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1 **EcPV2 DNA in equine squamous cell carcinomas and normal**
2 **genital and ocular mucosa**

3

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26 **Abstract**

27 Squamous cell carcinoma (SCC) represents the most common malignant tumour of the eye
28 and external genitals in horses. Comparable to humans, papillomaviruses (PV) have been
29 proposed as etiological agents of cancer in horses and recently, *Equine papillomavirus type 2*
30 (EcPV2) has been identified in genital SCCs. Hitherto it had never been demonstrated in
31 ocular SCCs .

32 The first goal of this study was to determine the prevalence of EcPV2 DNA in tissue samples
33 from equine genital and ocular SCCs, genital papillomas and penile intraepithelial neoplasia
34 (PIN) lesions, using EcPV2-specific PCR. The second goal was to investigate the possibility
35 of latent EcPV2 infection in the genital and ocular mucosa of healthy horses on swabs
36 obtained from the eye, penis, vulvovaginal region and cervix. EcPV2 DNA was detected in
37 all genital SCCs (17/17), genital papillomas (8/8), PIN lesions (11/11) and ocular SCCs (9/9).
38 In healthy horses, EcPV2 DNA was detected in 43% (17/40) of penile swabs, 53% (9/17) of
39 vulvovaginal swabs, 47% (8/17) of cervical swabs and 57% (32/56) of ocular swabs. This
40 study confirms the presence of EcPV2 DNA in equine genital SCCs. Moreover, we
41 demonstrate for the first time its involvement in other genital lesions and in ocular SCCs and
42 latent EcPV2 infections in normal genital (including cervical) and ocular equine mucosa. The
43 close relatives of EcPV2 are associated to cutaneous lesions, and this virus is not related to
44 high-risk human papillomaviruses causing cervical cancer. Thus, similar viral tropism does
45 not imply close evolutionary relationship.

46

47 **Keywords:** horse / squamous cell carcinoma / EcPV2 / papillomavirus / mucosa

48

49

50 1. Introduction

51 Squamous cell carcinoma (SCC) represents 20% of all equine tumours, making it the second
52 most common neoplasm in horses. SCC is a malignant epithelial tumour and is most often
53 associated with the eyes (Fig. 1) and the external male genitals (Fig. 2), but it can develop in
54 any epithelial tissue of the body (Macfadden and Pace, 1991). In many horses, penile SCCs
55 are accompanied by large, confluent, pink to yellowish plaques (Fig. 2), often referred to as
56 “precancerous lesions” (Brinsko, 1998). Similar to the frequently described penile
57 intraepithelial neoplasia (PIN) in men, the same term can be used in horses. Furthermore,
58 according to our own clinical observations, many genital SCCs are accompanied by genital
59 papillomas, which can also be considered as precancerous lesions.

60 Papillomaviruses (PVs) are small, epitheliotropic, non-enveloped double-stranded circular
61 DNA viruses. In humans, more than 100 different PV types have been fully sequenced, some
62 of them associated with both benign and malignant clinical conditions, ranging from
63 spontaneously regressing cutaneous and genital warts, to invasive anogenital and skin cancer
64 (de Villiers et al., 2004). Comparable to humans, PVs have been proposed as etiological
65 agents of cancer in horses. *Bovine papillomavirus type 1* (BPV1) and less commonly *type 2*
66 (BPV2) are associated with equine sarcoids, a common fibroblastic skin tumour in horses.
67 The presence of PVs in equine SCCs was first investigated in 1984, but could not be
68 demonstrated at that time (Junge et al., 1984). In 2004 the complete nucleotide sequence of
69 the first equine PV, *Equus caballus papillomavirus 1* (EcPV1), isolated from a cutaneous
70 papilloma, was determined (Ghim et al., 2004). In 2007 EcPV1 DNA was demonstrated in a
71 high proportion of cutaneous papillomas but could not be isolated from genital papillomas
72 (Postey et al., 2007). However, the existence of a different PV already suggested in 1986 by
73 Obanion et al., was strongly supported since both PCR and immunohistochemical analysis
74 respectively indicated the presence of PV DNA and PV antigens in genital lesions (Postey et

75 al., 2007). Recently a novel equine papillomavirus (EcPV2) has been identified in genital
76 SCCs and papillomas, but it could not be demonstrated in ocular SCCs yet (Scase, 2005;
77 Scase, 2007). The first goal of this study was therefore to determine the prevalence of EcPV2
78 DNA in tissue samples from equine genital and ocular SCCs, genital papillomas and PIN
79 lesions.

80 Humans are exposed since early life to PV infection, and most of these PV infections in
81 humans are asymptomatic (Antonsson et al., 2000). The same appears to be true in several
82 animal species, including virtually all mammals (Antonsson and Hansson, 2002). Since latent
83 PV infections have also been shown in cattle and horses (Campo et al., 1994; Antonsson and
84 Hansson, 2002; Bogaert et al., 2008) the second goal of this study was to investigate the
85 possibility of latent EcPV2 infections in the genital and ocular mucosa of healthy horses.
86 Finally, although cervical cancer has never been described in horses, we also investigated the
87 presence of EcPV2 in the cervix of clinically normal horses, regarding the importance of
88 high-risk HPVs in cervical cancer in women.

