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Novel canine papillomavirus type 18 found in pigmented plaques

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A variety of neoplasias of the skin and mucous membranes in humans and animals can be ascribed to papillomavirus infections. Some of these papillomavirus induced neoplasias can undergo malignant transformation, while most remain benign. Papillomavirus infections are often even asymptomatic. Therefore the understanding of these viruses is important for appropriate prevention, diagnostic and therapeutic approaches. The entire genomic sequence of a new canine papillomavirus was determined from DNA detected in pigmented plaques in a pug. The novel canine papillomavirus type 18 (CPV18) falls into the genus *Chipapillomavirus* whose members have all been isolated form dogs with pigmented plaques, some of them also with squamous cell carcinomas. A small partial sequence of a 2009 isolate from a dog in California is identical with CPV18.

Papillomaviruses (PVs) are non-enveloped, double-stranded DNA viruses with a circular genome of about 8.000 base pairs (bp). They are generally host species-specific with some exceptions. Numerous of the known PVs are associated with benign and malignant neoplasias of the skin and mucous membranes in humans and animals, but there is evidence, that asymptomatic infections are more common [1,9,14]. More than 200 human and 140 animal PVs have been characterized, illustrating broad genetic diversity (http://pave.niaid.nih.gov/) [2,6,9]. The identification and study of new animal PVs are important because new PVs are potential pathogens in veterinary medicine, some animal PVs may serve as models for human disease and tools for basic biology, and the analysis of animal PVs helps provide insights into PV evolution. Canine PVs (CPVs) have been found associated with classical *exo*phytic papillomas such as the common canine oral papillomatosis, with *endo*phytic papillomas, with pigmented plaques and in rare cases with squamous cell carcinomas (Table 1) [13]. Pigmented plaques are most often seen in pugs where CPV4 seems to be the most prevalent type involved [17,18,22,23,26]. Overall seventeen complete genomes and several partial sequences of different CPVs have been communicated. The different CPVs belong to either of three different papillomavirus genera *Lambda*, *Tau* or *Chi* [2,5,11,15–23,25,26,28–31]. These three genera are not monophyletic and hence only distantly related. The aim of this study was the analysis and description of a new potentially pathogenic CPV.

A 3 year old, female spayed pug was presented to Tufts Cummings Veterinary Medical Center due to the development of multiple pigmented plaques on the medioventral abdomen and inner thigh (Fig. 1). The dog was the only pet in the household but had frequent contact with other dogs. After thorough physical examination two 8 mm punch biopsies for histopathological analysis and a cytobrush sample for microbiological assessment were taken from the lesions. The skin biopsies were fixed in 4% buffered formalin. After embedding in paraffin, 4 μm sections were stained with haematoxylin and eosin (HE) for histopatholgical examination. The cytobrush was moistened with sterile 0.9% NaCl solution, rubbed for thirty seconds on the affected area and stored in an 1.5 ml Eppendorf tube at −20 °C until DNA extraction.

Total DNA was isolated from the cytobrush sample using a Qiagen DNeasy Blood and Tissue kit. The extract was tested for the presence of host and papillomavirus DNA by PCR using the primer combinations dogGAPDHf/dogGAPDHr, canPVf/FAP64 and CP4/CP5 [10,12,14]. Circular DNA was amplified from the total DNA extract by rolling circle amplification (RCA) using a TempliPhi Amplification kit. The RCA product was digested and entirely cloned into the *Spe*I site of a pBluescript II KS+ vector [17]. The sequences of the pure and uncloned RCA product and the genomic clones were determined independently, and the primary sequences assembled in DNA Star (DNASTAR, Inc.). Comparison of

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Table 1
List of carnivore papillomaviruses and associated lesions.

