Infection, Genetics and Evolution 42 (2016) 20–29

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Characterization of novel human papillomavirus types 157, 158 and 205 from healthy skin and recombination analysis in genus γ-Papillomavirus

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ARTICLE INFO

Article history:
Received 11 December 2015
Received in revised form 14 April 2016
Accepted 15 April 2016
Available online 21 April 2016

Keywords:
Novel human papillomavirus
HPV157
HPV158
HPV205
Genus γ-PV
Recombination

ABSTRACT

Gammapapillomavirus (γ-PV) is a diverse and rapidly expanding genus, currently consisting of 79 fully characterized human PV (HPV) types. In this study, three novel types, HPV157, HPV158 and HPV205, obtained from healthy sun-exposed skin of two immunocompetent individuals, were amplified by the "*Hanging droplet*" *long PCR* technique, cloned, sequenced and characterized. HPV157, HPV158 and HPV205 genomes comprise 7154-bp, 7192-bp and 7298-bp, respectively, and contain four early (E1, E2, E6 and E7) and two late genes (L1 and L2). Phylogenetic analysis of the L1 ORF placed all novel types within the γ-PV genus: HPV157 was classified as a new member of species γ-12 while HPV158 and HPV205 belong to species γ-1. We then explored potential recombination events in genus γ-PV with the RDP4 program in a dataset of 74 viruses (71 HPV types with available full-length genomes and the 3 novel types). Two events, both located in the E1 ORF, met the inclusion criterion (*p*-values <0.05 with at least four methods) and persisted in different ORF combinations: an inter-species recombination in species γ-8 (major and minor parents: species γ-24 and γ-11, respectively), and an intra-species recombination in species γ-7 (recombinant strain: HPV170; major and minor parents: HPV-109 and HPV-149, respectively). These findings were confirmed by phylogenetic tree incongruence analysis. An additional incongruence was found in members of species γ-9 but it was not detected by the RDP4. This report expands our knowledge of the family *Papillomaviridae* and provides for the first time *in silico* evidence of recombination in genus γ-PV.

1. Introduction

Papillomaviruses (PVs) are small non-enveloped DNA tumour viruses with a circular genome of nearly 8 Kbp that belong to the *Papillomaviridae* family (de Villiers et al., 2004). PVs are mainly host-specific and have been found in >20 different mammalian as well as bird and reptile species (Bravo et al., 2010). By convention, a novel PV type shows <90% nucleotide identity in the L1 gene with respect to any known PV type, while PV types belonging to new species within a genus share 60% to 70% nucleotide identity with PV types of any other known species (de Villiers, 2013). Definition of a new PV type requires the isolation and characterization of the full-length genome in order to verify the typical genetic organization of PVs (de Villiers, 2013).

Human PV (HPV) types are classified into mucosal/genital and cutaneous types based on clinical manifestations. Taxonomically, HPVs are distributed into five genera (α-, β-, γ-, μ- and ν-PV) according to the phylogenetic relationships of their complete L1 gene sequences. Mucosal/genital HPV types are grouped into the α-PV genus while cutaneous HPV types are distributed into the α-, β-, γ-, μ- and ν-PV genera (de Villiers et al., 2004).

In the last decade, the number of HPV types that has been characterized has considerable grown, from 96 types when the family *Papillomaviridae* was defined (de Villiers et al., 2004) to 198 types in the last HPV classification update (Bzhalava et al., 2015). Among the genera containing HPVs, genus γ-PV has been growing rapidly with 79 completely sequenced types, surpassing genera α- and β-PV, with 65 and 51 recognized HPV types, respectively (Bzhalava et al., 2015).

In contrast to the mucosal/genital HPVs, the involvement of cutaneous HPV types in human carcinogenesis is still unclear. Cutaneous HPVs have been found in benign skin warts and in non-melanoma skin cancer (NMSC) (Forslund et al., 2007; Hosnjak et al., 2015), but also on healthy

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http://dx.doi.org/10.1016/j.meegid.2016.04.018
1567-1348/

skin worldwide (Antonsson et al., 2003; Ekstrom et al., 2011; Foulongne et al., 2012). Ultraviolet (UV) radiation is a well-known risk factor for NMSCs but there may also be other risk factors associated to the development of this disease. In this sense, cutaneous HPVs are considered as the infectious agents acting as co-factors for the development of NMSC (Akgul et al., 2006).

Cutaneous HPV types represent about 75% of the HPVs described up to date, genus γ-PV being the highest divergent genus (http://www.hpvcenter.se/). In the last few years, several studies have found γ-PV types not only in skin specimens but also in different mucosal sites, including the oral and nasal cavity, penis and anal canal (Bottalico et al., 2011; Forslund et al., 2013; Sichero et al., 2013). In addition, novel evidence shows that the new γ-PV type HPV-197 is the most commonly detected virus in skin tumour specimens (Arroyo Muhr et al., 2015). These findings are due to improvements in the methods used for the detection of cutaneous HPV types and further efforts may eventually lead to the identification of additional types associated with skin cancer (Forslund et al., 2003a; Kocjan et al., 2015). However, there is still a high amount of cutaneous HPV types whose genomes need to be described (putative types). Approximately 150 putative new types belonging to new species of γ-PV genus have been described (Chouhy et al., 2013a). Although the number of cutaneous HPV and putative HPV types has considerably increased, knowledge about their phylogeny is insufficient. It has been shown that PVs have a slow evolutionary rate (Rector et al., 2007), suggesting that genetic drift is mainly responsible for viral diversity (Van Doorslaer, 2013). An additional force which is generally considered to be responsible for shaping the evolutionary history of PVs is co-evolution. In this way, PVs are thus believed to be well adapted to their hosts and to evolve slowly (Bravo and Felez-Sanchez, 2015). Nevertheless, the inconsistent tree topologies, either globally or with respect to specific associations between PVs and their hosts, might arise from singular events in the past, such as recombination. At present, several studies have reported evidences of recombination in types from genus α-PV, detecting statistically supported recombination events between the early and the late regions of the genome (Narechania et al., 2005; Ronco et al., 2014; Varsani et al., 2006). Moreover, Shah *et al* (Shah et al., 2010) proposed that multiple recombination events may have occurred among the E1 and/or L1 genes of the α-, β-, and γ-PV primate genera. As the rate of discovery of new HPVs is increasing, continuous efforts are needed to get deeper insights in the evolutionary history of these viruses, especially those belonging to genus γ-PV.

In this study, three novel γ-PV types were isolated from healthy sun-exposed skin of immunocompetent individuals by a highly sensitive amplification strategy (Chouhy et al., 2013b), fully cloned, sequenced and phylogenetically characterized. The new types were submitted in the *International HPV Reference Center* and officially designated HPV157, HPV158 and HPV205. In addition, we explore recombination as an evolutionary force influencing the genetic diversity of genus γ-PV by using bioinformatic tools.