89

90 **2. Methods**

91 2.1. Study population and sample collection

92 *Affected horses*

93 This group consisted of 26 horses, including 19 geldings, one stallion and six mares. The
94 median age was 14 years (range 3-24 years). There were six ponies, seven Haflingers, ten
95 Warmbloods, one Standardbred, one Appaloosa and one Arabian horse. Forty-five tissue
96 samples derived from 25 horses with one or more genital or ocular SCC, PIN lesion or
97 papillomas were included in the study. These horses were patients referred to the Department
98 of Surgery and Anaesthesiology of Domestic Animals of Ghent University (Belgium) for
99 treatment of the SCC, PIN lesion or papilloma. The samples enclosed twelve penile and two

100 preputial SCCs (P1-P14), two vulval SCCs (V1-V2), one anal SCC (A1), eleven PIN lesions
101 (PIN1-PIN11), seven penile papillomas (PPA1-PPA7), one vaginal papilloma (VPA1) and
102 nine ocular SCCs (O1-O9). Additional samples included one nasal SCC (N1) from a 26th
103 patient, a mouth lesion (M1) in one of the horses with a penile SCC (patient #2), as well as
104 two superficial inguinal lymph nodes (L1-L2) with metastases from a patient with a penile
105 SCC (patient #1). Table 1 summarizes the samples, sex, age and breed of the patients.

106 Samples were collected after surgical tumour excision or after debulking of the tumoural mass
107 before cryosurgery, immunotherapy or chemotherapy, except for the samples of the inguinal
108 lymph nodes, which were obtained after euthanasia of the horse. All samples were collected
109 by excising a representative part of the mass using a sterile scalpel and forceps. When several
110 samples were obtained from the same horse, each sample was processed with a different set of
111 instruments. Samples were stored dry at -18 °C.

113 *Healthy horses*

114 The stallions (N=22) and geldings (N=18) were patients referred to the Department of
115 Surgery and Anaesthesiology of Domestic Animals of Ghent University (Belgium) for non-
116 oncological surgery between August 2007 and April 2008. From each of them an ocular and
117 a penile swab were obtained under general anaesthesia, except from one stallion in which
118 only a penile swab was available. A single cotton-tipped swab was used to sample the third
119 eyelid, cornea and conjunctiva of both eyes. A second swab was used to sample the genital
120 region: the penis was protruded and the distal part of the urethra, the sinus urethralis and fossa
121 glandis, the glans penis, the penile shaft and the preputium were sampled with the same swab.
122 All precautions were taken to avoid cross contamination and samples were stored dry at -18
123 °C. Age, breed or colour were not recorded in six stallions. The remaining stallions had a
124 median age of three years (range 1-12 years); there were two ponies, ten Warmbloods, two

125 Standardbreds, two Paint Horses, one Lusitano and one Thoroughbred, with a colour
126 distribution of two black, eight bay, four chestnut, four grey and three skewbald horses. The
127 gelding group had a median age of eleven years (range 2-19 years). There were 13
128 Warmbloods, two Standardbreds, one Quarter horse, one Andalusian horse and one Anglo-
129 Arabian horse, with a colour distribution of two black, three bay, six chestnut, six grey and
130 one skewbald horse.

131 The mares (N=17) were patients referred to the Department of Obstetrics, Reproduction and
132 Herd Health of Ghent University for artificial insemination between August 2007 and April
133 2008. From each of them an ocular, a vulvovaginal and a cervical swab were taken. Ocular
134 swabs were taken as described for stallions and geldings. Before obtaining the genital swabs,
135 the vulva was scrubbed with povidonum iodinated 7.5% soap. A single cotton-tipped swab
136 was used to sample the vulva, the vestibulum vagina, the glans clitoridis and the fossa clitoridis.
137 To sample the cervix, a tube speculum was inserted and the sample was taken using a uterus
138 biopsy forceps with a small sterile tampon between the tips. The portio vaginalis, the ostium
139 uteri externum and the canalis cervicalis were sampled with this single tampon. Samples were
140 stored dry at -18 °C. The age and breed of 5 mares were not recorded. The remaining mares
141 had a median age of 6.5 years (range 4-19 years); there were four Warmbloods and eleven
142 Standardbreds, with a colour distribution of 15 bay and two chestnut horses.

143

144 2.2. DNA isolation and PCR

145 DNA was isolated using the Puregene Genomic DNA isolation kit (Gentra Systems) as
146 described previously (Bogaert et al., 2008). For detection of EcPV2 DNA, PCR was
147 performed using an EcPV2 specific primer set: EcPV2 forward primer 5'-
148 GCGGACTGCGCGTCACAAGAGGGGC -3' and reverse primer 5'-
149 ACGCAAGCACCACCCACTGCTTGGCA -3'. This primer set amplified a 679 base pair

150 (bp) fragment of the E1 gene (position 215-893) in the genomic sequence of EcPV2,
151 (GenBank accession number EU503122). PCR was performed in a 10 µL reaction mixture,
152 containing 200 µM of each dNTP, 0.5 µM of forward and reverse primer, 0.5 U Fast Start *Taq*
153 DNA polymerase (Roche) and 1.5 mM MgCl with 2.5 µl of template DNA. Amplification
154 was performed as follows: 95°C for 5 min, 40 cycles of 95°C for 1 min, 65°C for 1 min, 72°C
155 for 1 min and finally 72°C for 10 min. Negative controls with DNA from two equine
156 papillomas and two equine sarcoids (with confirmed EcPV-1 and BPV-1 infections
157 respectively) as well as a non-template control with H₂O, were included in each experiment.
158 PCR products were separated by electrophoresis on a 2% agarose gel and visualised by
159 ethidium bromide staining.

160

161 2.3. Sequencing of PCR products

162 In order to confirm the identity of the PCR products, amplicons of one penile, vulval, ocular
163 and nasal SCC and amplicons of one ocular, penile, vulvovaginal and cervical swab from
164 healthy horses were purified using the GeneClean II kit (Bio 101 Systems), ligated into pCR®
165 2.1 vector and transformed in *Escherichia coli* bacteria (One shot® INVαF' Chemically
166 Competent *E. coli*, Invitrogen) using the TA Cloning kit (Invitrogen). Bacteria were incubated
167 for blue-white colony screening on agar plates containing ampicilline and X-gal. Three white
168 colonies of each sample were amplified and the plasmid DNA was purified using the Qiagen
169 Plasmid Maxi Kit. Sequencing was performed at least twice per sample with universal T7
170 primers, using a Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).
171 Cycle sequencing reaction products were purified using ethanol precipitation and sequenced
172 with an ABI prism 310 genetic analyzer (Applied Biosystems).

