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Short Communication

Genital warts in Burmeister's porpoises: characterization of *Phocoena spinipinnis* papillomavirus type 1 (PsPV-1) and evidence for a second, distantly related PsPV

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We identified sequences from two distantly related papillomaviruses in genital warts from two Burmeister's porpoises, including a PV antigen-positive specimen, and characterized *Phocoena spinipinnis* papillomavirus type 1 (PsPV-1). The PsPV-1 genome comprises 7879 nt and presents unusual features. It lacks an E7, an E8 and a bona fide E5 open reading frame (ORF) and has a large E6 ORF. PsPV-1 L1 ORF showed the highest percentage of nucleotide identity (54–55 %) with human papillomavirus type 5, bovine papillomavirus type 3 (BPV-3) and *Tursiops truncatus* papillomavirus type 2 (TtPV-2). This warrants the classification of PsPV-1 as the prototype of the genus *Omikronpapillomavirus*. PsPV-1 clustered with TtPV-2 in the E6 and E1E2 phylogenetic trees and with TtPV-2 and BPV-3 in the L2L1 tree. This supports the hypothesis that PV evolution may not be monophyletic across all genes.

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses constituting the family *Papillomaviridae*. Their genome of about 8 kb comprises an early (E) region encoding non-structural proteins, a late (L) region encoding two capsid proteins and a non-coding upstream regulatory region (URR) controlling virus replication and transcription (Howley & Lowy, 2001; Münger *et al.*, 2004). PVs cause benign hyperproliferative epithelial lesions of the skin and mucosa (warts, papillomas and condylomas). High-risk PVs may induce invasive carcinomas (Lowy & Howley, 2001). Over 200 different PV types have been found in human beings and animals. They have been classified into 18 genera identified by Greek letters according to phylogenetic criteria (de Villiers *et al.*, 2004; ICTV, 2006).

Genital warts caused by PVs are common in Burmeister's porpoises (*Phocoena spinipinnis*) from Peru (Van Bressem *et al.*, 1996; Cassonnet *et al.*, 1998). Here we report on the detection of two PV sequences in genital tumours of two porpoises and on the cloning and characterization of *Phocoena spinipinnis* papillomavirus type 1 (PsPV-1), the first PV isolated in cetaceans and the first genital PV detected in mammals belonging to an order other than the Primates (Cassonnet *et al.*, 1998). Methods are described in Supplementary Methods, available in JGV Online.

Seven genital warts were collected from four porpoises from Peru (Supplementary Table S1, available in JGV Online). Group-specific PV antigen was detected in a wart of porpoise JAS-44 using antibodies against disrupted virions of human papillomavirus (HPV) type 1.

Total DNA extracted as described previously (Kawashima *et al.*, 1990) was analysed by PCR using primers located in the L1 open reading frame (ORF) (Kawashima *et al.*, 1990; Ting & Manos, 1990; de Roda Husman *et al.*, 1995). A 560 bp fragment amplified by the IPC primers was detected in the PV antigen-positive JAS-44 wart after Southern blot hybridization with HPV-11 and HPV-16 L1

The GenBank/EMBL/DDBJ accession nos of the sequences reported in this paper are AJ238373, AJ006300 and AJ006301.

Supplementary data are available with the online version of this paper.

