Bos grunniens papillomavirus type 1 (BgPV-1): a novel *Deltapapillomavirus* associated with fibropapilloma in yak

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Running title: BgPV-1 associated with fibropapilloma in yak

Contents Category: Animal viruses – Small DNA

SUMMARY

Papillomaviruses (PVs) have been widely identified among vertebrates, but have not yet been reported to infect yaks. We report for the first time a novel *Deltapapillomavirus* which was associated with fibropapilloma in yak herds in the Qinghai-Tibetan Plateau. Six skin papilloma samples were collected and examined using histopathology, immunohistochemistry and PCR assays. The samples were identified as fibropapilloma and were found to contain PV-antigens. Sequencing of the diagnostic PCR products and the full-length genome revealed that all samples were infected with the same PV type. The whole viral genome was 7946 bp in length and possessed the common PV genomic organization. The virus was identified as a new PV type and designated *Bos grunniens* papillomavirus type 1 (BgPV-1) based on the nucleotide sequence alignment of the L1 ORF. It is classified in the Delta-4 species of the *Deltapapillomavirus* genus based on phylogenetic analysis of the L1 ORF. The identification of this new PV type provides further information about the pathology, development of diagnostic methods and evolutionary studies of the family *Papillomaviridae*.

INTRODUCTION

Papillomaviruses (PVs) are a heterogeneous group of non-enveloped DNA viruses associated with a wide spectrum of proliferative epithelial and fibroepithelial lesions, including benign skin and mucosal papillomas as well as cervical, oral and other epithelial cancers (Antonsson & Hansson, 2002; Parrish, 2011; zur Hausen, 2002). PVs are widespread in nature and have been detected not only in most mammals (de Villiers *et al.*, 2004), but also in amniotes such as birds, snakes and turtles (Drury *et al.*, 1998; Herbst *et al.*, 2009; Lange *et al.*, 2011). Over 100 different types have been identified in humans (Bernard *et al.*, 2010). PVs are classified in the *Papillomaviridae* family and so far 30 genera have been designated according to nucleotide sequence diversity in the L1 gene open reading frame (ORF) and to their biological and pathological properties (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). Since almost all PVs are highly specific to their natural hosts and cannot infect even closely related species (Bernard *et al.*, 2010; Chow *et al.*, 2010), they have often been designated according to their host species.

There are at least 12 PV types in the *Bos* genus, officially named *Bos taurus* papillomavirus 1 to -12 (BPV-1 to 12) and commonly called bovine papillomaviruses (Hatama *et al.*, 2011; Hatama *et al.*, 2008; Parrish, 2011; Zhu *et al.*, 2012). To date, all reported BPVs have been detected in *Bos taurus* with no reports in other *Bos* species. Fifteen novel putative BPV types (BAA2 to 4, BAPV3 to 5, BAPV7 to 10, BAPV11MY and BPV/BR-UEL2 to 5) have been detected by PCR of healthy skin swabs or cutaneous warts from cattle kept in Sweden, Japan and Brazil (Antonsson & Hansson, 2002; Claus *et al.*, 2008; Ogawa *et al.*, 2004).

The yak, *Bos grunniens,* is herbivorous and predominantly inhabits the Qinghai-Tibetan Plateau, colloquially known as “the roof of the world”. Yaks are regarded as one of the world’s most remarkable domestic animals as they thrive in extremely harsh deprived conditions while providing a livelihood for local people (Gerald *et al.*, 2003). Although PVs have been widely reported in

vertebrates, they have not yet been reported in yaks. We describe a novel *Deltapapillomavirus* which was associated with fibropapilloma in yaks. The virus was designated *Bos grunniens* papillomavirus type 1 (BgPV-1) according to the criteria of the Papillomavirus Study Group for the International Committee on Taxonomy of Viruses (ICTV).

RESULTS

PCR diagnostic assay

DNA was extracted from papilloma samples collected from infected domestic yaks. PV-DNA was detected by PCR using primer pairs FAP59/FAP64 (Forslund *et al.*, 1999) and MY09/MY11 (Manos *et al.*, 1989). All 6 samples were found to be positive with FAP59/FAP64 but negative with MY09/MY11. FAP59/FAP64 PCR products were cloned and sequenced and confirmed to be 431 bp. Sequence alignment of all 6 samples revealed 100% nucleotide sequence identity. Sequence similarity analysis with BLAST tool of the National Center for Biotechnology Information (NCBI) showed that the putative PVs subgenomic fragments were related to BPVs.