173 Amplicon sequences were aligned using CAP3 (freely distributed by Xiaoqiu Huang at
174 <http://seq.cs.iastate.edu/>), using the published EcPV02 sequence as a reference. All amplified

175 sequences corresponded to EcPV2, as determined by Basic Local Alignment Search Tool
176 analyses. For phylogenetic analyses, a representative selection of 84 PV sequences was
177 chosen, and the E1, E2 and L1 genes concatenated, as described (Gottschling et al., 2007a).
178 Briefly, the sequences were aligned at the amino acid level with MUSCLE
179 (<http://www.drive5.com/muscle/>), visualised for manual correction with Se-AL (freely
180 distributed by Andrew Rambaut at <http://tree.bio.ed.ac.uk/software/seal/>) and back-translated
181 to the nucleotide level using PAL2NAL (<http://www.bork.embl.de/pal2nal>). Maximum
182 Likelihood (ML) phylogenetic analysis was performed with RAxML (Stamatakis, 2006)
183 using the GTR+ Γ 4 model of evolution and the CAT approximation of rate heterogeneity,
184 introducing three partitions that corresponded to each of the codon positions. After 1,000
185 bootstraps the final tree topology was optimised without resorting to the CAT approximation.
186 EcPV02 could be unambiguously ascribed to the delta+epsilon PV superclade, including
187 viruses infecting Carnivora (*Canis familiaris*, CPV4 and CsPV3), Perissodactyla (*Equus*
188 *caballus*, EcPV1) and Cetartiodactyla (*Bos taurus* BPV1, BPV2, BPV5 and BPV8; *Ovis*
189 *aries*, OvPV1 and OvPV2; *Rangifer tarandus*, RPV; *Odocoileus virginianus*, DPV; *Alces*
190 *alces*, EEPV; and *Capreolus capreolus*, CcPV). An additional phylogenetic search was then
191 performed with these sequences and the *Rousettus aegyptiacus* PV, RaPV, as outgroup.
192 Maximum likelihood was computed with the above-described settings. Bayesian phylogenetic
193 analysis was performed with BEAST v1.4.8 (<http://beast.bio.ed.ac.uk>) (Drummond and
194 Rambaut, 2007) with the GTR+ Γ 4 model of evolution, and for both strict clock and
195 uncorrelated log normal relaxed clock, introducing three partitions that corresponded to each
196 of the codon positions, and unlinking parameters across codon positions. Two independent
197 chains of 50 million steps were calculated, writing every 1,000 steps, and analysed with a
198 burn-in of ten million steps. Compatibility of both chains was assessed by calculating the
199 corresponding Bayes factor, and both chains were combined into one. The reference

200 cladogram for the animal species included in the taxon sample was pruned out from a
201 mammalian tree constructed after the supertree methodology (Bininda-Emonds et al., 2007),
202 using TREEPRUNER (freely distributed by Olaf Bininda-Emonds at [http://www.uni-](http://www.uni-oldenburg.de/molekularesystematik/33997.html)
203 [oldenburg.de/molekularesystematik/33997.html](http://www.uni-oldenburg.de/molekularesystematik/33997.html)).

204

205 2.4. Statistical analysis

206 The data obtained from the healthy horses were statistically analysed using SPSS 15.0. The
207 positivity from the swabs depending of the sample location was analysed by Fisher's exact
208 tests (2x2 tables). The χ^2 -test for an RxC contingency table was used to examine differences
209 in presence of EcPV2 between sexes, breeds, coat colours and ages. Differences were
210 considered statistically significant if *P*-values were below 0.05.

211

212 **3. Results**

213

214 3.1. EcPV2 DNA analysis

215 *Affected horses*

216 Amplicons of the expected size were detected in all penile, preputial, vulval and anal SCCs
217 (17/17), in all PIN lesions (11/11), in all genital papillomas (8/8) and in all ocular SCCs (9/9)
218 (Fig. 3) The two metastatic lymph nodes, the mouth lesion and the nasal SCC also tested
219 positive. DNA of equine papillomas, equine sarcoids and H₂O, were successfully used as
220 negative controls.

221

222 *Healthy horses*

223 In total, 56 ocular swabs, 40 penile swabs, 17 vulvovaginal swabs and 17 cervical swabs were
224 obtained from the healthy horses.

225 In stallions and geldings, EcPV2 DNA was detected in 71% and 67% respectively (15/21 and
226 12/18) of ocular swabs, compared to 29% (5/17) of positive ocular swabs in mares. Forty-five
227 percent (10/22) of penile swabs tested positive in stallions, compared to 40% (7/18) in
228 geldings. Regarding the 17 vulvovaginal and cervical samples, 53% (9/17) and 47% (8/17)
229 tested positive, respectively. Results are summarized in Table 2 and a typical gel image is
230 provided in Fig. 3. Positivity in one body location was not significantly correlated to
231 positivity in another location. Results are summarized in Table 3. There was no significant
232 effect of sex, breed, coat colour or age on positivity.

233

234 3.2. Sequencing results

235 Primers designed using the EcPV2 sequence could amplify in all cases a DNA fragment of ca.
236 680 bp, which after sequencing could be identified without ambiguity as belonging to this PV
237 type. The EcPV2 sequence deposited in the databases (GenBank EU503122) lacks the N-
238 terminus of the E1 open reading frame, which is otherwise highly conserved among PVs. In
239 all of the amplicons sequenced in this study there was a consistent mismatch with the
240 deposited EcPV2 sequence, which restored the E1 frame. This corrected sequence has been
241 deposited under GenBank accession number GU809241.