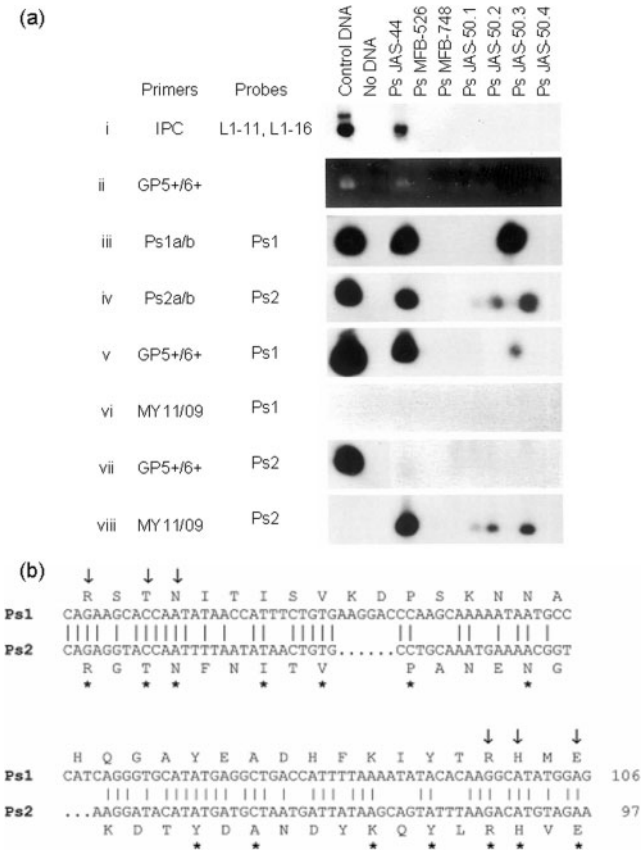

Fig. 1. (a) Detection of PV sequences in DNA wart preparations using primers IPC (row i), GP5+/GP6+ (rows ii, v and vii), MY11/MY09 (rows vi and viii), Ps1 a/b (row iii) and Ps2 a/b (row iv). PCR products were detected by EB (row ii) or SB hybridization using L1-11 and L1-16 (row i), Ps1 (rows iii, v and vi) or Ps2 (rows iv, vii and viii) probes. Controls were HPV-18 DNA fragments amplified using IPC (row i, 562 bp), GP5+/GP6+ (rows ii, v and vii, 145 bp) or MY11/MY09 (rows vi and viii, 455 bp) primers, and fragments amplified from JAS-44 DNA preparation using Ps1 a/b (row iii, 106 bp) and Ps2 a/b (row iv, 97 bp) primers. Sample code is given at top of figure. (b) Alignment of nucleotide and amino acid sequences of Ps1 and Ps2. Vertical bars indicate identical nucleotides. Asterisks show shared amino acids. Arrows point to amino acids conserved among PVs.

probes (L1-11 and L1-16; Kawashima *et al.*, 1990) (Fig. 1a row i). DNA fragments were amplified from this sample after a single-step PCR with GP5+/GP6+ primers (Fig. 1a row ii) or a nested PCR using MY11/MY09 primers for the first round and GP5+/GP6+primers for the second (data not shown). Direct sequencing of the GP5+/GP6+ amplicons after the single-step (Ps1; GenBank accession no. AJ006300) and nested PCRs (Ps2; accession no. AJ006301) disclosed a 106 and a 97 bp long ORF, respectively (without the primers). Ps1 and Ps2 shared 56.6 % identical nucleotides. Their deduced polypeptide sequences (35 and 32 aa, respectively) presented 40 % identical amino acids, including six highly conserved residues diagnostic of PV L1 proteins (Fig. 1b) (Chan *et al.*, 1997). The Ps1 nucleotide sequence was more closely related to the L1 sequence of HPV-90 (59.6 %) and the Ps2 sequence to the L1 of the manatee papillomavirus, TmPV-1 (66.3 %). Both have a high percentage of sequence identity with bovine papillomavirus (BPV) type 3, a member of the genus *Xipapillomavirus* (Ps1, 57.4 %; Ps2, 67.6 %). Ps1 presented the highest percentages of amino acid identity with HPV-4 (50 %), HPV-102 (48 %) and *Capra hircus* PV-1 (47 %), while Ps2 had highest identities with *Equus caballus* PV-1 (62 %), *Capra hircus* PV-1 (55 %) and *Tursiops truncatus* papillomavirus type 2 (TtPV-2, 51 %), using the NCBI BLASTP server. Divergences between PVs infecting the same host are not uncommon. *Canis familiaris* PV-2 is more closely related to bovine xipapillomaviruses and human gammapapillomaviruses than to canine oral PV and *Felis domesticus* PV (lambdapapillomaviruses) (Yuan *et al.*, 2007). Presence of PV sequences in wart DNA preparations was further examined using Ps1a/b and Ps2a/b primers and Ps1 and Ps2 probes. In addition to JAS-44, Ps1 sequences were detected in one out of four vaginal lesions from porpoise JAS-50 (Fig. 1a row iii) and Ps2 sequences in the three other JAS-50 specimens (Fig. 1a row iv). Ps1 sequences were found in the one-step GP5+/GP6+ PCR products (Fig. 1a rows v and vii) and Ps2 sequences in the MY11/MY09 PCR products (Fig. 1a rows vi and viii). Thus, GP5+/GP6+ and MY11/MY09 primers identified two putative, distantly related, genital *Phocoena spinipinnis* PV (PsPV) types. PV sequences were not detected in two other genital warts (Fig. 1a), probably because of insufficient amounts of DNA.