2. Materials and methods

2.1. Skin samples

The skin samples used for this study were collected in a previous study whose aim was directed to estimate the HPV prevalence and type spectrum in skin diseases and in healthy skin areas in a group of immunocompetent individuals from Argentina (Chouhy et al., 2010). The selected samples contained one or more putative HPV types from the γ-PV genus according to the CUT and/or FAP primer system screening (Chouhy et al., 2010).

2.2. "Hanging droplet" long PCR, cloning and DNA sequencing

The general strategy for generating HPV DNA genomic halves was previously shown (Chouhy et al., 2013b). E1 γ-PV primers (E1Gamma-R and E1Gamma-F) (Chouhy et al., 2013b) and different sets of L1 primers [FAP6085-F and FAP64-Rv (Forslund et al., 2003b), and CUT1B-Rv (Chouhy et al., 2010)] were used. Amplifications were performed using the previously described "*Hanging droplet*" *long PCR* technique (Chouhy et al., 2013b). A thermal cycler Mastercycler Personal (Eppendorf) was used in all the experiments involving PCR.

Bands of approximately 4 Kbps were excised from the gel and purified using a NucleoSpin Extract II kit (Macherey-Nagel). All the amplicons were cloned using a pGEM®-T Easy Cloning kit (Promega). DNA sequencing was performed by the Sanger technology using sequencing facilities (University of Maine DNA Sequencing Facility, US). Sequences were compared to available HPV-sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/) by using the BLAST server.

2.3. Identification of the full-length genome of HPV157

HPV157 was isolated from a swab sample collected from a sun-exposed healthy skin area from a 64-year-old female with a seborrheic keratosis lesion in the head, and corresponded to the previously reported putative type GC08 (Accession Number FJ969904) (Chouhy et al., 2010). The complete genome sequence of the novel HPV-157 type was obtained by generating overlapping amplicons as reported (Chouhy et al., 2013b). One of the overlapping amplicons was obtained with the E1Gamma-F/CUT1BRv primer pair in the described amplification conditions (Chouhy et al., 2013b). The other overlapping amplicon was obtained with GC08-F (5′-ggacctttaggaattggcagtacagg-3′) and GC08-R (5′-acctcttgaggcacagacacg-3′) specific primers, both designed using the 3´ and 5´ regions of the GC08 genomic half obtained with the E1Gamma-F/CUT1BRv primer pair as sequence template, and the FastPCR program (Kalendar et al., 2009). Reaction mixtures and cycling conditions were as previously described (Chouhy et al., 2013b), except for the reaction mix of the second round of amplification, in which 1.2 μM of each specific primer were used. The annealing temperatures of the GC08-F/GC08-R primer pair were 52 °C and 50 °C in the first and second rounds of amplification, respectively. Amplicons of approximately 4 Kbps were purified, cloned and sequenced as described above. Each GC08 genomic half was fully sequenced by primer walking on both DNA strands for complete identification. DNA clones and the corresponding sequences were submitted to the *International HPV Reference Center* at the *German Cancer Research Centre* (Heidelberg, Germany) for official designation as HPV157, and the sequences were reconfirmed.

2.4. Identification of the full-length genome of HPV158

HPV158 was isolated from the same swab sample from which HPV157 was obtained. HPV158 corresponded to the recently identified putative type GC23 (Accession Number JX429975) (Chouhy et al., 2013b). The complete genome sequence of the novel HPV158 type was obtained as reported (Chouhy et al., 2013b). One of the overlapping amplicons was achieved with the FAP6085F/E1Gamma-R primer pair in the same amplification conditions previously reported (Chouhy et al., 2013b). The other overlapping amplicon was obtained with GC23-F (5′-cctgataccggaaaatccatgttttgc-3′) and GC23-R (5′-ccaaaccaagcgctcatgc-3′) specific primers, both designed using the 3´ and 5´ regions of the GC23 genomic half obtained with the FAP6085F/E1Gamma-R primer pair as a sequence template, and the FastPCR program (Kalendar et al., 2009). Reaction mixtures and cycling conditions were the same as described for HPV157. The annealing temperatures of the GC23-F/GC23-R primer pair were as described for HPV157. Amplicons of approximately 4 Kbps were purified, cloned and sequenced as described above. DNA clones and the corresponding sequences were submitted to the *International HPV Reference Center* at the *German Cancer Research Centre* (Heidelberg, Germany) for official designation as HPV158, and the sequences were reconfirmed.

2.5. Identification of the full-length genome of HPV205

HPV205 was isolated from a swab sample collected from a sun-exposed healthy skin area from a 46-year-old female without skin pathologies. HPV205 corresponded to a new putative HPV type named EP01 [nucleotide (nt) position 5486 to 5858 of HPV205 (Accession number: KT698167)] that was identified for the first time in this work with the CUT primers as previously reported (Chouhy et al., 2010). The complete genome sequence of the novel HPV205 type was obtained as reported (Chouhy et al., 2013b). One of the overlapping amplicons was obtained with the FAP64-R/E1Gamma-F primer pair in the same amplification conditions previously reported (Chouhy et al., 2013b). The other overlapping amplicon was obtained with 27 A-F (5′-ggagggcctttgggtatt-3′) and 27 A-R (5′-acgcgtttcttaggttttgat-3′) specific primers, both designed using the 3′ and 5′ regions of the EP01 genomic half obtained with the FAP64-R/E1Gamma-F primer pair as a sequence template, and the FastPCR program (Kalendar et al., 2009). Reaction mixtures and cycling conditions were as described for HPV157 and HPV158. The annealing temperatures of the 27 A-F/27 A-R primer pair were 50 °C in both rounds of amplification. Amplicons of approximately 4 Kbps were purified, cloned and sequenced as described above. DNA clones and the corresponding sequences were submitted to the *International HPV Reference Center at the Karolinska Institutet* (Stockholm, Sweden) for official designation as HPV205, and the sequences were reconfirmed.

2.6. ORF and phylogenetic analysis

The ORFs of the three novel types were determined by using the ORF finder function of SnapGene 1.1.3 (GSL Biotech LLC). A multiple sequence alignment of the L1 ORF of HPV157, HPV158, HPV205 and 188 previously characterized HPV types available at http://www.hpvcenter.se/and http://pave.niaid.nih.gov/was constructed using the ClustalW algorithm of the MEGA6 software package at the nt level. The phylogenetic relationships were inferred by Bayesian analysis using Beast version 1.7.5 (Drummond et al., 2012). To do so, Markov Chain Monte Carlo (MCMC) simulations were performed during 10^7 generations, sampling one state every 1000 generations, with a burn-in of 10%. The JModelTest v2 (Darriba et al., 2012) determined that GTR + I + G was the best fit evolutionary substitution model for each run. The priors were set as default according to the chosen evolutionary model. Statistical convergence of MCMC was assessed visually by the traceplot and by calculating the effective sample size using TRACER v1.4 (available from http://beast.bio.ed.ac.uk/Tracer). The maximum clade credibility tree across all the plausible trees generated by BEAST was then computed using the TreeAnnotator program available in the BEAST package.

