equidae* (namely Zeta, Dyoiota, Dyorho, and the donkey Dyochi [19]) and with *Carnivora* PVs forming a separate but related branch including Chi, Dyonu, Dyoeta and Iota; 3) Alpha, Upsilon, Omicron, Dyodelta and Omega genera, including human mucosal, most giant panda viruses, and the dolphins and polar bear PVs; 5) a large group including human Beta and Gamma genera together with PVs isolated from various mammals (Tau, Pi, Dyoxi, Phi, Xi, and Dyoeta); 6) *Carnivora* PVs of the Lambda genus related to Dyophi, Kappa, Mu, and Sigma viruses.

In trees (Figs. 3, 4*)*, bovine PVs of the Xi genus are included in a statistically supported monophyletic branch, to which the goat ChPV1 is basal. BPV30, consistently with bovine PVs taxonomy, groups with other viruses of the genus *Xipapillomavirus*, and represents a new PV type

Table 2
BPV reference genotypes showing the highest CLUSTALX score with the whole-genome, LCR and individual BPV30 genes. Most similar virus and percentage scores are reported in parentheses both for nucleotides and proteins.

Sequence	Whole Genome	LCR	E10	E7	E1	E4	E2	L2	L1
Nt	BPV12 (72)	BPV12 (72)	BPV12 (67)	BPV12 (75)	BPV12 (78)	BPV12 (70)	BPV12 (71)	BPV12 (73)	BPV12 (78)
Aa			BPV12 (61)	BPV12 (74)	BPV12 (81)	BPV12 (55)	BPV12 (66)	BPV12 (77)	BPV12 (84)