To identify unique restriction enzyme recognition sites in PsPV genomes, the JAS-44 DNA wart preparation was cleaved by several restriction endonucleases and analysed by Southern blot hybridization with Ps1 or Ps2 probes. Only Ps1 probe gave a faint signal, allowing the detection of a 7.9 kb fragment generated by *Eco*RI and *Eco*RV. The PsPV-1 genome was inserted into bacteriophage λ ZAP II DNA at the *Eco*RI site and subcloned (Short et *al.*, 1988; Longuet *et al.*, 1996). No recombinant phage-containing Ps2 sequence was isolated. The complete nucleotide sequence of the PsPV-1 genome was determined (GenBank accession no. AJ238373). It consists of 7879 bp and has a G+C content of 46.4 mol%. The ORFs encoding the early proteins E1, E2, E4 and E6 and the late proteins L1 and L2 are located on the same DNA strand. E4 ORF is collinear to E2 and has no potential ATG start codon.

The URR located between the stop codon of L1 and the first ATG of E6 is 621 bp long (positions 7366–107). It contains five potential promoter sequences (TATAA, TATAT or TATATAT) at positions 7408, 7569, 7860, 10 and 58, and the putative late AATAAA polyadenylation signal at position 7538. Two consensus binding sites (BSs) for the viral E2 protein (ACC-N6-GGT; Androphy *et al.*, 1987) are found at positions 7830 and 40, flanking an E1-BS (ATGATTGTTAACAATTAT) (Ustav *et al.*, 1991) located at position 7874. PsPV-1 URR also contains

possible BSs for transcription factors found in the URR of genital HPVs (O'Connor *et al.*, 1995), i.e., GC-box binding protein (SP1; positions 7671, 7712, 7735, 7753, 45), activator protein 1 (AP1; positions 7368, 7738, 7803), nuclear factor 1 (NF1; position 7752), octamer-binding protein 1 (Oct-1; position 7489), Ying Yang 1 (YY1; position 7405) and CCAAT/enhancer-binding protein (C/EBP; positions 7377, 7426, 7475, 7616). Several potential regulatory signals were found downstream of the URR: potential promoter sequences (TATAA at positions 581, 786, 1548, 1836 and 6861; TATATA at positions 783, 4087, 5137 and 6066), E2-BSs (positions 1376, 2943 and 5201) and potential polyadenylation signals (AATAAA, position 3448; ATTAAA, position 4199).

The PsPV-1 genome organization displays several atypical features. No E7 ORF was found. This was confirmed by sequencing the PCR products obtained after amplification of the region encompassing the 3′ end of E6 and the 5′end of E1 directly from JAS-44 and JAS-50 DNA preparations. An E7 ORF is also missing in two other cetacean PVs, TtPV-1 and TtPV-2 (Rector *et al.*, 2006; Rehtanz *et al.*, 2006). E7 proteins promote proliferation of the basal and parabasal cells of squamous epithelia and allow vegetative viral DNA replication in growth-arrested, terminally differentiating keratinocytes (Howley & Lowy, 2001). In high-risk HPVs, E7 proteins fulfil this function by binding to members of the retinoblastoma (Rb) tumour suppressor protein family (Oh *et al.*, 2004). A Leu-X-Cys-X-Glu sequence (Leu-Lys-Cys-Thr-Glu), critical for Rb protein-binding by HPV E7 (Howley & Lowy, 2001), is present in a putative 26 aa long peptide encoded by a short ORF overlapping the 5′ end of PsPV-1 E1. It could be a remnant of an ancestral PV E7 ORF (Terai *et al.*, 2002).