Virus	Genus	Host	Associated lesions reported	Genebank ID
CPV1	Lambda	Dog	Oral papillomatosis, *endo*phytic papillomas, *exo*phytic papillomas, squamous cell carcinoma	D55633
CPV6	Lambda	Dog	*Endo*phytic papillomas	FJ492744
CcrPV1	Lambda	Spotted Hyena	Oral papillomatosis	HQ585856
FcaPV1	Lambda	Cat	*Exo*phytic papillomas	AY057109
ElPV1	Lambda	Sea Otter	Oral papillomatosis	KJ410351
LrPV1	Lambda	Bobcat	Oral papillomatosis	AY904722
PcPV1	Lambda	Puma	Oral papillomatosis	AY904723
PlPV1	Lambda	Raccoon	*Exo*phytic papillomas	AY763115
PlpPV1	Lambda	Asiatic Lion	Oral papillomatosis	AY904724
UuPV1	Lambda	Snow Leopard	Oral papillomatosis	DQ180494
CPV2	Tau	Dog	*Endo*phytic papillomas, *exo*phytic papillomas, squamous cell carcinoma	AY722648
CPV7	Tau	Dog	*Exo*phytic papillomas, squamous cell carcinoma	FJ492742
CPV13	Tau	Dog	Oral papillomatosis	JX141478
CPV17	Tau	Dog	Oral squamous cell carcinoma	KT272399
FcaPV3	Tau	Cat	Bowenoid in situ carcinoma	JX972168
FcaPV4	Tau	Cat	Gingivitis	KF147892
MpPV1	Tau	European Polecat	No lesion	KF006988
CPV3	Chi	Dog	Pigmented plaques, squamous cell carcinoma	DQ295066
CPV4	Chi	Dog	Pigmented plaques	EF584537
CPV5	Chi	Dog	Pigmented plaques	FJ492743
CPV8	Chi	Dog	Pigmented plaques	HQ262436
CPV9	Chi	Dog	Pigmented plaques	JF800656
CPV10	Chi	Dog	Pigmented plaques	JF800657
CPV11	Chi	Dog	Pigmented plaques	JF800658
CPV12	Chi	Dog	Pigmented plaques	JQ754321
CPV14	Chi	Dog	Pigmented plaques	JQ701802
CPV15	Chi	Dog	No details reported	JX899359
CPV16	Chi	Dog	Pigmented plaques, squamous cell carcinoma	KP099966
CPV18	Chi	Dog	Pigmented plaques	KT326919
UmPV1	Omega	Polar Bear	Oral papillomatosis	EF536349
FcaPV2	Dyotheta	Cat	Bowenoid in situ carcinoma	EU796884
VvPV1	Treiseta	Fox	No lesion	KF857586
ZcPV1	Dyonu	Sea lion	*Exo*phytic papillomas	HQ293213

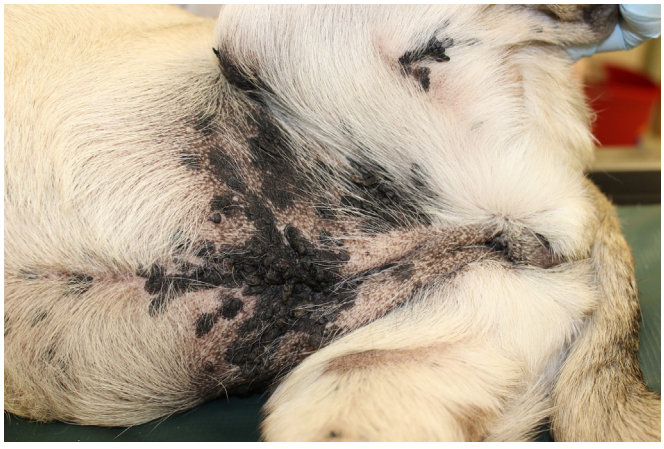

Fig. 1. Three year old female pug with numerous coalescing pigmented plaques and exophytic nodules (from 0.5 cm to 3 cm) on the medioventral abdomen and inner thighs.