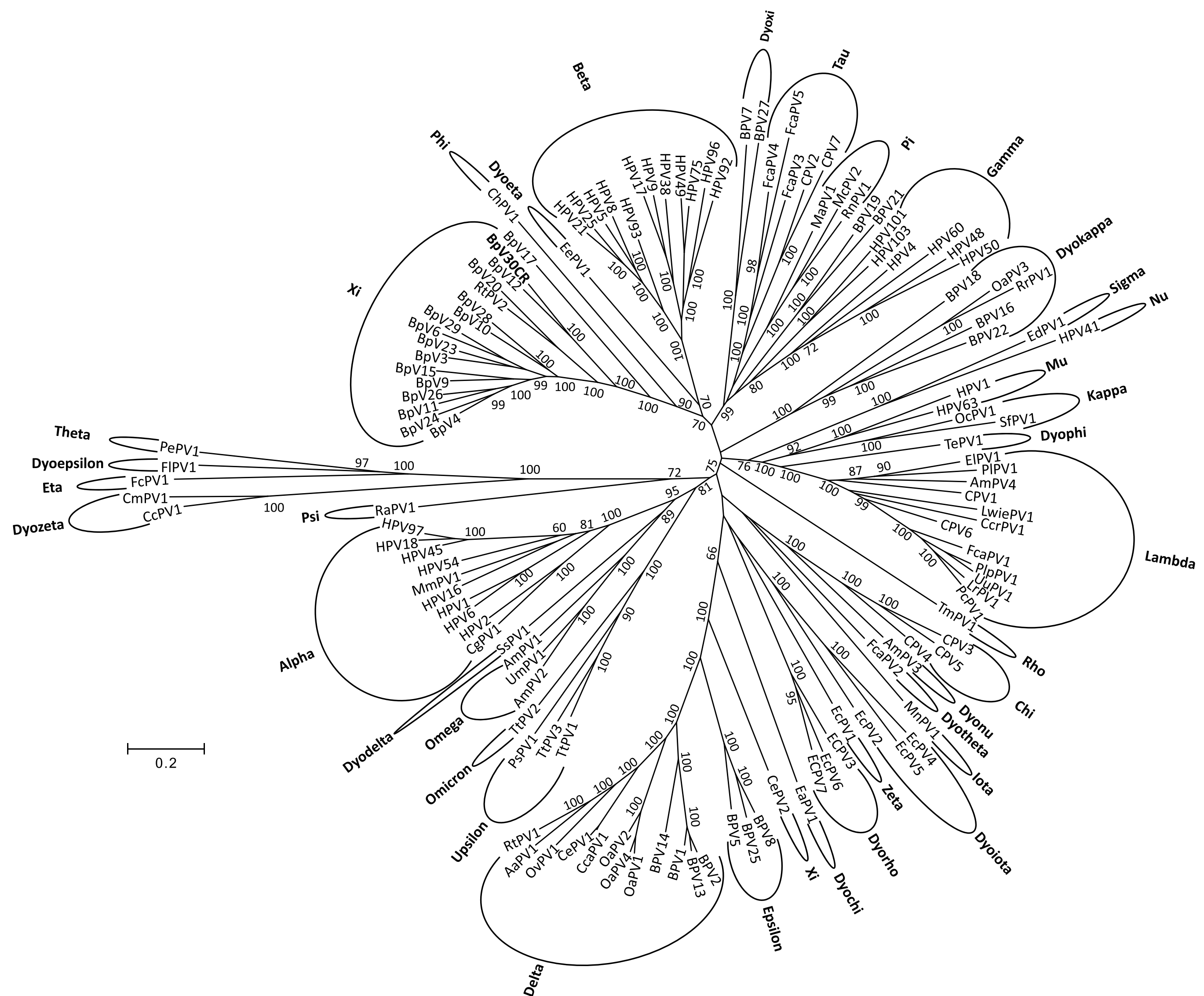


Fig. 3. : Molecular Phylogenetic analysis by Maximum Likelihood method. A Maximum likelihood tree including BPV30 and 127 PV-types, chosen in this study as representatives of different papillomavirus genera and species is shown. Phylogeny was inferred by an E1–E2–L1 nucleotide sequence compiled alignment. Genera are indicated by Greek letters, according to PAVE episteme website (http://pave.niaid.nih.gov). Numbers on branches indicate bootstrap values (only values above 70 are shown). Branch lengths are drawn to scale. The tree with the highest log likelihood (−307274.85) is shown.

within the *Xipapillomavirus* 2 species. Viral divergence times, calculated considering an overall evolutionary rate of the feline PV viral coding genomes of 1.95×10^{-8} (95% confidence interval 1.32×10^{-8} to 2.47×10^{-8}), indicated that BPV30 originated about 18.73 (5−31) Myr ago.

4. Discussion

Bovine papillomaviruses are widely studied among animal papillomaviruses both for their great veterinary significance and their tremendous value as an *in vivo* model for the study of human papillomavirus and cancer [7,9].

Infection by *Xipapillomavirus* is mostly related to the appearance of cutaneous proliferative lesions in bovines and can relate to leather depreciation and impaired milk production, by giving rise to obstruction of the teat and hygiene limitations often leading to hemorrhagic mastitis. Bovine papillomas or warts are exophytic and hyperplastic benign lesions of both cutaneous and mucosal epithelia; they may appear in young cattle and may persist and lead to transformation into malignant neoplasia, like in the case of BPV4 in the presence of persistent ingestion of bracken fern acting as environmental carcinogenic cofactors [7].

In this study, we reported a novel *Xipapillomavirus* (BPV30) within the species *Xipapillomavirus* 2 associated with cutaneous teat papilloma from a Jersey cow in Costa Rica. This is the first papillomavirus reported in cows for this Country. BPV30 genome sequence has been transferred to the PAVE episteme for official designation of this new type (cloned genome is available upon request). BPV30 has a typical *Xipapillomavirus* genome and shares viral features with BPV12. Indeed, BPV12 is the most similar among animal papillomavirus both at amino acid and nucleotide levels for both early and late regions. Also, BPV30 L1 shows 99% similarity with MT674603, a L1 ORF rescued in Northeast Brazil from a papillomatous lesion of a bovine [15], and this could indicate a larger BPV30 geographical distribution posing a potential threat to bovine productions in Central and South America. BPV30, similarly to BPV12, does not contain a canonical E6 ORF but encodes a hydrophobic protein in the same genomic position (E10; formerly known as E8). This confirms what observed by Van Doorslaer and McBride [37], who