Histopathology and immunohistochemistry

Histopathology and immunohistochemistry were performed on six samples Qh-1 to 6 (one or two samples each farm) to identify the papilloma type and detect PV antigens. The morphological lesions of neoplastic tissues from six cases were almost identical and the neoplastic masses consisted of a mixed proliferation of epithelial and mesenchymal components (Fig. 2a and 2b). The epidermis showed moderate to severe irregular papillary proliferation of the prickle cell layer (Fig. 2b) accompanied by vacuolar changes and orthokeratotic hyperkeratosis. The proliferating epithelium did not invade into the dermis. In five cases, pale intranuclear inclusion bodies were occasionally observed in the prickle cells. PV-antigens were detected by immunohistochemistry and were present as intranuclear inclusions (Fig. 2d). In the mesenchymal tissues, there was diffuse irregular

proliferation of fibroblasts with moderate collagen production. There was little cellular atypia or polymorphism in the proliferating cells, including prickle cells and fibroblasts. Based on these histopathological characteristics and the presence of PV antigens, the neoplastic masses examined were diagnosed as fibropapillomas associated with papilloma virus infections.

Whole genome organization and sequence similarity

Full-length genome sequences were amplified and sequenced from samples Qh-1 to -6. Each of the samples contained a unique complete PV genome sequence which was confirmed by rolling-circle amplification (RCA) and long PCR. Full-length genomes of all six strains were 7946 bp with a G+C content of 44.4%. Sequence alignment revealed 99.9% to 100% nucleotide sequence identity among them, and there was no specific variation which caused significant difference in ORF, motifs, and so on. They possessed a typical genome organization consisting of the long control region (LCR), and early and late regions which is similar to other known PVs. Because the genome was circular, the first nucleotide of the E6 ORF was assigned position 1. One strain (Qh-3) was selected as the representative for genomic analysis in this study due to the extremely high homology between the six strains (99.9% to 100%). The LCR was 924 bp, located at nt 7023-7946 (Fig. S1), and has only one non-coding-region. The early region contains ORFs of E6 (414 bp), E7 (381 bp), E1 (1821bp), E2 (1239 bp), E4 (342 bp) and E5 (135 bp) in that order. The late region consists of two ORFs of L2 (1404 bp) and L1 (1494 bp) which encode the minor and major capsid proteins, respectively. These cooperate with each other to package the viral DNA into the virion.

Whole genome sequence alignments revealed that the closest related PVs were BPV-1 (82.3 %) and BPV-2 (82.1 %). When each ORF was compared with other PVs the nucleotide identities were E6 (≤83.8 %), E7 (≤87.9 %), E1 (≤88.0 %), E2 (≤91.7 %), E4 (≤90.1 %), E5 (≤91.1 %), L2 (≤73.6 %) and L1 (≤80.8 %). In 1995, the definition of new PV types was released from the International Papillomavirus Workshop held in Quebec. A PV strain can be recognized as a new type

112 if the complete genome has been cloned and the DNA sequence of the L1 ORF shares less than 90%
113 homology with the closest known PV type (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). Based on
114 this criterion, the PV strain identified in this study should be designated as a new PV type. Because it
115 was isolated from *Bos grunniens*, it was designated as BgPV-1.

116 The L1 ORF is the most conserved region in PVs and, according to the current genus
117 classification system, most types within a PV genus share more than 60 % nucleotide identity in this
118 region (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). Sequence alignments revealed that the L1 ORF
119 of BgPV-1 shares 62.0 % to 81.3 % nucleotide identity with other members of *Deltapapillomavirus*,
120 and 62.4 % to 64.0 % with *Epsilonpapillomavirus* (Table 1). Therefore, the genus for BgPV-1 cannot
121 be defined based solely on the nucleotide identity of the L1 ORF, but phylogenic tree was
122 constructed with optimized alignments based on the nucleotide sequence of the L1 ORF.

123 **Phylogenetic analysis of BgPV-1**

124 The L1 ORF has been used to identify new PV types and new genera have been defined by
125 phylogenetic analysis over the last 20 years (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). In the
126 present study, we identified a new PV type, BgPV-1, based on the L1 ORF sharing less than 90%
127 identity with other PVs. Moreover, the phylogenetic relationship of BgPV-1 was established based
128 on multiple alignments of L1 ORF with representative types of each PV genera and the virus was
129 clearly classified into the *Deltapapillomavirus* genus (Fig. 3). There are six *Deltapapillomavirus*
130 species according to previous reports, consisting of Delta-1 (AaPV-1 and RtPV-1), Delta-2 (OvPV1),
131 Delta-3 (OaPV1 and OaPV2,), Delta-4 (BPV-1 and BPV-2), Delta-5 (CcaPV-1) and Delta-6 (CdPV-1
132 and CdPV-2) (Bernard *et al.*, 2010; Ure *et al.*, 2011). BgPV-1 was grouped into the Delta-4 branch
133 which also contains BPV-1 and BPV-2 (Fig. 3).