2.7. Recombination analysis

The dataset used to examine the phylogenetic relationships of the three novel viruses included 71 reference γ-PV types with available full-length genomes up to March 2015. Among them, 62 types were officially recognized HPVs and available at http://www.hpvcenter.se/, while 9 viruses corresponded to types identified by metagenomic sequencing that were deposited in http://pave.niaid.nih.gov/.

Due to the ambiguous alignments in non-coding regions and the high evolutionary distances of the E6-E7 region (Garcia-Vallve et al., 2005), only E1, E2, L2 and L1 ORFs were used (Bravo et al., 2010; Garcia-Perez et al., 2014; Gottschling et al., 2011). Multiple sequence alignments were created separately for each ORF using CLUSTAL W (Larkin et al., 2007) according to the amino acid sequences (BLOSUM protein weight matrix) to preserve codon identity. Determination of potential recombinant events, parental sequences and localization of recombinant breakpoints were performed triplet-by-triplet on each ORF separately and combining an increasing number of ORFs, as follows: *i*) E1, *ii*) E2, *iii*) L2, *iv*) L1, *v*) E1-E2, *vi*) E1-E2-L1, *vii*) E1-E2-L2 and *viii*) E1-E2-L2-L1. Recombination analysis was performed using the Recombination Detection Program (RDP) 4.1 (Martin et al., 2015), and the algorithms embedded in it: GENECONV (Padidam et al., 1999), BootScan (Martin et al., 2005) MaxChi (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCan (Gibbs et al., 2000), and 3Seq (Boni et al., 2007). *p*-values <0.05 were regarded as statistically significant. Bonferroni multiple comparison correction was set in the general options. All other parameters were set as default RDP settings. Only statistically significant events over four programs were taken into account for further analysis (inclusion criterion).

In order to confirm the potential recombinant events obtained with RDP program, phylogenetic tree incongruence analyses were performed using the Bayesian method (as described earlier) and Maximum Likelihood using RaxML program (Stamatakis et al., 2005) with an evolutionary substitution model set as GTR + G + I with rapid bootstrap of 2000 replicates.

2.8. Computational resources

This research was performed in part through the computational resources provided by the High Performance Computing (HPC) Cluster available at the "Centro Científico Tecnológico CONICET-Rosario" (http://cluster.rosario-conicet.gov.ar), Rosario City, Santa Fe province, Argentina.

2.9. Nucleotide sequence accession numbers

Novel HPV types have been submitted to GenBank with the following accession numbers: HPV157 (KT698166), HPV158 (KT698168), and HPV205 (KT698167).

3. Results

3.1. Characterization of the novel types HPV157, HPV158 and HPV205

The "*Hanging droplet*" *long PCR* technique (Chouhy et al., 2013b) was used as screening strategy to obtain the complete viral genomes of the novel HPV types. Phylogenetic analyses and pairwise comparisons of the L1 ORFs confirmed HPV157, HPV158 and HPV205 as γ-PV types (Fig. 1), showing nucleotide identities ranging from 79.8% to 59.3% with viruses representing current γ-PV species (data not shown). HPV157 represents a new member of species γ-12, showing the highest identity to HPV148 (76.4%). Instead, HPV158 and HPV205 belong to species γ-1, showing the highest identities to HPV4 (72.7%) and HPV173 (85%), respectively.

The main genomic features of the three novel types are depicted in Table 1. ORF analysis and sequence alignment comparisons showed the typical genomic organization of cutaneous HPVs, with six ORFs (E6, E7, E1, E2, L2 and L1). As other members of γ-PV genus, the intergenic region located between E2 and L2 genes was very short (HPV157: 1-bp, nt position 3723; HPV158: 1-bp, nt position 3715; HPV205: 20-bp, nt position 3632–3652), and thus there was no possible space for an E5 ORF in any of the new viruses.

No canonical E4 ORF nested within E2 could be identified in the novel types. However, short sequences of 118–128 amino acids in length (HPV157: nt position 2994–3248; HPV158: nt position 3021–3275; HPV205: nt position 3036–3392), with remnants of the proline-rich stretches that characterize E4, were detected. Although no start codons were found in these sequences, the putative E4 proteins of the novel viruses are likely to be translated from a spliced mRNA consisting of the first few codons of the E1 ORF joined to the E4 ORF (the E1^E4 spliced mRNA), as shown for other HPVs (Doorbar, 2013; Kovanda et al., 2011). The degree of similarity of these potential E4 proteins with respect to already known types