PsPV-1 E6 ORF encodes a 211 aa protein, larger than that usually observed in PVs (about 150 aa) (Howley & Lowy, 2001). A large E6 protein (206 aa) was also found in TtPV-2 (Rehtanz *et al.*, 2006). The sequence of the PsPV-1 E6 protein can be aligned with E6 proteins of human and animal PVs over a 128 aa region (Ile-9 to Cys-136). This region harbours two conserved zinc-finger motifs CXXC-X29-CXXC separated by 36 aa (Cys-27 to Cys-136), as well as other conserved residues, such as Leu-12, Leu-34, Leu-47, Glu-86, Arg-99, Glu-111 or Lys-112 (Van Ranst *et al.*, 1992; Howley & Lowy, 2001). These motifs and residues play a crucial role in the structure and activities of HPV E6 proteins (Nominé *et al.*, 2006). In high-risk genital HPVs, a phenylalanine residue (Phe-47 for HPV-16) is important for the recruitment of the p53 protein to the ubiquitin ligase E6AP and for its E6-mediated degradation (Nominé *et al.*, 2006). This residue is not conserved in PsPV-1 (Thr-44) or TtPV-2 (Tyr-63). The 75 aa long carboxyl-terminal end of PsPV-1 E6 has a high content (34.6 %) of serine and threonine but no cysteine. It shows no significant similarity with any PV protein, except for a stretch of 28 aa (positions 167–194) that shares 32 % identical amino acids with TtPV-2 E6. In contrast to TtPV-2 (Rehtanz *et al.*, 2006), the extreme carboxyl terminus of PsPV-1 E6 does not harbour a PSD-95/Disc-large/ZO-1 (PDZ) domain-binding motif X-S/T-X-V/L (where X represents any amino acid) found in E6 proteins of genital oncogenic HPVs (Kiyono *et al.*, 1997). The interaction of the E6 oncoproteins with specific PDZ proteins leads them to proteolytic degradation and probably plays an important role in pathogenesis (Münger *et al.*, 2004).

PsPV-1 lacks the bona fide E5 ORF present in the E2–L2 intergenic region of alphapapillomaviruses and deltapapillomaviruses. In contrast, TtPV-2 has an E5 ORF encoding a putative 103 aa long protein with a 52.4 % Ile + Leu + Val content. PsPV-1 also lacks the E8 ORF observed in gammapapillomaviruses, kappapapillomaviruses and mupapillomaviruses devoid of an E5 ORF (Bravo & Alonso, 2004; García-Vallvé *et al.*, 2005; Nonnenmacher *et al.*, 2006) and in xipapillomaviruses lacking an E6 ORF (Jackson *et al.*, 1991). E5 and E8 ORFs encode highly hydrophobic proteins that share similar properties and are allegedly essential for wart formation (Orth, 2006). Two short ORFs, tentatively named E5a and E5b, overlap the 3′ end of the PsPV-1 E2 ORF. They lack a start codon and have a coding capacity for polypeptides of 37 (E5a) and 34 (E5b) aa. According to the TMHMM 2.0 program (Krogh

Table 1. Percentage nucleotide (amino acid) identity of the different PsPV-1 ORFs with the corresponding ORFs of different PV types

ORF	HPV-16	HPV-6	RhPV-1	HPV-1	HPV-5	BPV-1	BPV-3	BPV-5	EcPV-1	MnPV	TtPV-2
E6	29 (20)	31 (27)	30 (22)	25 (16)	27 (20)	26 (16)	No E6	25 (19)	30 (24)	27 (19)	41 (32)
E6*	39 (27)	45 (39)	33 (24)	37 (24)	36 (26)	36 (22)	No E6	34 (25)	38 (29)	39 (28)	35 (28)
E1	52 (43)	53 (43)	53 (45)	51 (43)	48 (40)	46 (35)	48 (41)	46 (37)	50 (44)	48 (41)	55 (45)
E2	41 (29)	41 (32)	38 (30)	39 (30)	35 (28)	33 (29)	43 (31)	38 (29)	41 (33)	36 (31)	44 (32)
E4	27 (19)	26 (15)	25 (18)	30 (20)	22 (14)	27 (18)	32 (14)	29 (18)	24 (12)	24 (15)	33 (23)
L2	38 (30)	37 (30)	36 (32)	39 (32)	42 (36)	34 (28)	41 (36)	36 (29)	34 (32)	37 (28)	39 (30)
L1	50 (44)	51 (48)	52 (49)	51 (47)	55 (50)	51 (44)	55 (51)	50 (44)	51 (45)	47 (46)	54 (48)

*Identity calculated using only the first 140 amino-terminal amino acids of PsPV-1 E6 and the corresponding 420 nt at the 5′ end of the PsPV-1 E6 ORF.