134

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DISCUSSION

The yak predominantly inhabits the Qinghai-Tibetan Plateau, providing a livelihood for the local people. Almost everything from the yak is either used directly or sold to provide an income to sustain the lives of the herdsmen and their families. The yak hide and pelt in particular have great importance in the local economy (Gerald *et al.*, 2003). The occurrence of papillomatosis destroys or reduces the quality of the hide and pelt causing economic losses. Although papillomatosis has been causing problems in yaks, there has so far not been any genomic information relating to yak PVs. Here, we report for the first time the characterization of a novel *Deltapapillomavirus* BgPV-1 which was associated with fibropapilloma in yaks.

In the present study, a total of 6 skin papillomas were detected with the PCR assay and whole-genomes from all of them were sequenced. It was suggested that the infection was caused by the same PV strain based on the high nucleotide sequence identities. We presume that BgPV-1 is the major cause of skin fibropapillomatosis in yaks in Qinghai-Tibetan Plateau because the samples were collected from different areas more than 50 kilometers apart and the infected yaks were born and grew up in their own farms without interchange with other farms. In addition, almost all PVs are reported to be highly specific to their natural hosts and cannot infect even closely related species (Bernard *et al.*, 2010; Chow *et al.*, 2010).

To date, a total of ten PV types belonging to the *Deltapapillomavirus* have been identified, including AaPV-1, BPV-1, BPV-2, CcaPV-1, CdPV-1 CdPV-2, OaPV1, OaPV2, OvPV1 and RtPV-1. All of them were reported to be associated with fibropapilloma in animals including elk, cattle, deer, camel and sheep (Ahola *et al.*, 1986; Chen *et al.*, 1982; Erdelyi *et al.*, 2008; Groff & Lancaster, 1985; Moreno-Lopez *et al.*, 1987; Ure *et al.*, 2011). In this study, we report a novel *Deltapapillomavirus*, BgPV-1, which was also associated with fibropapilloma. This result provides further information for the characterization of *Deltapapillomavirus*.

BgPV-1 possesses a typical genome organization consisting of three regions known as the LCR, early and late regions which are similar to other known PVs. Some PVs, such as BPV-4, possess two non-coding-regions: the first one is located upstream of the early region and the second is between the L2 and L1 ORFs (Patel *et al.*, 1987). In the BgPV-1genome, LCR has only one region located between ORFs L1 and E6 (Fig. S1). The early region of PVs encodes non-structural viral proteins involved in viral DNA replication, transcription and cell transformation. Depending on the individual virus, the early region can encode up to eight proteins (E1-E8) (Howley & Lowy, 2007). BgPV-1 has ORFs for E6, E7, E1, E2, E4 and E5, but an E8 was not predicted which is similar to other delta-PVs. Most regulatory element motifs for virus replication and transcription were found in BgPV-1 when compared with BPVs and other PVs. The pRbBD in E7 was reported to work with ZnBD to be responsible for immortalization and transformation of host cells (Chan *et al.*, 2001; Liu *et al.*, 2006), and is thought to be a biological marker of epitheliotropic papillomavirus. PVs possessing pRbBD usually cause epithelial papillomas (Chan *et al.*, 2001; Dahiya *et al.*, 2000; Dick & Dyson, 2002), while PVs without pRbBD, including *Delta-* and *Epsilonpapillomavirus*, cause fibropapilloma (Erdelyi *et al.*, 2008; Narechania *et al.*, 2004; Tomita *et al.*, 2007). The absence of pRbBD in BgPV-1 suggests that it should be a fibrotropic papillomavirus, which is consistent with the results of histological and immunohistochemical examination. Therefore, the result of this study contributes one more piece of evidence that supports the above conclusion.

METHODS

Sample collection and diagnosis

A total of 6 skin papilloma samples were collected from 6 infected domestic yaks (Fig. 1) at three farms (two samples from each farm) which were more than 50 kilometers apart from each other in Qinghai-Tibetan Plateau of China. Whole papilloma biopsies from infected yaks were

184 excised surgically using a local anesthetic. Half of each sample was fixed with formalin for
185 histopathology and immunohistochemistry, and the other half was stored at -20 °C for DNA
186 extraction and genomic analysis. DNA was extracted with blood and tissue kit (Qiagen). Diagnostic
187 PCR assays were performed with primer pairs FAP59/FAP64 (Forslund *et al.*, 1999) and MY09/11
188 (Manos *et al.*, 1989). PCR products were cloned into the pMD20-T vector (Takara) and sequenced
189 using a Bigdye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an
190 ABI Prism 3130 genetic analyzer (Applied Biosystems). Sequences were aligned with Seqman
191 DNAStar software (Lasergene). Similarity analysis was performed using the BLAST tool
192 (http://blast.ncbi.nlm.nih.gov) of NCBI.