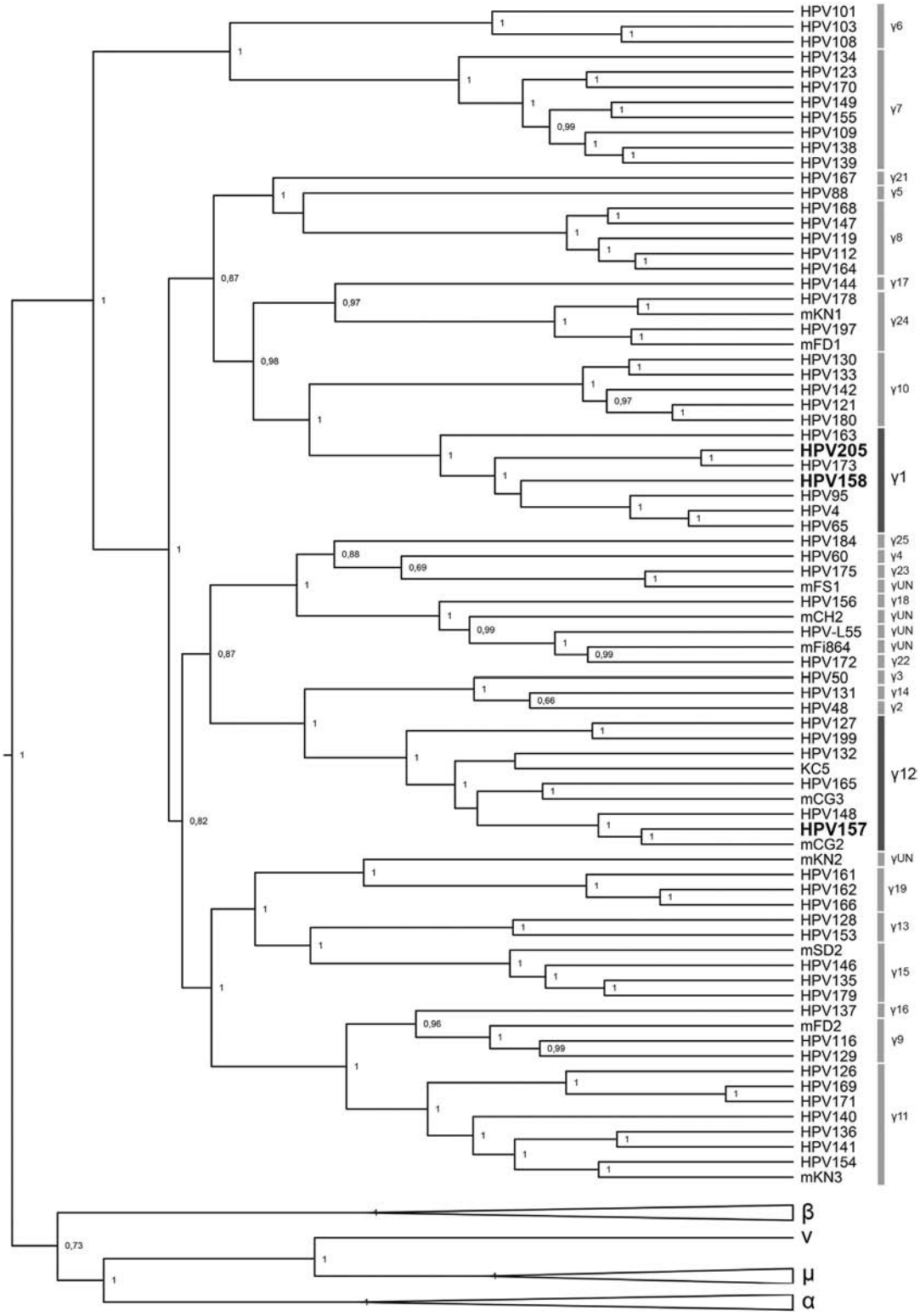

Fig. 1. Phylogeny of the novel HPV types found in this study with respect to reference HPVs. Phylogenetic analysis of the L1 ORF sequences from the 3 novel HPV types identified in this study and reference HPVs from α-, β-, γ-, μ-, and ν-PV genera. Bayesian posterior probability values (BPP) > 0.50 are shown. Novel types HPV157, HPV158 and HPV205 are shown in bold; the corresponding species are depicted in dark grey. γ-un: species not included in the current HPV taxonomy (Bzhalava et al., 2015).

was dissimilar. The putative HPV157 E4 protein showed the highest degree of similarity (73%) with the HPV148 E4 protein. Instead, the putative HPV158 and HPV205 E4 proteins had lower degrees of similarity, sharing 52% and 46% with HPV95 and HPV65 E4 proteins, respectively.

In addition to the mentioned ORFs, the genomes of the three new HPV types contained a non-coding upstream regulatory region (URR) with typical TATA box (TATAA) and E2 binding sites. Also, multiple potential binding sites for transcriptional regulatory factors such as AP-1, NF-1 and SP-1 were also present within the URR in the three viral sequences.

Table 1
Main genomic features and putative proteins of HPV157, HPV158 and HPV205 novel γ-PV types.

Genomic features		HPV157	HPV158	HPV205
Genome GC content (%)		37.6	38.2	37.5
Genome length (bp)		7154	7192	7298
ORFs		E6, E7, E1, E2, L2, L1	E6, E7, E1, E2, L2, L1	E6, E7, E1, E2, L2, L1
URR	Length (bp)	467	464	500
	Nucleotide sequence (pre-stop codon)	nt 6688–7154	nt 6729–7192	nt 6799–7298
	TATA box	1	1	2
	E2 binding site ACC(N)$_6$GGT	3	2	1
E6	Protein size (aa)	138	140	140
	Nucleotide sequence (pre-stop codon)	nt 1–417	nt 1–423	nt 1–423
	Zinc binding domains CXXC(X)$_{29}$CXXC[†]	2 separated by 36 aa	2 separated by 36 aa	2 separated by 36 aa
	PDZ binding domains X(T/S)X(L/V)[#]	PTRL (5–8 aa)	LSLV (47–50 aa)	
			RTSV (81–84 aa)	PTGL (6–9 aa)
		CSLV (33–36 aa)	TSVL (82–85 aa)	LSLV (47–50 aa)
			ESLL (86–89 aa)	LSAL (66–69 aa)
E7	Protein size (aa)	93	95	97
	Nucleotide sequence (pre-stop codon)	nt 417–698	nt 420–707	nt 420–713
	Zinc-binding domains CXXC(X)$_{29-30}$CXXC[†]	CXXC(X)$_{29}$CXXC	CXXC(X)$_{30}$CXXC	CXXC(X)$_{30}$CXXC
	pRB binding site LXCXE[‡]	1	Absent	Absent
E1	Protein size (aa)	601	600	603
	Nucleotide sequence (pre-stop codon)	nt 682–2487	nt 694–2496	nt 700–2511
	ATP binding site GXXXXGK(T/S)[§]	GPPDSGKS	GPPDTGKS	GPPDTGKS
E2	Protein size (aa)	400	391	392
	Nucleotide sequence (pre-stop codon)	nt 2420–3622	nt 2438–3613	nt 2453–3631
L2	Protein size (aa)	506	520	527
	Nucleotide sequence (pre-stop codon)	nt 3624–5144	nt 3616–5178	nt 3653–5236
L1	Protein size (aa)	510	512	515
	Nucleotide sequence (pre-stop codon)	nt 5155–6687	nt 5190–6728	nt 5251–6798

URR: upstream regulatory region; aa: amino acid; nt: nucleotides.
[†] Zinc-binding domain consensus sequence (Ullman et al., 1996).
[#] PDZ binding domain consensus sequence (Fanning and Anderson, 1999).
[‡] pRB binding site consensus sequence (Radulescu et al., 1995).
[§] ATP-binding site consensus sequence (Titolo et al., 1999).

3.2. Recombination analysis

We next explored potential recombination events in genus γ-PV including the three novel viruses and 71 HPV types with available full-length genomes. For that purpose, an initial screening for recombination evidence with RDP4 program (Martin et al., 2015) on each ORF separately was performed. In a second screening, the number of ORFs increased gradually. Table 2 shows statistically supported recombination events (p-values <0.05) identified in this analysis with at least four methods (inclusion criterion). L1 ORF separately did not show any recombination event. Instead, statistically supported events in the E1 ORF were detected by fewer than four methods (data not shown), and therefore this gene separately did not meet our inclusion criterion. Of all recombination events that met the inclusion criterion (Table 2), only two persisted in different ORF combinations (Table 2, bold). The first event represents an inter-species recombination that was detected in all the types grouped in the γ-8 species, and involved around 200 bps in the E1 ORF (recombinant region: nt 1045 to 1253 in the E1-E2-L2-L1 alignment with respect to HPV112 reference type). The major and minor parents might be ancestor viruses belonging to species γ-24 and γ-11, respectively. The second occurrence was an intra-species recombination between viruses from species γ-7. HPV170 was identified as a recombinant type, with the recombinant region involving around 420 bps in the E1 ORF (nt 1422 to 1846 in the E1-E2-L2-L1 alignment). RDP4 identified HPV-109 and HPV-149 types as the major and minor parents, respectively.