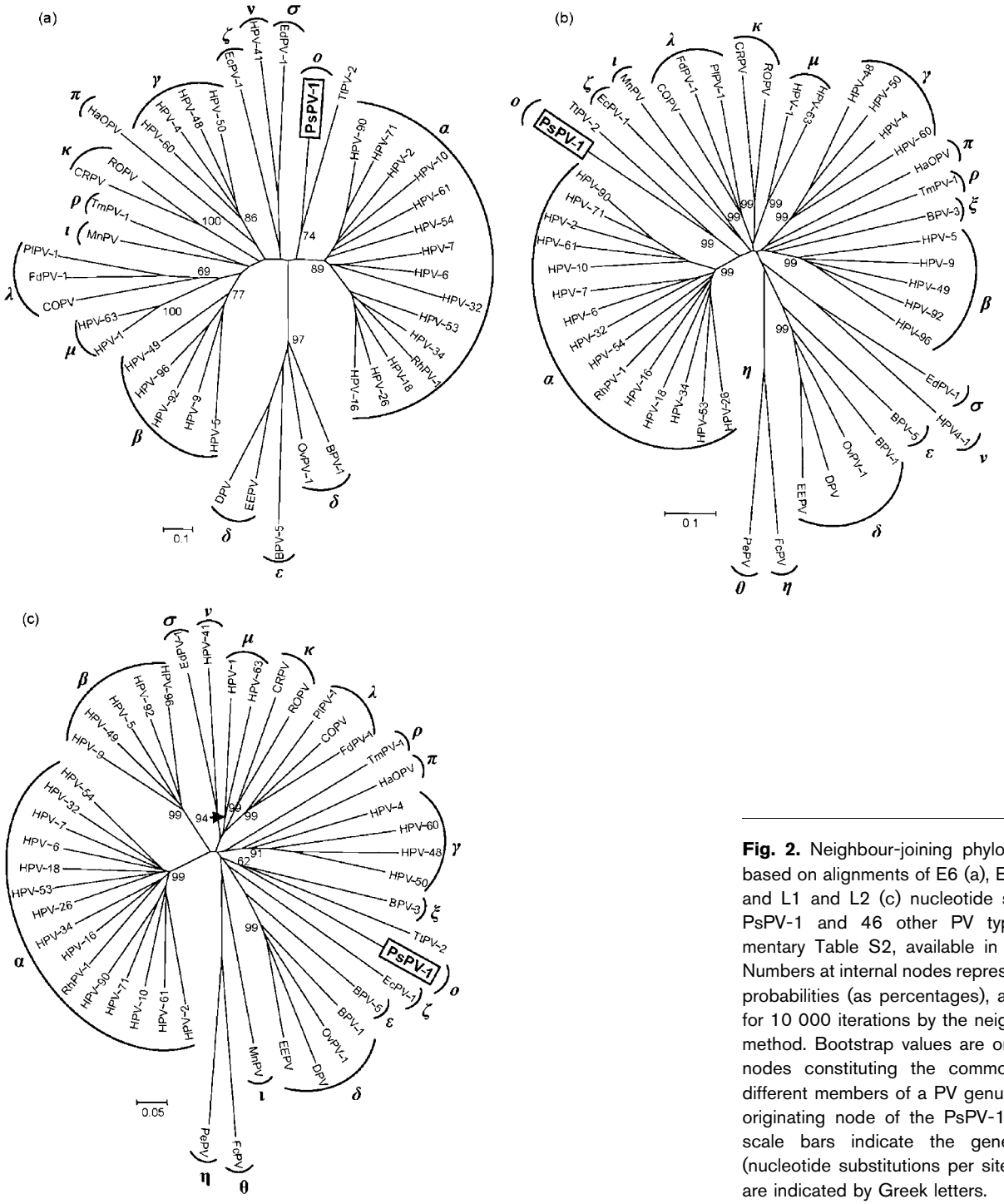

Fig. 2. Neighbour-joining phylogenetic trees based on alignments of E6 (a), E1 and E2 (b), and L1 and L2 (c) nucleotide sequences of PsPV-1 and 46 other PV types (Supplementary Table S2, available in JGV Online). Numbers at internal nodes represent bootstrap probabilities (as percentages), as determined for 10 000 iterations by the neighbour-joining method. Bootstrap values are only shown for nodes constituting the common branch of different members of a PV genus, and for the originating node of the PsPV-1 branch. The scale bars indicate the genetic distance (nucleotide substitutions per site). PV genera are indicated by Greek letters.

et al., 2001), E5b may contain an 18 aa helical transmembrane domain (Val-7 to His-23). Whether spliced transcripts allow the expression of these ORFs remains to be determined.