193 **Histopathology and immunohistochemistry**

194 Tissue samples were fixed with 10% formalin and embedded in paraffin using a routine
195 procedure. All sections were stained with hematoxylin and eosin (HE), and Masson's trichrome.
196 Immunohistochemistry for detection of PV antigens and identification of the epithelial and
197 mesenchymal components of the neoplastic tissues was performed by the Envision polymer method
198 (Dako-Japan) using standard reagents. Rabbit antiserum against BPV (Quartett, Berlin, Germany),
199 mouse monoclonal antibodies against cytokeratin (AE1/AE3, Dako-Japan) and vimentin (V9,
200 Dako-Japan) were used as primary antibodies and visualized using 3.3'-diaminobenzidine (DAB).
201 For BPV positive control, another papilloma sample from cattle was used. For negative control,
202 immunostaining was conducted without primary antigen. Mayer's hematoxylin was used as a
203 counterstain.

204 **Whole genome cloning and sequencing**

205 A rolling-circle amplification (RCA) protocol was used to enrich the circular PV DNA with
206 TempliPhi 100 Amplification kit (Amersham Biosciences) according to the manufacturer's
207 instructions. Primer walking sequencing was carried out using the RCA products as template.

208 Sequence information obtained from the 431 bp FAP59/FAP64 fragment was used to design a pair of
209 specific primers: BgPV1-1F (5'-CACAAACAACAGATGACAGGAA-3') and BgPV1-1R (5'-
210 CTGTCCCCGAGATACCTGAATA -3'). These were designed to amplify the remaining part of the
211 genome and PCR was performed with LA Taq kit (Takara) according to the manufacturer's protocol.
212 Subsequently, PCR products were gel purified using a QIAquick gel extraction kit (Qiagen). The
213 purified products were sequenced using the primer walking method. In order to confirm the correct
214 sequence, the whole genome was sequenced at least three times for each reaction using a Bigdye
215 Terminator v3.1 cycle sequencing kit (Applied Biosystems). The whole genome was cloned into the
216 pMD20-T vector (Takara) and sequenced. Sequencing was performed in an ABI Prism 3130 genetic
217 analyzer (Applied Biosystems). To reconstitute the full-length genome sequence, overlapping
218 sequences were assembled into contiguous sequences using Seqman DNAStar software (Lasergene).
219 **Genome organization and phylogenetic analysis**
220 For genome organization analysis, putative ORFs and their corresponding amino acids were
221 predicted using the ORF finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and similarity
222 analysis was performed using BLAST. Peptide motif analysis was performed with the TFSEARCH
223 tool of Parallel Protein Information Analysis system
224 (http://www.cbrc.jp//research/db/TFSEARCHJ.html). Multiple nucleotide sequence alignments and
225 phylogenetic tree construction were performed with MEGA version 5.05 (Tamura *et al.*, 2011).
226 Analysis for L1 ORFs were performed with the Maximum Likelihood method based nucleotide
227 sequences. The GenBank accession numbers of representative PV strains are as follows: AaPV1
228 (M15953), BPV-1 (X02346), BPV-2 (PPB2CG), BPV-3 (AJ620207), BPV-4 (X05817), BPV-5
229 (AJ620206), BPV-6 (AJ620208), BPV-7 (DQ217793), BPV-8 (DQ098913), BPV-9 (AB331650),
230 BPV-10 (AB331651), BPV-11 (AB543507), BPV-12 (JF834523), CcaPV1 (EF680235), CcPV-1
231 (EU493092), CdPV-1 (HQ912790), CdPV-2 (HQ912791), ChPV-1 (DQ091200), CPV-1 (D55633),

CPV-2 (AY722648), CPV-5 (FJ492743), EcPV-1 (AF498323), EcPV-2 (EU503122), EdPV-1 (AY684126), EePV1 (FJ379293), FcPV-1 (AY057109), FdPV-2 (EU796884), FlPV-1 (EU188799), HPV-1 (V01116), HPV-4 (X70827), HPV-5 (M17463), HPV-16 (K02718), HPV-41 (X56147), MaPV-1 (E15111), MnPV-1 (U01834), OaPV1 (U83594), OaPV2 (U83595), OvPV1 (M11910), PePV-1 (AF420235), PsPV-1 (AJ238373), RaPV-1 (DQ366842), RtPV-1 (AF443292), SfPV-1 (K02708), SsPV-1 (EF395818), TmPV-1 (AY609301), TtPV-1 (EU240894), UmPV-1 (EF536349).