To reinforce the results obtained with the RDP4 tool, we generated phylogenetic trees from different parts of the E1-E2-L2-L1 alignment to visualize potential phylogenetic incongruences. In order to analyze the inter-species recombination event that may give rise to species γ-8, we performed Bayesian trees using the 200 bps recombinant region (Fig. 2, left) or the rest of the alignment (E1–E2–L2–L1 concatenated alignment without the recombinant region) (Fig. 2, right). As can be seen, the recombinant species γ-8 clusters near the minor parent (γ-11) in the left tree, while species γ-8 is closer to the major parent (γ-24) in the right tree.

As expected, similar results were obtained when using the same approach to examine the phylogenetic support in the potential intra-species recombination within species γ-7 (Fig. 3). The tree performed with the 420 bp recombinant region (Fig. 3, left) showed the putative recombinant type (HPV170) clustering together with the minor parent (HPV149), while in the tree performed using the rest of the alignment (E1-E2-L2-L1 concatenated alignment without the recombinant region) (Fig. 3, right), HPV170 was closer to the major parental virus (HPV109).

Curiously, an interesting phylogenetic incongruence was found among members of species γ-9 (Fig. 2). As can be seen (Fig. 2, right), species γ-9 formed the typical monophyletic clade observed in members belonging to the same species in the tree constructed without the recombinant region (see also Fig. 1). In contrast, HPV129 drastically changed its topological place, clustering with HPV175 (γ-23) in the tree obtained using the recombinant region (Fig. 2, left). However, this phylogenetic incongruence was not detected by any of the methods included in the RDP4 program.

4. Discussion

Hundreds of putative types have been identified around the world (Chouhy et al., 2013a) indicating that efforts directed toward identifying full-length genomes of putative HPV types are required to have a complete picture of the *Papillomaviridae* family and its pathological role in humans. In line with this, we report the characterization of three new viruses, designated HPV157, HPV158 and HPV205, using a highly sensitive approach for the identification of HPV genomes (Chouhy et al., 2013b). All novel viruses have genome organizations and putative proteins (Table 1) that resembled those of other γ-PV types (http://www.ncbi.nlm.nih.gov). Phylogenetic

Table 2
Exploring recombination events in genus γ-PV.

Putative recombinant virus	Species	Breakpoint localization (virus, GenBank accession N°)[a,*]	Gene	Parent sequences		Methods	*p*-Value	
				Major	Minor			
HPV155	γ-7	nt 3253–3460 (HPV155, JF906559)	E2	HPV149 (γ-7)	HPV109 (γ-7)	GENECONV	3.9×10^{-2}	E2 ORF
						BootScan	1.7×10^{-3}	
						MaxChi	2.6×10^{-5}	
						Chimaera	3.5×10^{-5}	
						SiScan	2.0×10^{-9}	
HPV116	γ-9	nt 4439–4727 (HPV116, FJ804072)	L2	HPV199 (γ-12)	UN (γ-1)	RDP	2.2×10^{-4}	L2 ORF
HPV129								
mFD2*						BootScan	1.0×10^{-3}	
HPV136	γ-11							
HPV168						MaxChi	4.6×10^{-4}	
HPV171						Chimaera	4.1×10^{-3}	
mKN3*						SiScan	4.8×10^{-5}	
HPV126								
HPV112	**γ-8**	**nt 1045–1244 (HPV112, EU541442)**	**E1**	**mFD1* (γ-24)**	**HPV140 (γ-11)**	**RDP**	$\mathbf{8.4 \times 10^{-4}}$	E1-E2 ORF
HPV147						**GENECONV**	$\mathbf{7.8 \times 10^{-3}}$	
HPV164						**BootScan**	$\mathbf{3.9 \times 10^{-3}}$	
HPV168						**MaxChi**	$\mathbf{2.2 \times 10^{-2}}$	
HPV176						**Chimaera**	$\mathbf{1.1 \times 10^{-2}}$	
						SiScan	$\mathbf{3.6 \times 10^{-2}}$	
HPV178	γ-24	nt 746–929 (HPV178, KJ130020)	E1	HPV140 (γ-11)	mSD2* (γ-15)	RDP	4.0×10^{-3}	
HPV197						GENECONV	2.6×10^{-2}	
mFD1*						BootScan	2.2×10^{-2}	
mKN1*						SiScan	1.5×10^{-4}	
HPV170	**γ-7**	**nt 1430–1846 (HPV170, JX413110)**	**E1**	**HPV109 (γ-7)**	**HPV149 (γ-7)**	**RDP**	$\mathbf{2.3 \times 10^{-6}}$	
						BootScan	$\mathbf{1.6 \times 10^{-4}}$	
						MaxChi	$\mathbf{2.9 \times 10^{-2}}$	
						Chimaera	$\mathbf{3.3 \times 10^{-2}}$	
						SiScan	$\mathbf{2.7 \times 10^{-5}}$	
HPV127	γ-12	nt 2442–2589 (HPV127, HM011570)	E1	HPV141 (γ-11)	HPV119 (γ-8)	RDP	2.3×10^{-4}	
HPV132						BootScan	1.7×10^{-2}	
HPV148						MaxChi	8.8×10^{-3}	
HPV157						Chimaera	2.8×10^{-2}	
HPV165								
HPV199								
mCG2*						SiScan	5.5×10^{-4}	
mCG3*								
HPV112	**γ-8**	**nt 1045–1253 (HPV112, EU541442)**	**E1**	**mFD1* (γ-24)**	**HPV140 (γ-11)**	**RDP**	$\mathbf{2.0 \times 10^{-4}}$	E1-E2-L2 ORF
HPV147						**GENECONV**	$\mathbf{4.5 \times 10^{-3}}$	
HPV164						**BootScan**	$\mathbf{1.4 \times 10^{-3}}$	
HPV168						**MaxChi**	$\mathbf{2.2 \times 10^{-2}}$	
HPV176						**SiScan**	$\mathbf{1.2 \times 10^{-3}}$	
HPV88	γ-5							
HPV116	γ-9	nt 4447–4656 (HPV116, FJ804072)	L2	HPV199 (γ-12)	UN (γ-10)	RDP	5.8×10^{-3}	
HPV129						BootScan	2.4×10^{-2}	
mFD2*						MaxChi	7.3×10^{-3}	
mKN3*						SiScan	1.2×10^{-2}	
HPV170	**γ-7**	**nt 1430–1855 (HPV170, JX413110)**	**E1**	**HPV109 (γ-7)**	**HPV149 (γ-7)**	**RDP**	$\mathbf{1.3 \times 10^{-6}}$	
						BootScan	$\mathbf{1.9 \times 10^{-4}}$	
						MaxChi	$\mathbf{4.5 \times 10^{-2}}$	
						Chimaera	$\mathbf{5.0 \times 10^{-2}}$	
						SiScan	$\mathbf{2.1 \times 10^{-5}}$	
HPV155	γ-7	nt 3253–3460 (HPV155, JF906559)	E2	HPV149 (γ-7)	HPV109 (γ-78)	BootScan	3.7×10^{-2}	
						MaxChi	7.9×10^{-5}	
						Chimaera	1.0×10^{-4}	
						SiScan	3.2×10^{-8}	
		nt 3817–4134 (HPV156, JX429973)	L2	HPV205 (γ-1)	UN (γ-7)	RDP	4.5×10^{-5}	
HPV156	γ-18					GENECONV	5.8×10^{-4}	
HPV172	γ-22					BootScan	1.7×10^{-5}	
HPV175	γ-23					MaxChi	2.5×10^{-2}	
mFi884	γ-UN					Chimaera	5.8×10^{-3}	
						SiScan	5.3×10^{-6}	
HPV112	**γ-8**	**nt 1045–1238 (HPV112, EU541442)**	**E1**	**mFD1* (γ-24)**	**HPV140 (γ-11)**	**RDP**	$\mathbf{1.1 \times 10^{-3}}$	E1-E2-L1 ORF
HPV147						**GENECONV**	$\mathbf{1.3 \times 10^{-2}}$	
HPV164						**BootScan**	$\mathbf{3.3 \times 10^{-4}}$	
HPV168						**MaxChi**	$\mathbf{2.1 \times 10^{-2}}$	
HPV176						**SiScan**	$\mathbf{6.8 \times 10^{-3}}$	
HPV112	γ-8	nt 2597–2787 (HPV112, EU541442)	E1/E2	HPV141 (γ-11)	HPV119 (γ-8)	GENECONV	3.2×10^{-5}	
HPV147						BootScan	2.8×10^{-2}	
HPV164						MaxChi	2.3×10^{-2}	
HPV168						Chimaera	1.8×10^{-3}	
HPV176						SiScan	2.4×10^{-4}	
HPV170	**γ-7**	**nt 1420–1848 (HPV170, JX413110)**	**E1**	**HPV109 (γ-7)**	**HPV149 (γ-7)**	**RDP**	$\mathbf{2.4 \times 10^{-6}}$	
						BootScan	$\mathbf{2.7 \times 10^{-4}}$	