Despite the absence of E5/E8 and E7 ORFs and the occurrence of an unusual E6 protein, PsPV-1 seems able to cause genital warts in porpoises. Two additional ORFs, E3 and L3, with a potential start codon and a coding capacity for 61 (E3) and 169 (L3) aa polypeptides, overlap E1 and L1, respectively (Supplementary Fig. S1, available in JGV Online). Seven ORFs with an ATG start codon and a coding potential for 68–173 aa long polypeptides were detected on the complementary DNA strand (Supplementary Fig. S1). TATA boxes were found 19–250 nt upstream of their first ATG and polyadenylation signals (AATAAA) at positions 2101 and 7022 in the 5′→3′ orientation. No predictable function was found by

bioinformatics analysis for these nine putative proteins. None of them showed any significant similarity with proteins encoded by other PVs, including putative proteins encoded by the ORFs overlapping E1 or L1 or located on the complementary strand of the TtPV-2 genome (P. Cassonnet, unpublished observation). Whether some of these ORFs encode proteins that may complement those that are missing remains to be determined.

We investigated sequence identities between PsPV-1 and the prototypes of major PV genera and species. The percentage identities determined after pairwise alignments of the E6, E1, E2, E4, L2 and L1 ORFs and proteins are shown in Table 1. The highest scores were noted for the E1 and L1 ORFs, as expected (Howley & Lowy, 2001). For the E6 and E1 ORFs, PsPV-1 showed the highest identity percentages with TtPV-2 (unnamed genus) and HPV-6 (*Alphapapillomavirus*). When only the 420 nt corresponding to the first 140 aa of the PsPV-1 E6 protein were considered, the identity percentages increased with HPV-6 and decreased with TtPV-2. The highest identity scores for L1 and L2 ORFs were obtained for HPV-5, BPV-3 and TtPV-2. The fact that PsPV-1 L1 shares only 55 % nucleotide identity with the most closely related L1 ORFs warrants its classification as the sole member of the genus *Omikronpapillomavirus* (de Villiers *et al.*, 2004).

We evaluated the evolutionary relationships between PsPV-1 and 46 PV prototypes of different genera and species. Separate phylogenetic trees were constructed for the E6, E1 and E2 and L1 and L2 ORFs (Fig. 2). In the early region, PsPV-1 clusters in a monophyletic branch with TtPV-2 (Fig. 2a, b), with high bootstrap values (99 %) for the E1E2 tree, whereas BPV-3 is included in the PsPV-1/TtPV-2 branch in the L1L2 region (Fig. 2c). The different phylogenies of the early and late genes of PsPV-1 further confirm that PV evolution may not be monophyletic across all genes (García-Vallvé *et al.*, 2005; Narechania *et al.*, 2005; Bravo & Alonso, 2006; Gottschling *et al.*, 2006). The close phylogenetic relatedness of PsPV-1 and TtPV-2 and common features of their genetic organization suggest that a common ancestor of the Delphinidae and Phocoenidae (suborder Odontoceti) was infected with an ancestral cetacean PV that co-evolved with its hosts. The presence of a bona fide E5 ORF in TtPV-2 and its absence in PsPV-1 suggest that either this gene was lost from the PsPV-1 genome, or it accessed the TtPV-2 genome after the separation of Delphinidae and Phocoenidae about 11–25 million years ago (Fordyce & Barnes, 1994; Waddell *et al.*, 2000; Nikaido *et al.*, 2001).

Our data indicate that at least two papillomaviruses cause genital warts in Burmeister's porpoises. A PV detected in a cutaneous wart from a harbour porpoise (*Phocoena phocoena*) (Van Bressem *et al.*, 1999) probably represents another PV infecting members of the Phocoenidae. The unusual features of PsPV-1, its association with genital warts and the existence of a putative second genital PsPV emphasize the need to further study cetacean PVs to obtain a deeper insight into PV gene history and mechanisms underlying PV diversification.

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