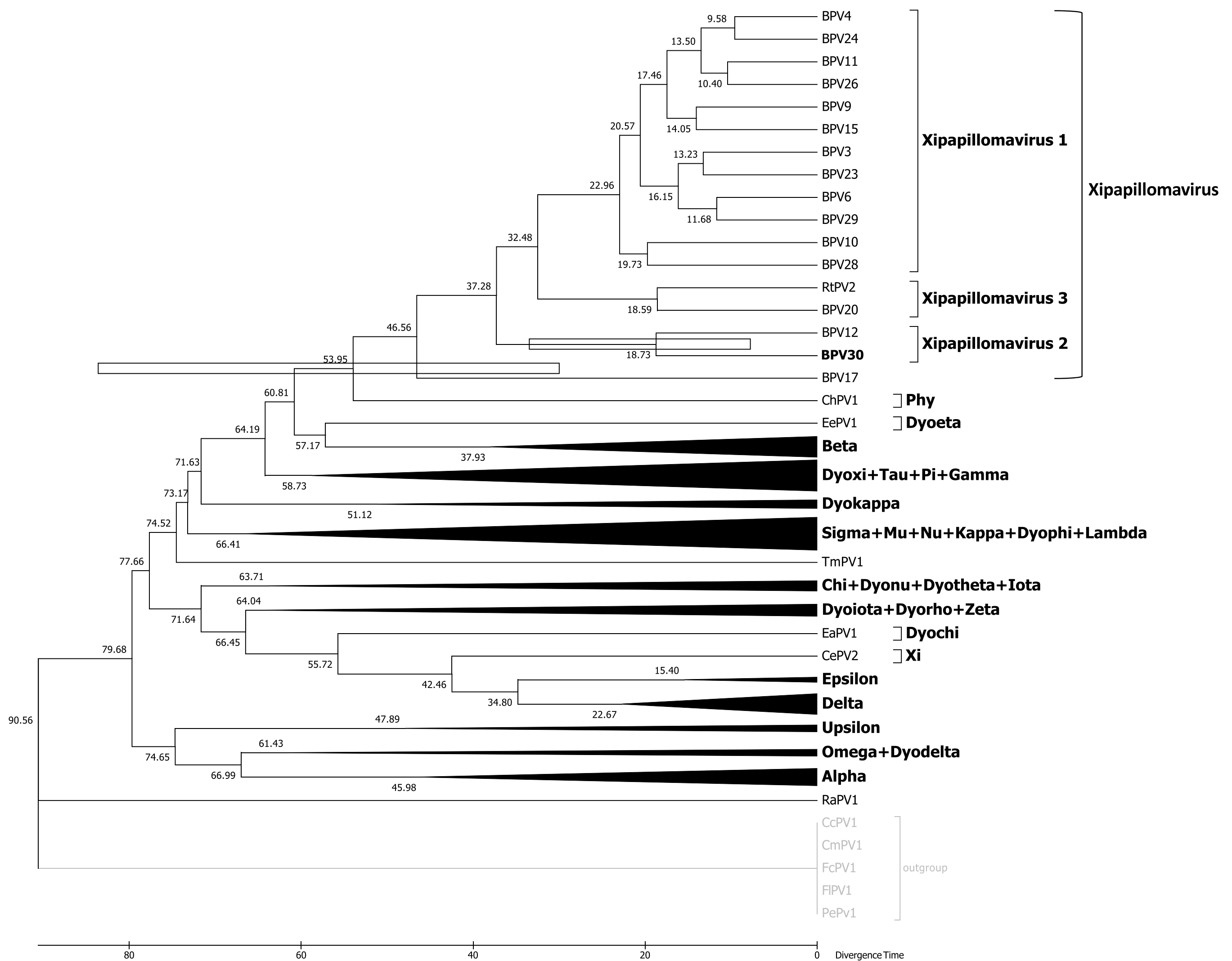


Fig. 4. Timetree analysis using the RelTime method. A timetree inferred using the RelTime method and the General Time Reversible model is shown. The timetree was computed using 1 calibration constraints. The estimated log likelihood value is − 307274.85. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.2187)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 4.29% sites). Divergence times confidence intervals (empty boxes) are only shown for selected nodes (BPV/BPV12; Xipapillomaviruses/ChPV1).

postulated repeated losses of E6 during *Xipapillomavirus* evolution. Indeed, the absence of E6 and the presence of E10 in BPV30 confirm a first loss occurring along the clade leading to BPV12. Papillomavirus E7 is an oncoprotein involved in cellular transformation by interfering with the retinoblastoma tumor suppressor protein pRb, a critical cell cycle regulator [11]. BPV30 E7 contains the conserved amino sequence motif LXCXE for binding pRb protein. Interestingly, this motif has been commonly observed in BPVs classified in *Xipapillomavirus* 1 and has been suggested to be of biological significance for epitheliotropic BPVs but not in fibropapilloma-associated BPVs [1,26].