(continued on next page)

Table 2 (*continued*)

Putative recombinant virus	Species	Breakpoint localization (virus, GenBank accession N°)[a,*]	Gene	Parent sequences		Methods	*p*-Value	
				Major	Minor			
						MaxChi	**9.3×10^{-3}**	
						Chimaera	**4.9×10^{-2}**	
						SiScan	**6.3×10^{-5}**	
HPV112	**γ-8**	**nt 1045–1253 (HPV112, EU541442)**	**E1**	**mFD1* (γ-24)**	**HPV140 (γ-11)**	**RDP**	**3.8×10^{-4}**	E1-E2-L2-L1 ORF
HPV147						**GENECONV**	**8.1×10^{-3}**	
HPV164						**BootScan**	**1.6×10^{-3}**	
HPV168						**MaxChi**	**3.0×10^{-2}**	
HPV176						**SiScan**	**1.3×10^{-2}**	
HPV109	γ-7	nt 5072–5445 (HPV170, JX413110)	L2	HPV119 (γ-8)	HPV173 (γ-1)	RDP	1.8×10^{-4}	
HPV123						GENECONV	1.5×10^{-3}	
HPV134						BootScan	6.5×10^{-3}	
HPV138						MaxChi	3.8×10^{-3}	
HPV139						Chimaera	6.4×10^{-4}	
HPV149								
HPV155						SiScan	9.5×10^{-6}	
HPV170								
HPV170	**γ-7**	**nt 1422–1846 (HPV170, JX413110)**	**E1**	**HPV109 (γ-7)**	**HPV149 (γ-7)**	**RDP**	**1.5×10^{-6}**	
						BootScan	**1.9×10^{-4}**	
						MaxChi	**1.7×10^{-2}**	
						Chimaera	**3.5×10^{-2}**	
						SiScan	**1.2×10^{-4}**	

Recombination events meeting the inclusion criterion (a minimum of 4 methods with *p*-values <0.05) are shown. The events persisting in the different ORF combinations are depicted in bold.

[a] Reference sequence used to position the recombination breakpoint.

analyses of the complete L1 ORF of the novel types considered them as new members of γ-1 (HPV158 and HPV205) and γ-12 (HPV157) species (Fig. 1).

The putative E6 proteins of HPV157, HPV205 and HPV158 contained two, three and four different putative PDZ-binding domains (Table 1), respectively (Fanning and Anderson, 1999). A specific characteristic of