Nucleotide sequence accession numbers

The nucleotide sequence of the BgPV-1 whole genome has been deposited in the GenBank sequence database under accession numbers: JX174437-JX174442

ACKNOWLEDGEMENTS

This study was supported by Grant-in-Aid for Scientific Research, Japan (KAKENHI 24580427), and by JSPS KAKENHI Grant Number 24·1466 from the Japan society for the Promotion of Science.

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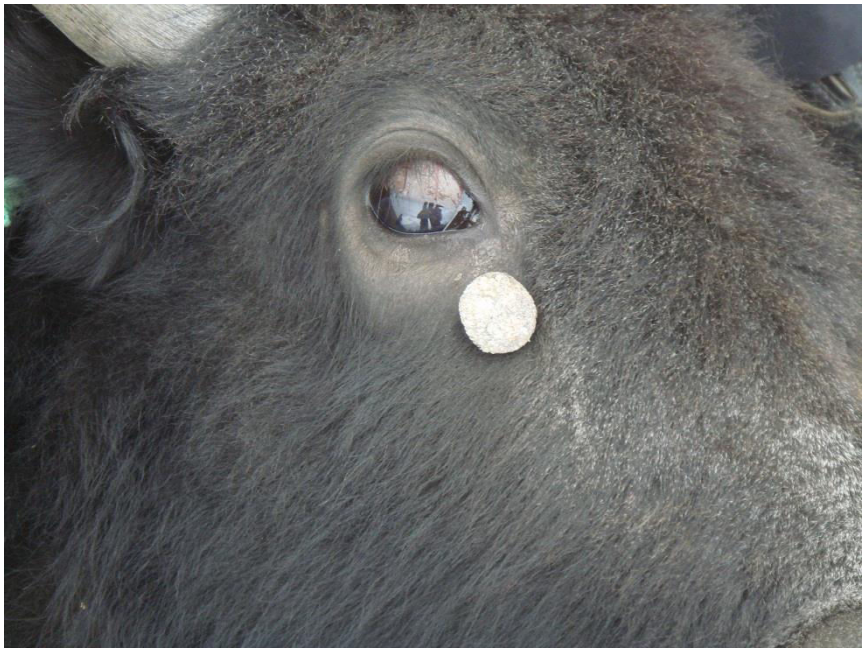
Fig. 1. Papillomatosis in domestic yaks infected with BgPV-1.

Fig. 2. Histopathological features and immunohistochemical staining of fibropapilloma excised from yaks. (a) HE stain, bar=2 mm. (b) Masson's trichrome, bar=2 mm. (c) cytokeratin-immunostaining, bar=2 mm. (d) PV-immunostaining, bar=100 μm. The neoplastic mass consists of a mixed proliferation of papillary epidermis (a and c), and fibrous connective tissues (b). In the prickle cell layer, there are intranuclear inclusion bodies (arrows), which are immunopositive for PV-antigen (d).

Fig. 3. Phylogenetic tree of BgPV-1 and PVs classified by genera *Alphapapillomavirus* to *Sigmapapillomavirus*. The tree was constructed with the Maximum Likelihood method in MEGA version 5.05 (Tamura *et al.*, 2011) based on the nucleotide sequence of L1 ORFs. The number on the scale bar shows the percent occurrence in 1000 bootstrap replicates. Abbreviations: AaPV (Alces alces PV), BPV (Bos taurus PV), CcaPV (Capreolus capreolus PV), CcPV (Caretta caretta PV), CdPV (Camelus dromedarius PV), ChPV (Capra hircus PV), CPV (Canis familiaris PV), EcPV (Equus caballus PV), EdPV (Erethizon dorsatum PV), EePV (Erinaceus europaeus PV), FcPV (Fringilla coelebs PV), FdPV (Felis domesticus PV), FlPV (Francolinus leucoscepus PV), HPV (Human PV), MaPV (Mesocricetus auratus PV), MnPV (Mastomys natalensis PV), OaPV (Ovis aries PV), OvPV (Odocoileus virginianus PV), PePV (Psittacus erithacus timneh PV), PsPV (Phocoena spinipinnis PV), RaPV (Rousettus aegyptiacus PV), RtPV (Rangifer tarandus PV), SfPV (Sylvilagus floridanus PV), SsPV (Sus scrofa PV), TmPV (Trichechus manatus latirostris PV), TtPV (Tursiops truncatus PV), UmPV (Ursus maritimus PV).

Fig. S1. Genomic map of BgPV-1. The circular genomes are represented in a linearized form. The ORFs are depicted as rectangles. Putative motifs in the LCR are indicated with different symbols and names.