Evolutionary analyses (Fig. 3) correctly assigned BPV30 to *Xipapillomavirus*, confirming a monophyletic origin of the PV species belonging to this genus.

In general, in timetrees divergence times for nodes considered together with their confidence intervals overlapped with previous observations [38]. However, our analyses were based on the assumption that evolutionary rates are constant for all papillomavirus genera and further investigations are needed to confirm data.

In timetrees (Fig. 4), BPV30 and BPV12 separated some 18 myr ago, in a time span roughly overlapping the origin of *Bos* genus [5]. *Xipapillomavirus* could have originated roughly 47 Myr ago (with the most recent estimate of 30 Myr) and this supports a distribution of this PV genus in all *Bovidae* host species (e.g belonging to genera *Capra*, *Ovis*, *Saiga*, *Gazella*) and in hosts less related to the family *Bovidae* such as host species belonging to *Antilocapridae*, *Giraffidae*, *Cervidae*, *Moschidae*. Notably, the recent discovery of *Cervus elaphus* Papillomavirus type 2 (CePV2, unpublished, Genbank ID KT932712), the first *Xipapillomavirus* in a *Cervidae* host, seems to confirm this hypothesis. Further investigation, and more research efforts are needed to establish *Xipapillomavirus* host range and distribution.

The identification of novel BPVs is fundamental to the development of specific prophylactic tools, which represent the most effective weapon to fight BPVs circulation, to prevent infections, and eventually controlling associated proliferative lesions.

Finally, unveiling novel PV species and hosts is paramount for the correct reconstruction of viral origin and evolution, which is still strongly influenced by the actual fragmentary knowledge of animal PVs diversity.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2022.101768.

References

[1] A. Alberti, S. Pirino, F. Pintore, M.F. Addis, B. Chessa, C. Cacciotto, T. Cubeddu, A. Anfossi, G. Benenati, E. Coradduzza, R. Lecis, E. Antuofermo, L. Carcangiu, M. Pittau, *Ovis aries* Papillomavirus 3: a prototype of a novel genus in the family *Papillomaviridae* associated with ovine squamous cell carcinoma, Virology 407 (2010) 352–359.

[2] R. Araldi, Bovine Papillomavirus: What We Know and What We Should Know, Lambert Academic Publishing, Germany, 2015, p. 124.

[3] R.P. Araldi, S.M.R. Assaf, R.F. Carvalho, M.A.C.R. Carvalho, J.M. Souza, R. F. Magnelli, D.G. Módolo, F.P. Roperto, R.C. Stocco, W. Beçak, Papillomaviruses: a systematic review, Genet. Mol. Biol. 40 (2017) 1–21.

[4] H.U. Bernard, R.D. Burk, Z. Chen, K. van Doorslaer, H. zur Hausen, E.M. de Villiers, Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments, Virology 401 (2010) 70–79.

[5] F. Bibi, A multi-calibrated mitochondrial phylogeny of extant *Bovidae* (*Artiodactyla*, *Ruminantia*) and the importance of the fossil record to systematics, BMC Evol. Biol. 13 (2013) 166, https://doi.org/10.1186/1471-2148-13-166.

[6] F. Bocaneti, G. Altamura, A. Corteggio, E. Velescu, F. Roperto, G. Borzacchiello, Bovine papillomavirus: new insights into an old disease, Transbound. Emerg. Dis. 63 (2014) 14–23.

[7] G. Borzacchiello, F. Roperto, Bovine papillomaviruses, papillomas and cancer in cattle, Vet. Res. 39 (2008) 1–10.

[8] M.S. Campo, Animal models of papillomavirus pathogenesis, Virus Res. 89 (2002) 249–261.