242

243 3.3. Phylogenetic reconstruction

244 The equine EcPV2 belonged unequivocally to the delta-epsilon-zeta PV superclade
245 (Gottschling et al., 2007a; Bravo and Alonso, 2007) (Fig. 4a), which encompasses PVs
246 infecting different hosts within Laurasiatheria: dog (CsPV3 and CPV4), horse (EcPV1 and
247 EcPV2), cow (BPV1, BPV2, BPV5 and BPV8), sheep (OvPV1 and OvPV2), European elk
248 (EPPV), roe deer (CcPV), deer (DPV), and white-tailed deer (RPV). Within this superclade,
249 PV infecting Canidae and infecting Cetartiodactyla were respectively monophyletic, i.e., they

250 share a recent common ancestor (Fig. 4b). However, PV that infect horse and also PV that
251 infect cow are not monophyletic respectively. Finally, there is also a global disagreement
252 between the topology of PV infecting Cervidae and the topology of the hosts they infect (Fig.
253 4c).

254

255 **4. Discussion**

256 In the present study EcPV2 DNA was detected in all (para-)genital SCCs, PIN lesions and
257 genital papillomas. This suggests an etiological role of EcPV2 in the development of these
258 genital lesions, comparable to the role of high-risk HPVs in anogenital cancers such as
259 cervical cancer. However, we assume that an EcPV2 infection is not sufficient to induce
260 tumoral transformation, since we observed 75% of healthy EcPV2 carriers. In humans, high-
261 risk-HPV infection is a necessary but not sufficient cause of cervical cancer. Other cofactors,
262 such as long term use of oral contraceptives, high parity, smoking, immunosuppression, or
263 other sexually transmitted infections, are necessary for progression from high-risk HPV
264 infection to cancer (Munoz et al., 2006). Known cofactors in the development of SCCs in
265 horses are repeated trauma, retention of smegma (Burney et al., 1992), solar radiation, breed,
266 hair colour and age (Valentine, 2006). The EcPV2 mRNA and protein expression pattern as
267 well as functional pathways should be studied in order to establish the precise role of EcPV2
268 in tumour development.

269 Our study is the first to demonstrate PV DNA in equine lymph nodes, as EcPV2 DNA was
270 detected in two metastatic lymph nodes. In humans, the relationship between HPV DNA in
271 lymph nodes and metastases has not yet been elucidated. The presence of HPV DNA in
272 histologically tumour-free lymph nodes could be a sign of (early) tumoral involvement and
273 therefore an important prognostic factor (Lukaszuk et al., 2007). Other researchers
274 hypothesise that HPV DNA detected in histologically tumour-free lymph nodes originates

275 either from immune cells that have taken up HPV particles or from free migrating HPV
276 particles, and therefore do not attribute a prognostic value to HPV in lymph nodes (Fule et al.,
277 2006). The significance of EcPV2 DNA in the lymph nodes in horses with SCCs cannot be
278 explained on the basis of the available data, since only two lymph nodes obtained from one
279 single patient have been analysed in this study.

280 EcPV2 DNA was also detected in a mouth lesion of a horse with a penile SCC. Histological
281 examination of this mouth lesion showed focal epithelial hyperplasia. According to the
282 owners of the horse, the horse was often licking and biting his penile SCC and after a few
283 months the mouth lesion appeared. The detection of EcPV2 DNA in this lesion suggests a
284 broad tropism of EcPV2, able to productively infect histologically different mucosal tissues in
285 different anatomical locations, as has been also reported for COPV, infecting dogs and
286 causing both oral and ocular lesions (Brandes et al., 2009). The possibility of transmission
287 from infected to non-infected horses is unknown. In women, cervical cancer is strongly
288 associated with genital high-risk HPV infections in their male sexual partners and vice versa
289 (Barrasso et al., 1987). In horses, sexual transmission could also be possible since EcPV2
290 DNA was detected in both penile and vaginal SCCs. However, also horses that never had
291 been used as breeding animals were included in this study, which suggests transmission routes
292 other than the sexual route. In this sense, the possibility of both intra- and inter-individual PV
293 inoculation among different anatomical locations (e.g. penis, scrotum, anus, cervix or hand)
294 has also been demonstrated in humans (Hernandez et al, 2008). An alternative hypothesis is
295 that the virus could be spread by insects, which is a suggested transmission route of BPV in
296 the pathogenesis of equine sarcoids (Finlay et al., 2009). Finally, vertical transmission from
297 mare to foal, before, during or directly after delivery, could also be an alternative transmission
298 route, as has also been described in humans and suggested in cows (dos Santos et al., 1998;
299 Rombaldi et al., 2008).

300 EcPV2 DNA was detected in 100% of ocular SCCs and the PCR amplified sequence of this
301 ocular PV DNA was identical to the genital PV DNA. This finding is in contrast to the results
302 of the study of Scase (2007), where EcPV2 DNA was only demonstrated in genital but not in
303 ocular lesions.

304 A nasal SCC analyzed in the present study also tested positive for EcPV2. Known
305 predisposing factors in equine nasal and paranasal tumours are epithelial changes due to
306 chronic inflammatory processes (Head and Dixon, 1999). In humans, risk factors in the
307 development of head and neck cancer, including tumours of the nose and paranasal sinuses,
308 are smoking and alcohol consumption and in addition there is a relationship between certain
309 HPVs and these cancers (Syrjanen, 2005).