Fig. 1



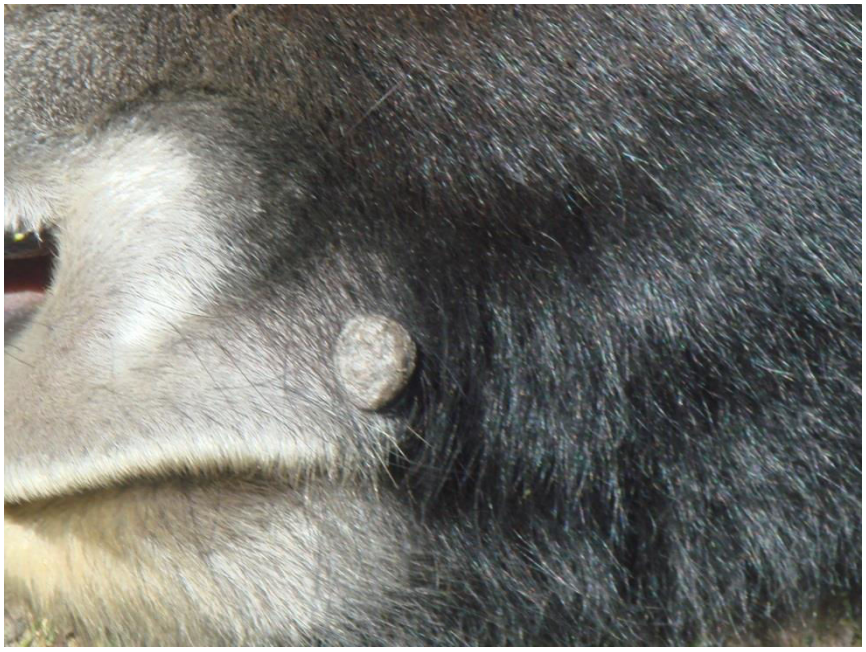


Fig. 2