RCA sequence and clone sequence ensured correctness and completeness especially around the *Spe*I cloning site. Pairwise sequence alignments were performed with the open reading frames

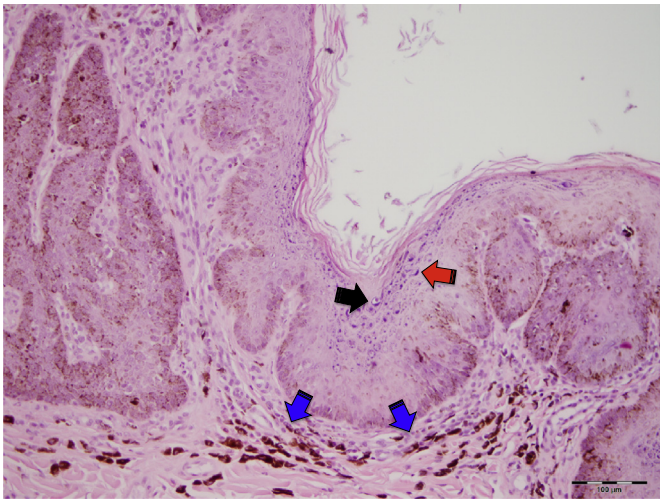

Fig. 2. Histologic section of one of the pigmented plaques. Epidermal hyperplasia, with melanosis, large keratohyalin granules (red arrow), as well as a few single scattered clear cells reminiscent of koilocytes (black arrow). The superficial dermis presented a mild inflammatory infiltrate and pigmentary incontinence (blue arrows).

(ORFs) of the obtained sequence using the Needleman-Wunsch algorithm. A phylogenetic tree for 80 PVs including the sequences of the CPVs as well as representatives of all thus far characterized PV genera was calculated (Supplement 1). For that purpose the predicted amino acid sequences of E1, E2, L2 and L1 were translated and aligned by using MAFFT before being back-translated to DNA sequences [17]. GBLOCK (version 0.91b) was used to remove regions of poor similarity and the resulting shortened sequences were combined into a single multiple sequence alignment by concatenating the sequences from each virus [3]. The optimal model of DNA evolution was evaluated for best fit of the dataset using MODELTEST (version 1.4.4); using the default settings [4,8]. Bayesian phylogeny of the E1-E2-L2-L1 concatenate was inferred using MRBAYES (version 3.2); Markov Chain Monte Carlo with GTR substitution matrix, variable gamma rates, invariant sites, two runs, four chains of 10 000 000 generations [26]. FcPV1 was defined as outgroup, to root the tree. Trees were sampled after every 1000 steps during the process to monitor phylogenetic convergence. The average standard deviation of split frequencies was below 0.0053 (MrBayes recommended final average < 0.01). This was performed on the Orchestra High Performance Compute Cluster at Harvard Medical School. The first 10% of the trees were discarded and the remaining ones combined using TreeAnnotator (version 1.8.2; http://beast.bio.ed.ac.uk) and displayed with FIGTREE (1.4.2; http://tree.bio.ed.ac.uk/) [24,25]. The code to run MrBayes as well as the files produced during this analysis are stored on GitHub: https://github.com/alosdiallo/Phylogeny_model_papillomavirus.

The histological examination revealed severe papillated epidermal hyperplasia, with melanosis, large clumped keratohyalin granules as well as a few single scattered clear cells reminiscent of koilocytes. The superficial dermis presented a mild inflammatory infiltrate and pigmentary incontinence (Fig. 2). The lesions were classified as canine viral pigmented plaques without signs of malignant transformation.

No differences between RCA product and clone were found upon comparison of the sequencing results. The circular sequence of the novel isolate consists of 7810 base pairs, which falls into the typical size range of *Chi*-PVs and has a GC content of 51% (Supplement 2) [11]. It contains the PV typical early (E1, E2, E4, E6, E7) and late (L1, L2) ORFs as well as non-coding region one between L1 and E6 (NCR1, 565 bp) and a second one between E2 and L2 (NCR2,

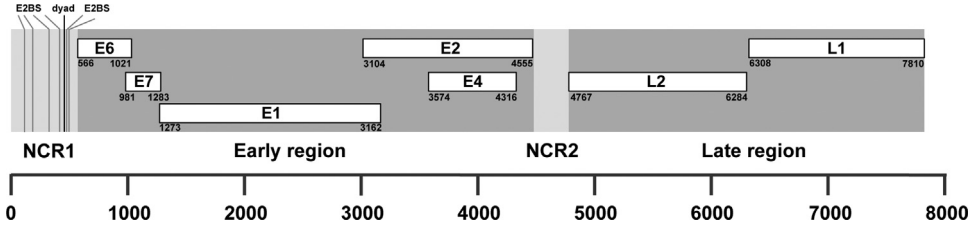

Fig. 3. Schematic representation of the linearized genome of CPV18 indicating the genome size and the organization of the open reading frames. Position 1 defined by the first base after the L1 stop codon.