310 In healthy horses, EcPV2 DNA was detected in 57% (32/56) of ocular swabs, 43% (17/40) of
311 penile swabs, 53% (9/17) of vulvovaginal swabs and 47% (8/17) of cervical swabs, which
312 indicates the existence of latent EcPV2 infections. This elevated prevalence is comparable to
313 that in humans where the majority of the population undergoes subclinical HPV infections at
314 least once in their life, while only a small part shows progression to clinical lesions (Koutsky
315 et al., 1988). We interpret therefore that, as in humans, spontaneous clearance will happen in
316 most horses, while evolution to precarcinogenic lesions will only occur in a limited number of
317 horses, of which only a small percentage will evolve to SCCs. A long-term follow-up study of
318 infected but clinically normal horses is required to gain insight in the evolution of latency.

319 In order to determine whether the presence of EcPV2 DNA in one location is related to its
320 presence in a second location, a statistical analysis was done to verify whether EcPV2
321 frequently infects several locations in the same horse. This could reflect either an inherent
322 susceptibility of certain horses, or be instead an indication of self-inoculation, as has been
323 suggested in humans (Hernandez et al., 2008). However, no significant correlation was found.
324 Since geldings, certain breeds (draft horses, Appaloosas, Paints and Pintos), pale coloured

325 horses and older horses are more likely to develop SCCs according to several authors (Burney
326 et al., 1992; Valentine, 2006), the effect of sex, breed, coat colour or age on positivity was
327 also statistically analyzed but no significant correlation was found. However, it should be
328 taken into account that only small groups of horses were sampled.

329 In the present study, EcPV2 DNA has been detected in the cervix of eight mares. This is the
330 first study demonstrating PV DNA in the equine cervix. Probably EcPV2 can reach the cervix
331 by sexual transmission or by using EcPV2-infected material during artificial insemination or
332 other gynaecological interventions. The fact that EcPV2 DNA is detectable in the cervix of
333 mares, but that cervical cancer has never been described in this species can have several
334 explanations. It is possible that the local immune milieu of the mare differs from the human
335 one and facilitates spontaneous clearance. In humans most women are exposed to at least one
336 HPV type during their sexual life, and spontaneous clearance of genital infection by high-risk
337 HPVs occurs within two years and only in less than 10% cervical (pre)cancer arises
338 (Schiffman et al., 2007). Additional factors could be the limited age reached by horses in
339 comparison to humans and the differential exposure to carcinogenic cofactors. In women the
340 evolution of high-risk HPV infection to cervical cancer takes many years to even decades.
341 The peak incidence of HPV infections occurs in their twenties, that of high grade cervical
342 intraepithelial lesions in their thirties and cervical cancer occurs mostly in their forties
343 (Kitchener et al., 2006). Another possibility is that mares do not develop cervical cancer
344 because of a different anatomohistological cervical structure in comparison with women. In
345 women cervical cancer occurs typically in the transformation zone of the cervix, which is
346 located at the transition from the multilayered squamous epithelium of the ectocervix to the
347 glandular epithelium of the endocervix (Schiffman et al., 2007). To date no investigation
348 about the transformation zone in mares has been carried out. Finally, an evolutionary
349 explanation for the non-existence of cervical cancer in mares despite cervical EcPV2 infection

350 might be that the virus-host interaction mechanisms between high-risk HPVs and those of
351 EcPV2 may be essentially different, even though they infect anatomically equivalent host
352 cells. High-risk HPVs involved in cervical cancer are phylogenetically very distant from
353 EcPV2. PVs in the delta-epsilon-zeta superclade, where EcPV2 belongs, are associated
354 mainly to cutaneous papillomas and fibropapillomas. This is the case of EcPV1, which was
355 isolated and characterized from cutaneous lesions (Ghim et al., 2004; Obanion et al., 1986)
356 but also that of PV infecting Bovidae and Cervidae, and also classified in this superclade
357 (Gottschling et al., 2007b; Bravo and Alonso, 2007). Thus, the most parsimonious
358 explanation is that the tropism of the common ancestor to the whole clade was cutaneous,
359 whereas the mucosal tropism of EcPV2 is derived, appeared and developed after viral
360 speciation, and is not shared by the rest of the members of the superclade. On the other hand,
361 high-risk HPVs belong to the alpha-omicron PV superclade, where the predominant tropism
362 is mucosal (Bravo and Alonso, 2007), and only a few PV, such as HPV3 or SsPV1, have
363 subsequently developed a cutaneous tropism. The last common ancestor to both superclades
364 could have existed before the radiation within mammals, around 95 millions of years ago
365 (Garcia-Vallve et al., 2005). The ancestors of both superclades specialised in different
366 tropisms, and only later, EcPV2 colonised separately the genital mucosal niche, in an event of
367 convergent evolution.

368

369 **5. Conclusion**

370 The present study confirms the presence of EcPV2 DNA in equine genital SCCs and is the
371 first to demonstrate its involvement in precancerous genital lesions, in ocular SCCs, in a nasal
372 SCC, in lymph nodes and in a mouth lesion. Moreover, latent EcPV2 infection in normal
373 genital (including cervical) and ocular equine mucosa could be demonstrated in healthy
374 patients.

375

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378 helping with the PCR reactions. We are grateful to Dr. Jan Van Doorselaere (KATHO,
379 campus Roeselare, Belgium) for performing the sequencing reactions.

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383 Cajal Program.