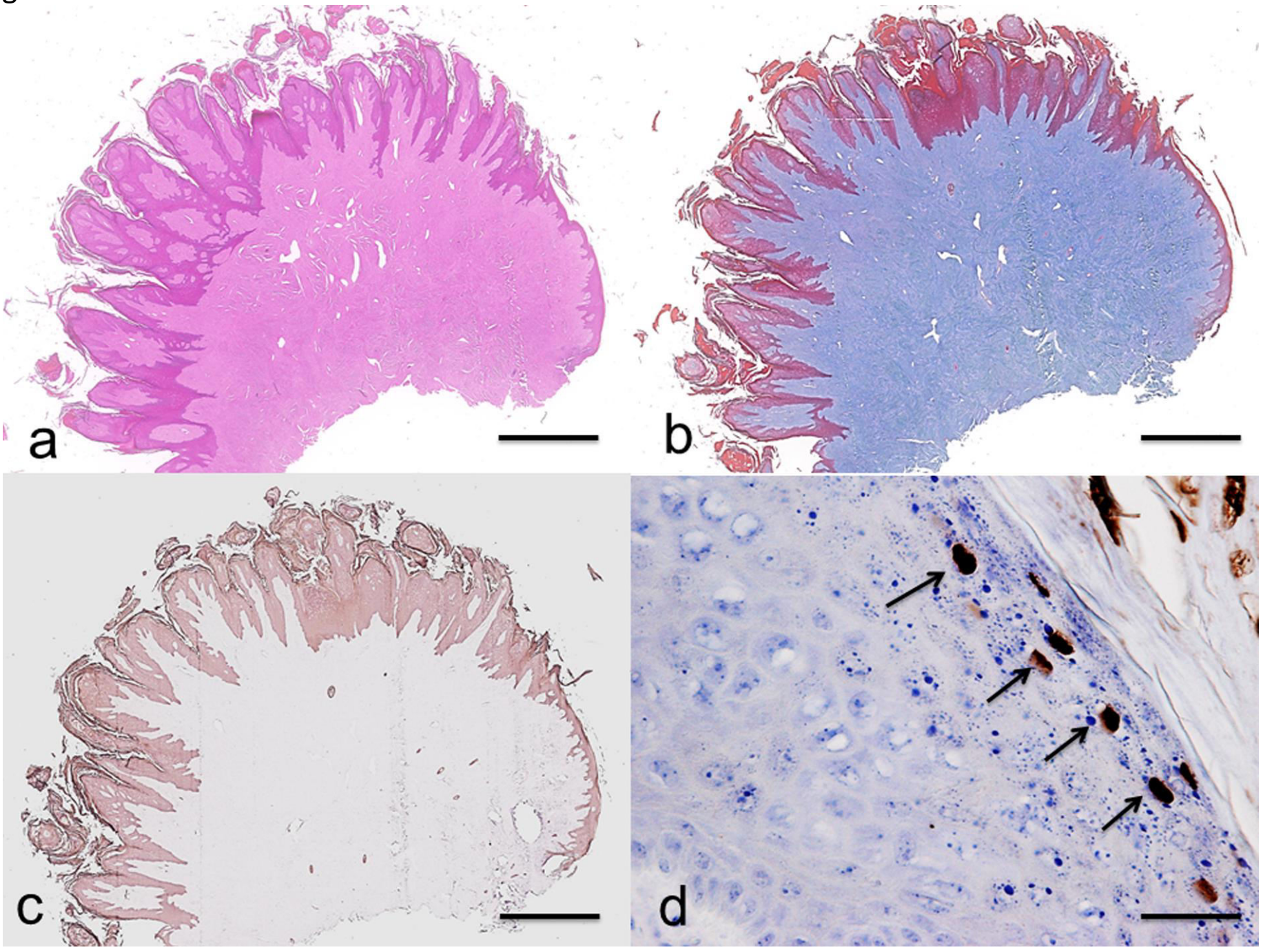
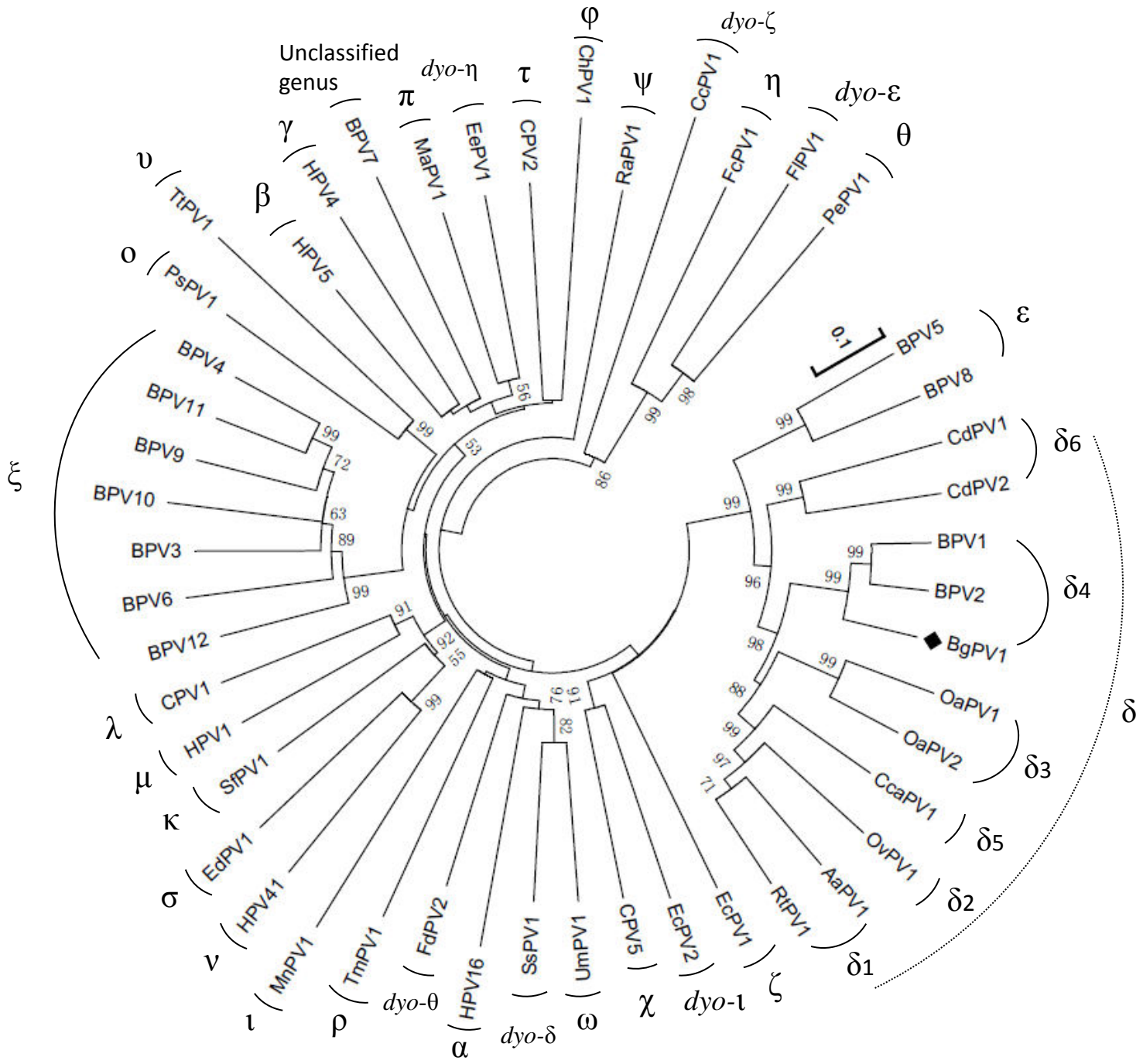