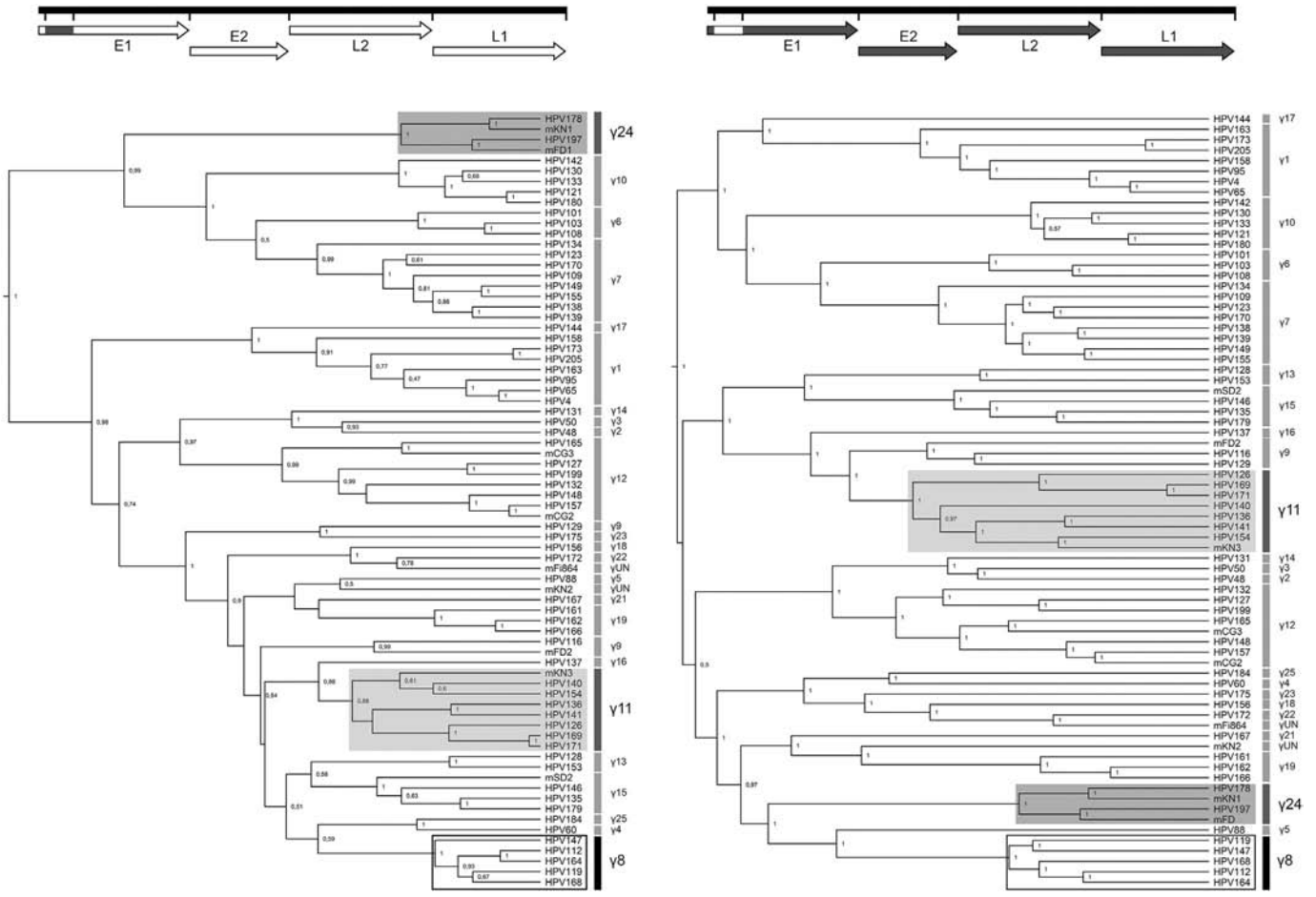

Fig. 2. Phylogenetic incongruence analysis of the putative inter-species recombination event found in genus γ-PV. Bayesian phylogenetic trees of the putative recombinant region (left) and the rest of the alignment (right). The HPV genomic organization is depicted (top), and the regions analyzed in each tree are shaded in dark grey. BPP > 0.50 are shown. Species containing the major (γ-24) and minor (γ-11) viral parents are shaded in dark grey and grey, respectively, while the putative recombinant species γ-8 is indicated with an empty square.

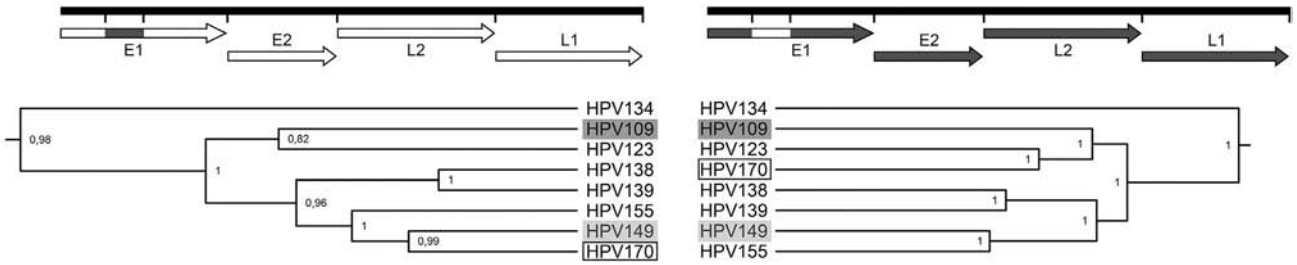

Fig. 3. Phylogenetic incongruence analysis of the putative intra-species recombination event found within species γ-7. Bayesian phylogenetic trees of the recombinant region (left) and the rest of the alignment (right). Only viruses grouped in species γ-7 are shown. The HPV genomic organization is depicted (top), and the regions analyzed in each tree are shaded in dark grey. BPP > 0.50 are shown. The major (HPV109) and minor (HPV149) viral parents are shaded in dark grey and grey, respectively, while the putative recombinant type (HPV170) is indicated with an empty square.

the oncogenic mucosal HPV types is a PDZ-binding motif on the carboxyl terminus of E6, which is important in cell proliferation, cell polarity and cell transformation (Cavatorta et al., 2004). Although the PDZ-binding domains were not found at the carboxyl terminus of the novel γ-PV types here described, it has been shown that the E6 proteins of some oncogenic β-PV types encode a conserved domain (YXDM) at the carboxyl terminus which alters the localization and signalling of the β1-integrin that was associated with increased cell migration, and therefore with a higher pathogenicity of these β-HPV types (Holloway and Storey, 2014). Binding partners and possible functions of the different conserved *C*-terminal motifs of the E6 proteins should be explored in order to investigate the pathological mechanism of some γ-PV types. In this regard, the latest HPV classification update has considered types of species γ-1 and the recently published HPV197 (γ-24) as prevalent viruses in NMSC (Bzhalava et al., 2015). These observations must be confirmed at the molecular level in order to define putative sequence signatures able to understand the mechanisms by which cutaneous HPVs potentially could contribute to skin carcinogenesis.

PVs have been found in most vertebrates investigated, and it is assumed that they developed together with various animal species over hundreds of millions of years (Bravo et al., 2010). This co-evolution implies that they can efficiently spread between individuals and cause chronic infections without inducing any tissue damage, at least under normal conditions. In this regard HPV157, HPV158 and HPV205 were isolated from sun-exposed healthy skin samples, but the tissue tropism and the potential clinical significance of their infections need to be determined.

Although an exponential growth in the number of new PV types has taken place in the past 10 years, no recombinant PV strain has been isolated from a biological sample. However, phylogenetic incongruities within α-PVs (Bravo and Alonso, 2004; Narechania et al., 2005), υ-PVs and o-PVs (Rector et al., 2008), as well as *in silico* indications for ancient recombination events in α-PVs (Angulo and Carvajal-Rodriguez, 2007; Varsani et al., 2006), have suggested that recombination may occur in this viral family. Moreover, BPCV1, a novel virus detected in papillomas and carcinomas of the western barred bandicoot, shares genomic features from both PVs and polyomaviruses (Woolford et al., 2007), suggesting that recombination may have occurred during the evolution of PVs. Considering that data about recombination in the genus γ-PV is scarce, we explored whether this evolutionary force could influence the genetic diversity of this genus using an updated dataset and the three novel types.