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- 483
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- 485

486 **Table 1.** Overview of the samples, sex, age and breed of affected horses.

| Patient | Samples | Sex | Age (years) | Breed |
|---------|--------------------------|----------|-------------|---------------|
| 1 | P1, PPA1, L1, L2 | Gelding | 24 | Shetland pony |
| 2 | P2, PPA2, PPA3, PPA4, M1 | Gelding | 9 | Pony |
| 3 | P3, PIN1 | Gelding | 22 | Warmblood |
| 4 | P4, PPA5, PIN2 | Gelding | 13 | Pony |
| 5 | P5, PIN3 | Gelding | 11 | Warmblood |
| 6 | P6, PIN4 | Gelding | >20 | Shetland pony |
| 7 | P7, P8, PIN5 | Gelding | 20 | Warmblood |
| 8 | PIN6 | Gelding | 22 | Pony |
| 9 | P9, PIN7, PPA6 | Gelding | 17 | Pony |
| 10 | P10, PIN8, PPA7 | Gelding | 22 | Arabian horse |
| 11 | P11, PIN9 | Gelding | 12 | Warmblood |
| 12 | PIN10 | Gelding | 7 | Warmblood |
| 13 | P12 | Gelding | 14 | Appaloosa |
| 14 | P13 | Gelding | 20 | Warmblood |
| 15 | P14, PIN11 | Gelding | 18 | Warmblood |
| 16 | V1 | Mare | 20 | Standardbred |
| 17 | V2, A1, VPA1 | Mare | 12 | Haflinger |
| 18 | O1, O2 | Gelding | 9 | Haflinger |
| 19 | O3 | Gelding | 3 | Haflinger |
| 20 | O4 | Mare | 13 | Haflinger |
| 21 | O5 | Mare | 20 | Warmblood |
| 22 | O6 | Mare | 14 | Haflinger |
| 23 | O7 | Stallion | 10 | Warmblood |
| 24 | O8 | Mare | 14 | Warmblood |
| 25 | O9 | Gelding | 11 | Haflinger |
| 26 | N1 | Gelding | 11 | Haflinger |

487 L: Lymph node; M: Mouth lesion; N: Nasal SCC; O: Ocular SCC; P: Penile SCC; PIN: Penile

488 Intraepithelial Neoplasia lesion; PPA: penile papilloma; V: Vulval SCC;

489 VPA: Vulval papilloma

490

491 **Table 2.** Summary of the EcPV2 DNA analysis of samples obtained from healthy horses.

| Sample group | Penile swabs | | Vulvovaginal swabs | | Cervical swabs | | Ocular swabs | |
|------------------|--------------|----------------|--------------------|----------------|----------------|----------------|--------------|----------------|
| | <i>N</i> | <i>Pos (%)</i> | <i>N</i> | <i>Pos (%)</i> | <i>N</i> | <i>Pos (%)</i> | <i>N</i> | <i>Pos (%)</i> |
| Stallions | 22 | 10 (45%) | | | | | 21 | 15 (71%) |
| Geldings | 18 | 7 (40%) | | | | | 18 | 12 (67%) |
| Mares | | | 17 | 9 (53%) | 17 | 8 (47%) | 17 | 5 (29%) |

492 *N*: number of samples examined

493 *Pos (%)*: number (percentage) of samples positive for EcPV2 DNA

494

495

496 **Table 3.** Positivity in the different locations analyzed in healthy horses.

| Sample group | <i>N</i> | <i>n(1)</i> | <i>n(2)</i> | <i>n(3)</i> | <i>n</i> |
|---------------------|-----------------|--------------------|--------------------|--------------------|-----------------|
| Stallions | 22 | 9 | 8 | | 17 (77%) |
| Geldings | 18 | 11 | 4 | | 15 (83%) |
| Mares | 17 | 3 | 5 | 3 | 11 (65%) |
| Total | 57 | 23 | 17 | 3 | 43 (75%) |

497 *N*: number of horses examined498 *n(1)*, *n(2)*, *n(3)*: number of horses positive in respectively one, two or three locations499 *n*: total number of positive horses

500

501

502 **Fig. 1.** Limbal squamous cell carcinoma involving the cornea and sclera in a 6 years old
503 Haflinger.
504

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505 **Fig. 2.** Penile squamous cell carcinoma with PIN lesions in a 22 years old Arabian horse.

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508 **Fig. 3.** Gel image after PCR amplification of a 679 base pair (bp) fragment of the E1 gene of
509 EcPV2 in several samples of squamous cell carcinoma, a precancerous genital lesion and
510 normal equine mucosa
511 Lane 1: 1 Kb Plus DNA Ladder (Invitogen®), 2: H₂O template control, 3: equine skin
512 papilloma, 4: equine sarcoid, 5: penile SCC, 6: PIN lesion, 7: ocular SCC, 8: swab normal
513 penile mucosa, 9: swab normal ocular mucosa, 10: swab normal vulvo-vaginal mucosa, 11:
514 swab normal cervical mucosa.
515

516 **Fig. 4.** Phylogenetic relationships of EcPV2 among the Papillomaviridae family.

517 (A) Maximum- likelihood best-known tree for the concatenated E1E2L1 sequences, rooted

518 using PV sequences from Aves and from Reptiles. Node values indicate support after 1000

519 bootstrap cycles. Bar scale indicates substitutions per site. The position of EcPV2, nested

520 within the delta-epsilon-zeta PV superclade has been labeled with an arrow. (B) Bayesian

521 phylogenetic reconstruction of EcPV2 and its closest relatives in the superclade, rooted using

522 RaPV as outgroup. Node values indicated support after two combined chains of 50 million

523 steps each. Bar scale indicates substitutions per site. Viruses infecting horses have been

524 labeled with arrows. The taxonomic description of the hosts is given on the right. (C)

525 Cladogram for the host species listed in B.

526

527

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| 22 | O6 | Mare | 14 | Haflinger |
| 23 | O7 | Stallion | 10 | Warmblood |
| 24 | O8 | Mare | 14 | Warmblood |
| 25 | O9 | Gelding | 11 | Haflinger |
| 26 | N1 | Gelding | 11 | Haflinger |

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Table 3. Positivity in the different locations analyzed in healthy horses.