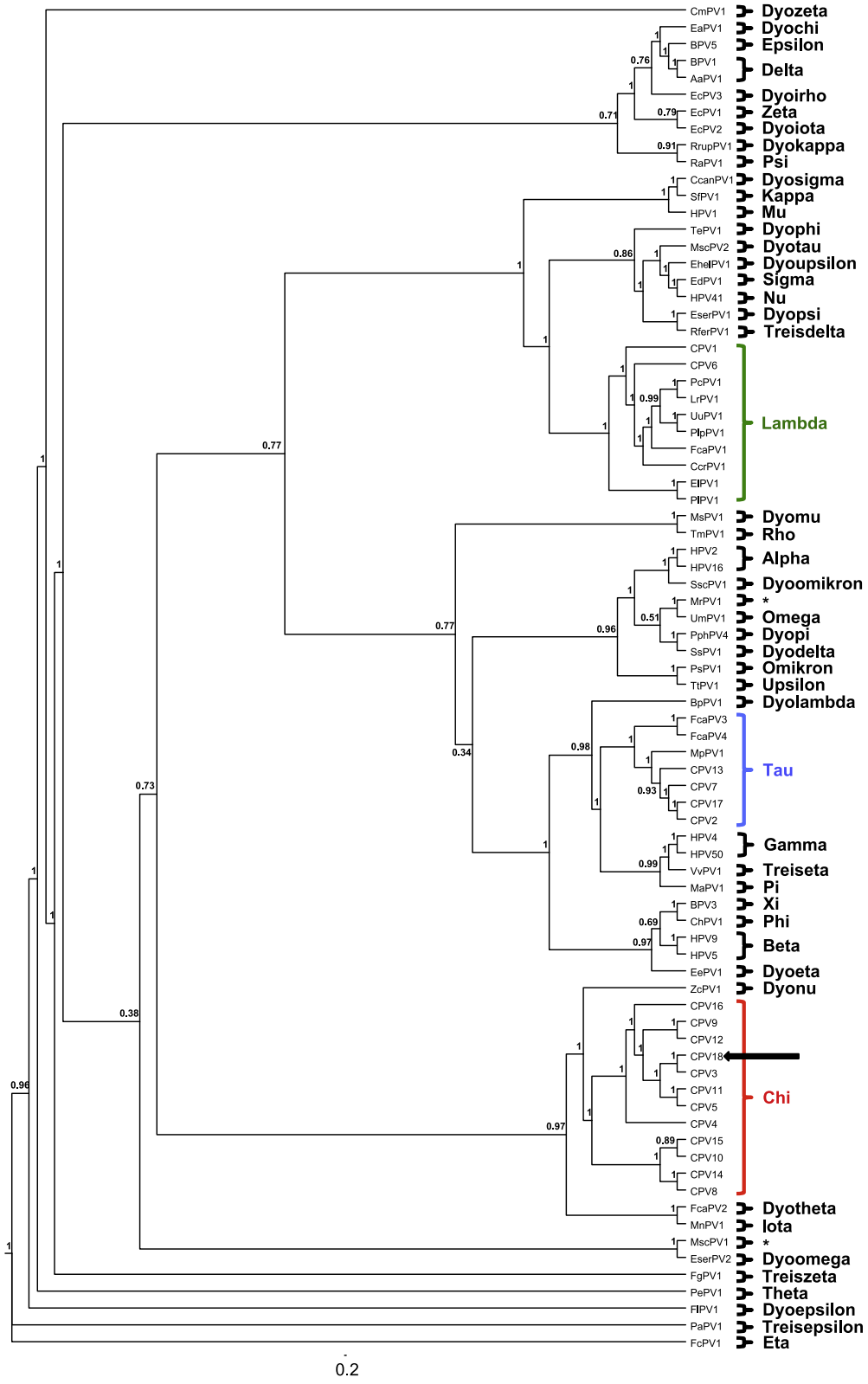

Fig. 4. Phylogenetic tree of 80 papillomaviruses including all currently assigned genera and carnivore infecting virus types. Tree based on the sequence information of E1, E1, L2 and L1. Genera containing canine papillomaviruses are highlighted in red (*Chi*), blue (Tau) and green (Lambda). Unclassified viruses are marked with an asterisk.