Fig. 3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ψ
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θ
ε
δ
λ
μ
κ
σ
ν
ι
ρ
α
ω
χ
ζ
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δ4
δ3
δ5
δ2
δ1
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PsPV1
HPV4
HPV5
BPV7
MaPV1
EePV1
CPV2
ChPV1
RaPV1
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FcPV1
FlPV1
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BPV4
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BPV9
BPV10
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BPV6
BPV12
CPV1
HPV1
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EdPV1
HPV41
MnPV1
TmPV1
FdPV2
HPV16
SsPV1
UmPV1
CPV5
EcPV2
EcPV1
RtPV1
AaPV1
OvPV1
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CdPV2
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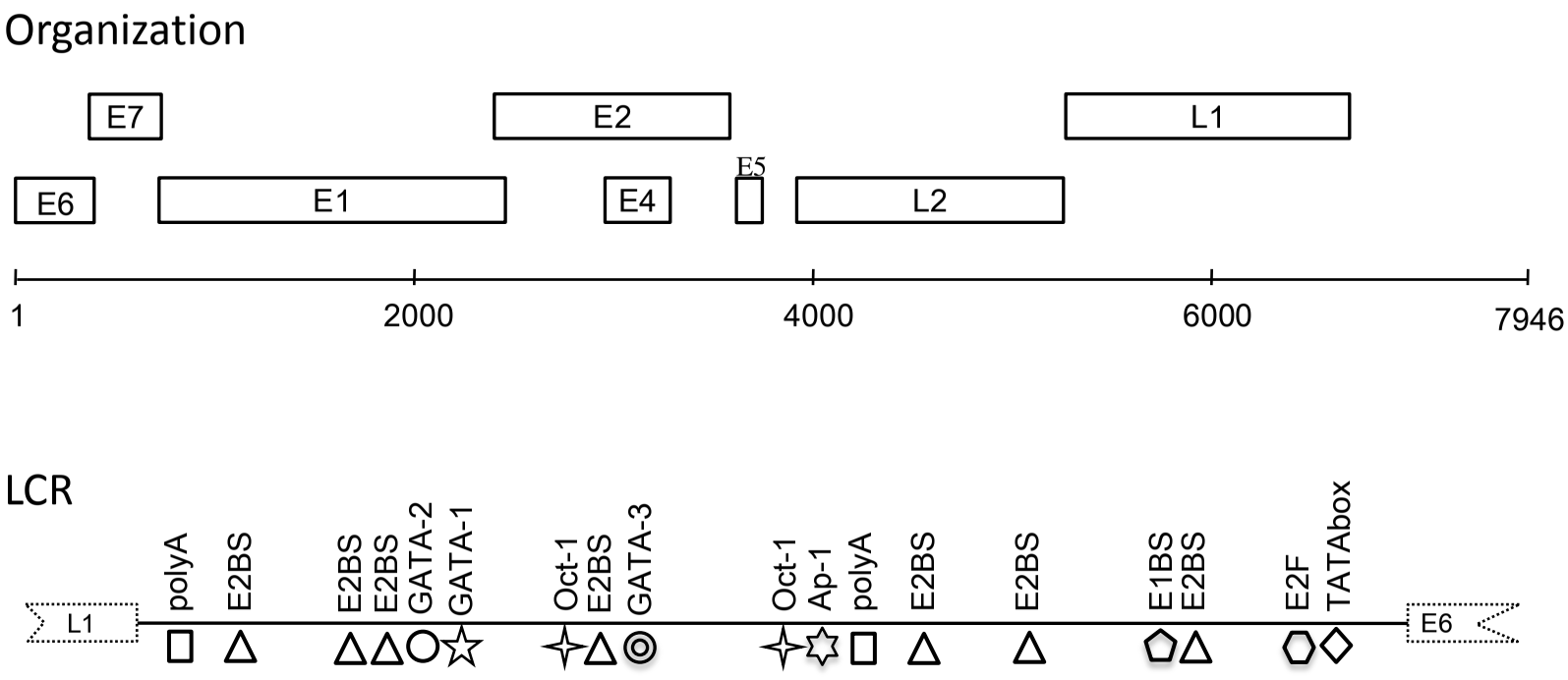
BPV5

Fig. S1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E2BS
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GATA-2
GATA-1
Oct-1
E2BS
GATA-3
Oct-1
Ap-1
polyA
E2BS
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E1BS
E2BS
E2F
TATAbox
E6