| Sample group | <i>N</i> | <i>n(1)</i> | <i>n(2)</i> | <i>n(3)</i> | <i>n</i> |
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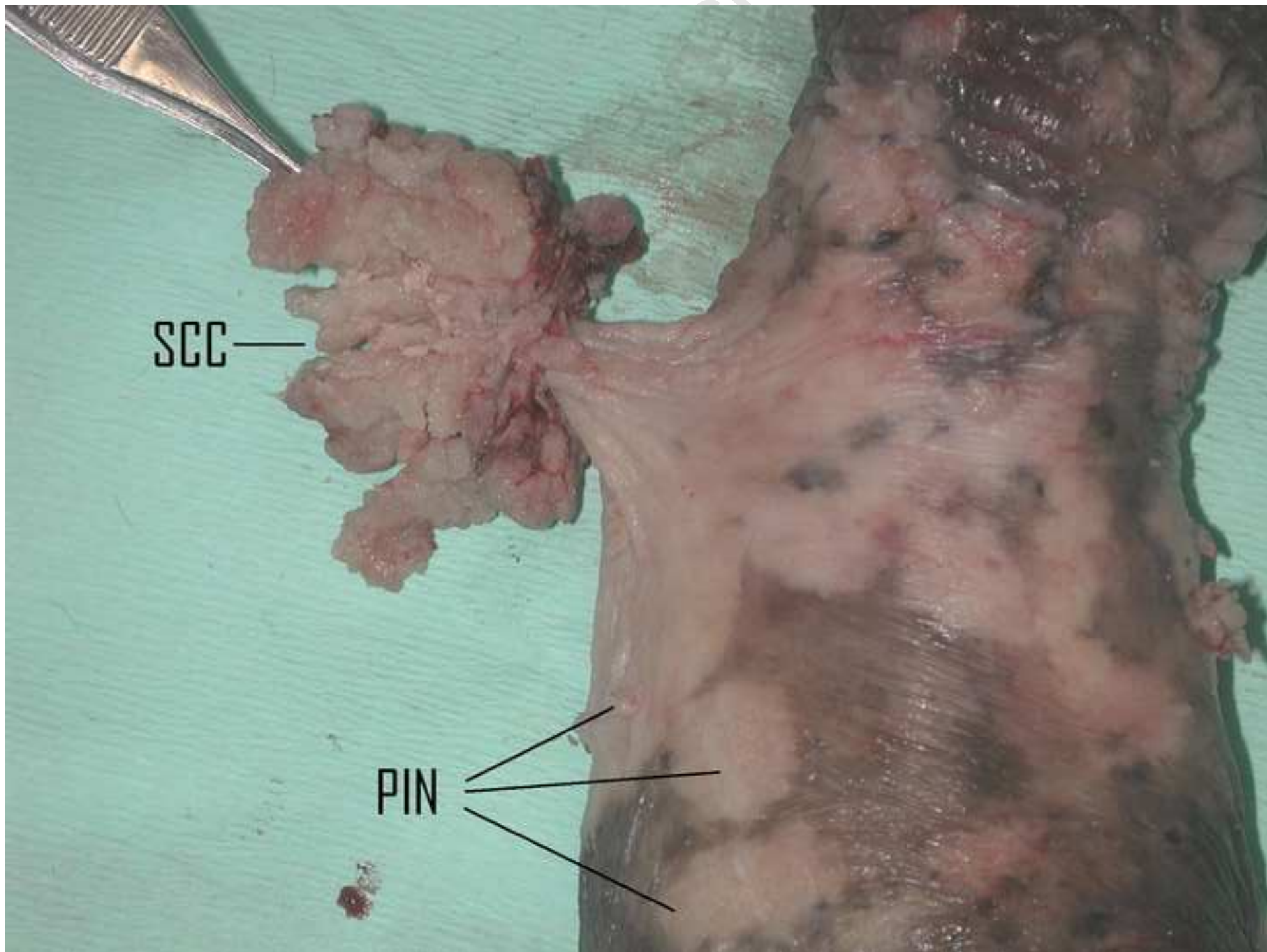
N: number of horses examined

n(1), *n(2)*, *n(3)*: number of horses positive in respectively one, two or three locations