211 bp) (Fig. 3). Various characteristic PV motifs can be identified: Dyad symmetry repeats (TTGTTGTTAACAACAA) flanked by four E2 binding sites (ACC-N_6-GGT) upstream and two downstream are located in an AT-rich area in the NCR1 putatively marking the origin of replication (Fig. 3) [9,27]. Polyadenylation signals (AATAAA) were found within L2 near the 5′ end and overlapping with the 3′ end of the L1 ORF. The E1 amino acid (aa) sequence contains a modified ATP-dependent helicase motif (GPPDTGKS). A pRB-binding motif (LXCXE) is present in E7. E7 also contains one zinc-binding motif ($CX_2CX_{29}CX_2C$), whereas E6 contains two.

The L1 nucleotide sequence, which is used for classification, shares 85% identity with CPV3 and 74% with CPV5. The sequence of L1 of this novel isolate is 100% identical with a 355 bp partial L1 sequence (JQ040499). The sequence derived from a sample of a pug with pigmented plaques collected in California in 2009 [18]. The highest degree of identity in the E1 aa sequence was found with CPV3 (92%), CPV5 and CPV11 (82%). At the aa level for E6 and E7, the highest similarity is observed with CPV3 (83%, 98%), CPV9 (77%, 85%) and CPV12 (77%, 83%). Upon the phylogenetic analysis based on joined the E1-E1-L2-L1 ORFs the novel papillomavirus sequence clustered with these *Chi*-CPVs (Fig. 4). Based on the overall analysis, the isolate was designated as a new PV type and recognized by the Papillomavirus Study Group of the ICTV as CPV18.

The clinical and histopathological findings strongly indicate a PV induced disorder. The detection of CPV18 and no other PV DNA suggests that this virus might be causative. The type of lesion found in the here-described case has repeatedly been associated with CPV infections. In all cases of canine pigmented plaques that were tested *Chi*-PV DNA was found and *Chi*-PVs were only found in respective pigmented lesions (Table 1). Viral gene expression and replication has been demonstrated for some *Chi*-PVs and a causal relationship between *Chi*-PV infection and the development of pigmented plaques seems likely based on the overall data [15, 17]. Considering, that the DNA of CPV18 and CPV4 have previously or repeatedly been found in pugs with pigmented plaques, a genetic predisposition in a lineage of this breed might be plausible. So far however, there is no data regarding this matter.

CPV18 also aligns well with the other *Chi*-PVs in terms of genome size and organization (Fig. 3, Supplement 2). *Chi*-PVs belong to the smallest known PVs overall in terms of their genome size and do neither have an E5 ORF nor a large second NCR. Within the *Chi*-PVs CPV18 falls into the *Chi* – 1 species group with CPV3 being its closest relative. This holds true upon phylogenetic analysis of all six ORFs individually (Supplement 3). While the species allocation within the *Chi*-PVs is very robust in the analysis, the exact position of the individual types within them varies somewhat from gene to gene; a phenomenon that has previously been observed in PVs in general [7]. Especially the trees based on E6 and E7 vary from the others and from each other (Supplement 3, Supplement 4). No signs of recombination events were found within the known *Chi*-PVs.

Taken together the findings identify CPV18 as a new member of the papillomavirus genus *Chi* (*Chi* – 1) that is putatively involved in the development of pigmented plaques in dogs.

Nucleotide sequence accession number. The nucleotide sequence of CPV18 is deposited in GenBank under accession no. KT326919.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.pvr.2016.08.001.

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