[9] M.S. Campo, Bovine papillomavirus: old system, new lessons? in: M.S. Campo (Ed.), Papillomavirus Research: from Natural History to Vaccine and Beyond Caister Academic Press, Wymondham, 2006, pp. 1–31.

[10] G. Dolz, R. Lecis, A. Solórzano-Morales, F. Aguilar-Vargas, T. Solórzano-Scott, R. Peña, R. Zobba, G. Tore, M. Pittau, A. Alberti, *Leopardus wiedii* Papillomavirus type 1, a novel papillomavirus species in the tree ocelot, suggests *Felidae Lambdapapillomavirus* polyphyletic origin and host-independent evolution, Infect. Genet. Evol. 81 (2020), 104239, https://doi.org/10.1016/j.meegid.2020.104239.

[11] J. Doorbar, W. Quint, L. Banks, I.G. Bravo, M. Stoler, T.R. Broker, M.A. Stanley, The biology and life-cycle of human papillomaviruses, Vaccine 30 (2012) 55–70.

[12] J. Doorbar, The E4 protein; structure, function and patterns of expression, Virology 445 (2013) 80–98.

[13] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (2004) 1792–1797.

[14] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.

[15] R.P. Figueirêdo, G.F. Santos, L. Oliveira B., L.A.B.O. Santos, D.M. Barreto, A. L. Cândido, A.C. Campos, E.O. Azevedo, M.V.A. Batista, High genotypic diversity, putative new types and intra-genotype variants of Bovine papillomavirus in Northeast Brazil, Pathogens 15 (2020) 748, https://doi.org/10.3390/pathogens9090748.

[16] O. Forslund, A. Antonsson, P. Nordin, B. Stenquist, B. Göran Hansson, A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin, J. Gen. Virol. 80 (1999) 2437–2443.

[17] M. Gottschling, M. Göker, A. Stamatakis, O.R. Bininda-Emonds, I. Nindl, I.G. Bravo, Quantifying the phylodynamic forces driving papillomavirus evolution, Mol. Biol. Evol. 28 (2011) 2101–2113.

[18] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol 33 (2016) 1870–1874.

[19] R. Lecis, G. Tore, A. Scagliarini, E. Antuofermo, C. Dedola, C. Cacciotto, G.M. Dore, E. Coradduzza, L. Gallina, M. Battilani, A.G. Anfossi, M. Muzzeddu, B. Chessa, M. Pittau, A. Alberti, *Equus asinus* papillomavirus (EaPV1) provides new insights into equine papillomavirus diversity, Vet. Microbiol. 170 (2014) 213–223.

[20] C.L. Lindsey, M.E. Almeida, C.F. Vicari, C. Carvalho, A. Yaguiu, A.C. Freitas, W. Beçak, R.C. Stocco, Bovine papillomavirus DNA in milk, blood, urine, semen, and spermatozoa of bovine papillomavirus-infected animals, Genet. Mol. Res. 8 (2009) 310–318.

[21] A. López-Bueno, C. Mavian, A.M. Labella, D. Castro, J.J. Borrego, A. Alcami, A. Alejo, Concurrence of Iridovirus, polyomavirus, and a unique member of a new group of fish papillomaviruses in lymphocystis disease-affected gilthead sea bream, J. Virology 90 (2016) 8768–8779.

[22] A.A. McBride, Mechanisms and strategies of papillomavirus replication, Biol. Chem 398 (2017) 919–927.

[23] A.A. McBride, Human papillomaviruses: diversity, infection and host interactions, Nat. Rev. Microbiol (2021), https://doi.org/10.1038/s41579-021-00617-5.

[24] J.S. Munday, Bovine and human papillomaviruses: a comparative review, Vet. Pathol. 51 (2014) 1063–1075.

[25] L. Muro, C. Botteira, A. Piccinin, Papilomatose bovina, Rev. Cient. Eletr. Med. Vet. 10 (2008) 1–7.

[26] A. Narechania, M. Terai, C.Z. Chen, R. DeSalle, R.D. Burk, Lack of the canonical pRB-binding domain in the E7 ORF of artiodactyl papillomaviruses is associated with the development of fibropapillomas, J. Gen. Virol. 85 (2004) 1243–1250.

[27] M. Nei, S. Kumar, Molecular Evolution and Phylogenetics, Oxford University Press, New York, 2000.