To use a distance based method like the RDP4 program (Martin et al., 2015) for the identification of recombination, a reliable alignment is absolutely essential. Recombination signal detection methods are extremely sensitive to misalignments and will usually identify misaligned regions of sequences as having a recombinant origin. With this restriction, and considering that genus γ-PV is highly divergent with currently 25 different species (Bzhalava et al., 2015), we aligned each ORF separately and in combination as concatenated sequences containing different ORFs. Other studies have shown that E1-E2-L1 ORF combination at amino acid level is well suited for simultaneous phylogenetic inference of the entire PV sequence data set (Gottschling et al., 2007, 2011). On the other hand, the inclusion of the L2 gene in the analyses is justified when the reconstruction of PVs is addressed at a lower taxonomic level (Gottschling et al., 2007). In line with it, we explored potential recombination events only in genus γ-PV (lower taxonomic level), and in all combinations of genes (E1-E2-L1, E1-E2-L2 and E1-E2-L2-L1) in order to validate our data. According to our approach, several recombination signals were detected in single or combined ORFs (Table 2). However, only two signals prevailed in almost every ORF combinations: a putative intra-species event in species γ-7, and a potential inter-species event between ancestor viruses of species γ-24 and γ-11 that might have originated the types currently grouped in γ-8 species. Both events appeared when the analysis was performed with the concatenated ORFs (E1-E2, E1-E2-L2, E1-E2-L1, E1-E2-L2-L1), but were not detected when the analysis was done with the single E1 ORF. This may be explained by the fact that there is less phylogenetic information in the single ORFs with respect to that contained in concatenated sequences.

As depicted in Table 2, we have detected putative recombination events within E1, E2 and L2 ORFs. Other studies exploring potential recombination events in genus α-PV have found statistically supported signals within the L2 gene (Angulo and Carvajal-Rodriguez, 2007; Varsani et al., 2006). In our recombination analysis of genus γ-PV, the two events that met the inclusion criterion and prevailed in the different ORFs combinations were located within the E1 ORF (viral DNA helicase). In order to confirm these findings, we investigated the major and minor parental sequence regions in separate phylogenetic analyses to explore possible incongruent tree topologies (Figs. 2 and 3). Incongruent tree topologies between early- and late-gene phylogenies have been explained by recombination events (Gottschling et al., 2007), which have been detected in genera α-PV (Narechania et al., 2005), γ-PV (Kohler et al., 2011), υ- and o-PVs (Rector et al., 2008; Robles-Sikisaka et al., 2012). In this work, we used the recombinant breakpoint obtained with RDP4 program instead of the early- and late-gene phylogeny, as this separation should contain more accurate recombination information. However, in the tree obtained using the recombinant region (Fig. 2, left), some of the more recent nodes had poor BPP support, probably because of the short length of the alignment used. Therefore, we built a Maximum Likelihood tree with RAxML program (Stamatakis, 2006; Stamatakis and Ott, 2008), confirming this tree topology (data not shown).

In the intra-species analysis of species γ-7, the recombination event was highly supported by high BPP values (Fig. 3). Previously, Kohler et al. found that HPV149 was the closest relative of HPV109 in the early gene analyses. However, HPV149 was closely related to HPV123 in the late-gene analyses (Kohler et al., 2011). In this report we performed a similar analysis with an updated database, and we identified HPV170 as a potential recombinant virus, being HPV109 and HPV149 the minor and major parental types, respectively (Table 2).

We also found a possible ancestral recombination that may have originated HPV129 (species γ-9) (Fig. 2). HPV129 was identified by Kohler et al., but they could not find any recombination event in this type (Kohler et al., 2011). The fact that our dataset contained HPV175 (minor parent), unavailable when HPV129 was fully characterized, made the identification of this putative recombination event possible. However, this finding deserves further investigation in order to better understand if this was a real evidence of ancient recombination, a proof of the changing evolutionary history of the genes, or an artifact due to imperfect sequence alignment. This fact embodies the current problem with recombination detection in PVs. Despite the multiple evidence suggesting recombination as an important phylodynamic force of PV evolution (Angulo and Carvajal-Rodriguez, 2007; Hu et al., 2009; Narechania et al., 2005; Shah et al., 2010; Varsani et al., 2006), we are still unable to find a trustworthy method to differentiate between recombination signals and alignment artifacts. In any case, the phylogenetic information of the genus γ-PV, though increasing every year, is still incomplete, and further analysis and characterization should be performed to successfully clarify its evolutionary history.

5. Conclusions

>150 putative γ-HPV types are waiting to be completely characterized (Chouhy et al., 2013a), as well as >30 putative PV types from non-human primates (Kohler et al., 2011) that cluster inside the genus γ-PV. This scenario suggests that HPVs may become polyphyletic if more novel viruses become available for phylogenetic analysis within the genus γ-PV (Kohler et al., 2011).

Here we described three novel types characterized from healthy sun-exposed skin of two immunocompetent individuals that exhibited genome organization and characteristics typical of γ-PVs. Phylogenetically, HPV157 was classified as a new member of species γ-12 while HPV158 and HPV205 belong to species γ-1.

To get deeper insight of the evolutionary processes that may influence the diversification of genus γ-PV we explored potential recombination events on an updated database, using a rigorous approach that included bioinformatics and phylogenetic analysis. Two putative events, both located in the E1 ORF, met the inclusion criterion and persisted in different ORF combinations: an inter-species recombination in species γ-8, and an intra-species recombination in species γ-7.

In conclusion, this report contributes with three new viruses to fill the gaps of the family *Papillomaviridae*, and provides *in silico* evidence of potential intra- and inter-species recombination in genus γ-PV.

Funding

This work was funded by the Agencia Nacional de Promoción Científica y Tecnológica (Grant PICT-2012-0652). Elisa M. Bolatti is supported by a PhD fellowship of CONICET, Germán R. Perez is supported by a PhD fellowship of ANPCyT (PICT 2008–0420) and Emma J. Stella is supported by a Post-Doctoral fellowship of ANPCyT (PICT 2012–0652). The funders had no role in the study design, data collection and analysis, nor with the preparation of the manuscript or the decision to publish.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

We would like to thank MDs Gustavo Piccirilli, Cristian Quatrocchi, Jesica Nipotti and Ramón Fernandez-Bussy Jr. for the collection of skin samples. Special thanks to Dr. Ethel-Michele de Villiers from the Deutsches Krebsforschungszentrum (Heidelberg, Germany) for her help in the taxonomic characterization of HPV-157 and HPV-158, and to Drs. Joakim Dillner and Carina Eklund from the Karolinska Institutet (Stockholm, Sweden) for their taxonomic assistance with HPV-205. Finally, we would like to thank María Robson, Mariana de Sanctis, Geraldine Raimundo, Carolina Perret and Romina Ricardo (Departamento de Inglés, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario) for the language correction and style of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.04.018.

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