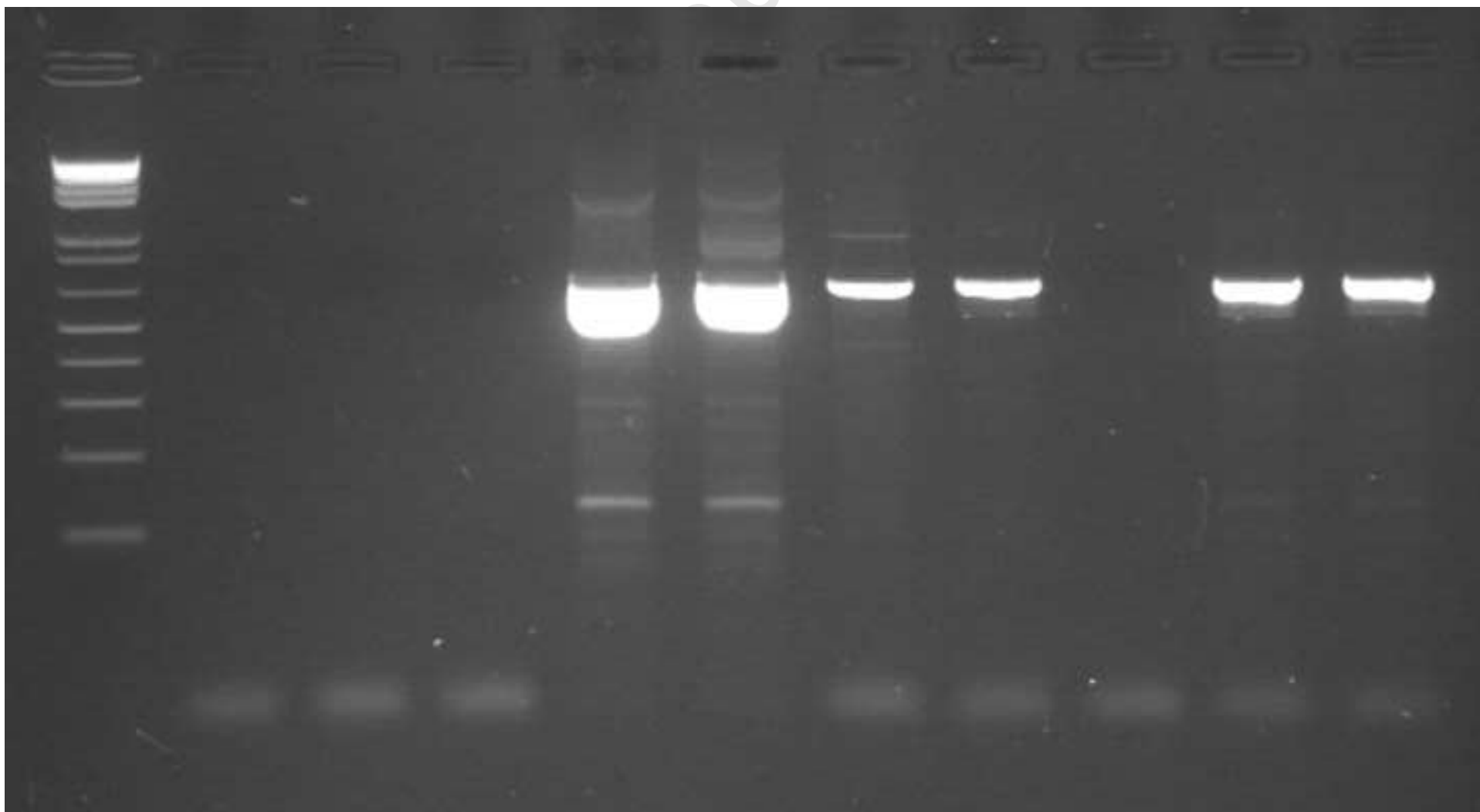
n: total number of positive horses



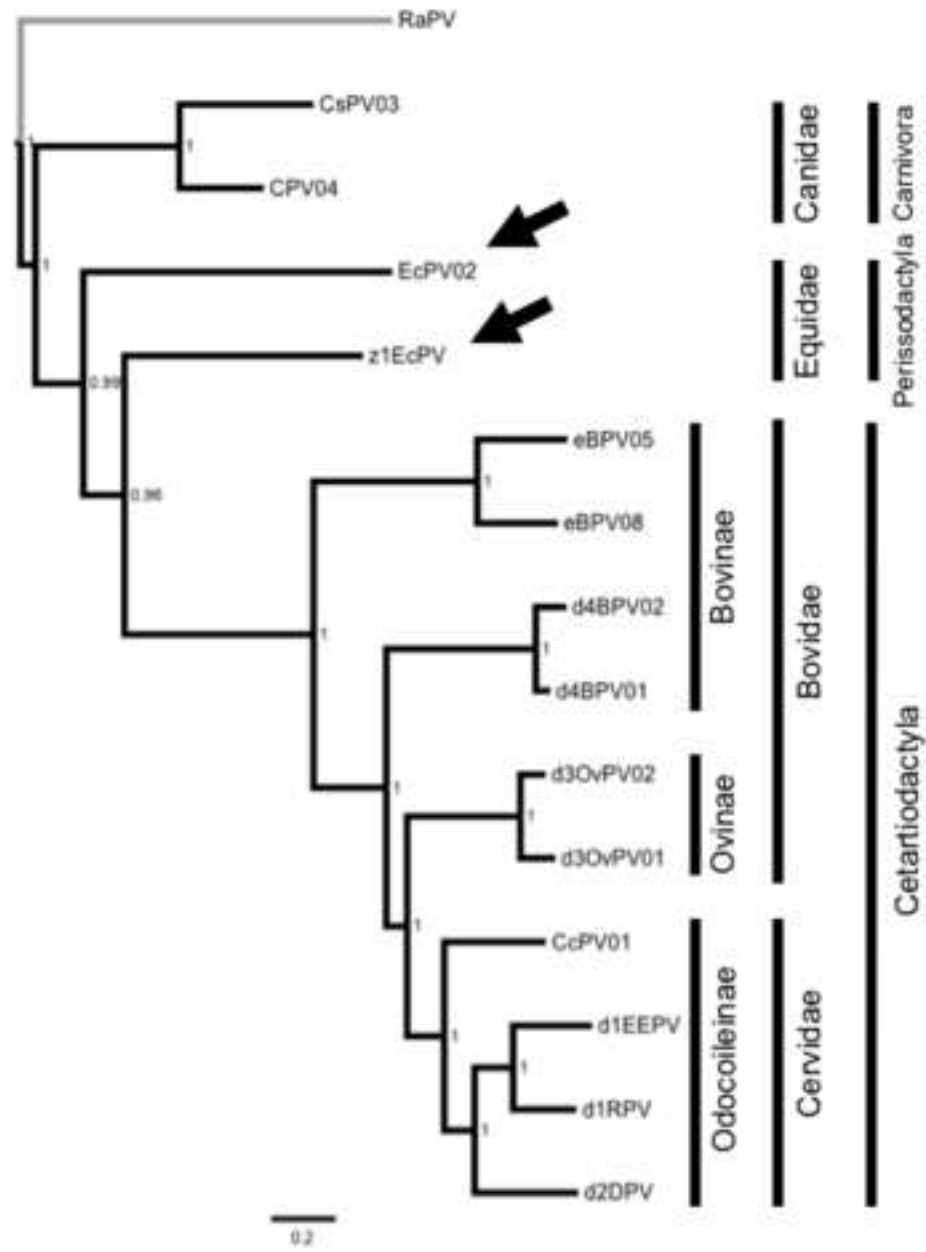
Figure 2
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