[28] A. Rector, R. Tachezy, M. Van Ranst, A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification, J. Virol. 78 (2004) 4993–4998.

[29] A. Rector, P. Lemey, R. Tachezy, S. Mostmans, S.J. Ghim, K. Van Doorslaer, M. Roelke, M. Bush, R.J. Montali, J. Joslin, R.D. Burk, A.B. Jenson, J.P. Sundberg, B. Shapiro, M. Van Ranst, Ancient papillomavirus-host co-speciation in Felidae, Genome Biol. 8 (2007) R57, https://doi.org/10.1186/gb-2007-8-4-r57.

[30] A. Rector, M. Van Ranst, Animal papillomaviruses, Virology 445 (2013) 213–223.

[31] E. Santos, M. Silva, N. Pontes, L. Coutinho, S. Paiva, R. Castro, A. Freitas, Detection of different bovine papillomavirus types and co-infection in bloodstream of cattle, Transbound. Emerg. Dis. 2014 (63) (2014) e103–e108.

[32] A. Scagliarini, L. Gallina, M. Battilani, F. Turrini, F. Savini, A. Lavazza, M. Chiari, E. Coradduzza, A. Peli, K. Erdélyi, A. Alberti, *Cervus elaphus* papillomavirus (CePV1): new insights on viral evolution in deer, Vet. Microbiol. 165 (2013) 252–259.

[33] G. Tore, C. Cacciotto, A.G. Anfossi, G.M. Dore, E. Antuofermo, A. Scagliarini, G. P. Burrai, S. Pau, M.T. Zedda, G. Masala, M. Pittau, A. Alberti, Host cell tropism, genome characterization, and evolutionary features of OaPV4, a novel *Deltapapillomavirus* identified in sheep fibropapilloma, Vet. Microbiol. 204 (2017) 151–158.

[34] G. Tore, G.M. Dore, C. Cacciotto, R. Accardi, A.G. Anfossi, L. Bogliolo, M. Pittau, S. Pirino, T. Cubeddu, M. Tommasino, A. Alberti, Transforming properties of ovine papillomaviruses E6 and E7 oncogenes, Vet. Microbiol. 230 (2019) 14–22.

[35] K. Van Doorslaer, Evolution of the papillomaviridae, Virology 445 (2013) 11–20.

[36] K. Van Doorslaer, R. DeSalle, M.H. Einstein, R.D. Burk, Degradation of human PDZ-proteins by human *alphapapillomaviruses* represents an evolutionary adaptation to a novel cellular niche, PLoS Pathog. 11 (2015), e1004980, https://doi.org/10.1371/journal.ppat.1004980.

[37] K. Van Doorslaer, A. McBride, Molecular archeological evidence in support of the repeated loss of a papillomavirus gene, Sci. Rep. (2016) 6, https://doi.org/10.1038/srep33028.

[38] K. Van Doorslaer, V. Ruoppolo, A. Schmidt, A. Lescroël, D. Jongsomjit, M. Elrod, S. Kraberger, D. Stainton, K.M. Dugger, G. Ballard, D.G. Ainley, A. Varsani, Unique genome organization of non-mammalian papillomaviruses provides insights into the evolution of viral early proteins, Virus Evolution 3 (Issue 2) (2017), https://doi.org/10.1093/ve/vex027.

[39] V. Vitiello, G.P. Burrai, M. Agus, A.G. Anfossi, A. Alberti, E. Antuofermo, S. Rocca, T. Cubeddu, S. Pirino, *Ovis aries* Papillomavirus 3 in ovine cutaneous squamous cell carcinoma, Vet. Pathol. 54 (2017) 775–782.

[40] V. Vitiello, G.P. Burrai, S. Pisanu, C. Cacciotto, M.F. Addis, A. Alberti, E. Antuofermo, T. Cubeddu, S. Pirino, Proteomic profiles and cytokeratin 13 as a potential biomarker of *Ovis aries* papillomavirus 3-positive and negative cutaneous squamous cell carcinomas, Res. Vet. Sci. 134 (2021) 112–119.

[41] X. Xia, Z.H. Xie, DAMBE: software package for data analysis in molecular biology and evolution, J. Hered. 92 (2001) 371–373.