Virus Research 262 (2019) 24–29

Contents lists available at ScienceDirect

Virus Research

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

Short communication

Evolutionary studies on the betaretrovirus RERV-H in the Leporidae family reveal an endogenization in the ancestor of *Oryctolagus*, *Bunolagus* and *Pentalagus* at 9 million years ago

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ARTICLE INFO

Keywords:
Rabbit
Leporids
Endogenous retroviruses
ERVs
RERV-H

ABSTRACT

RERV-H was first identified in human tissues and mistaken for a human exogenous retrovirus. However, the integration sites carried by this virus showed that it was instead a European rabbit (*Oryctolagus cuniculus*) endogenous retrovirus. The first clones retrieved from European rabbit samples represented defective proviruses, although estimation of proviral copy numbers found in the European rabbit genome ranged from hundreds to thousands. Screening for the presence of RERV-H showed the absence of the virus in two other lagomorphs, pika (*Ochotona*) and hares (*Lepus*), which diverged from rabbits about 35 and 12 million years ago, respectively.

Using a PCR-based approach, samples of seven different Lagomorph genera were tested for the presence of RERV-H. It was possible to amplify a proviral fragment corresponding to RNaseH from *Oryctolagus*, *Bunolagus* and *Pentalagus* genomic samples. The amplification of proviral DNA in species other than *Oryctolagus* revealed that this virus was endogenized in their common ancestor, roughly 9 million years ago. Using the European rabbit genome sequence OryCun2.0, it was possible to find multiple copies spread throughout the genome and several complete proviral genomes were retrieved. Some copies contained full open reading frames for all viral components. The lack of a complete genome in the other Lagomorph species did not allow further analyses of the provirus, although more deleterious mutations were found in *Bunolagus* and *Pentalagus* than in *Oryctolagus* RNaseH-amplified sequences. To what extent RERV-H and other endogenous viruses might have had an impact on the rabbit genome and its immune system remains elusive.

1. Introduction

Rabbit endogenous retrovirus H (RERV-H) was initially found in a sucrose gradient fraction from the human salivary gland of a patient with Sjögren's syndrome (Griffiths et al., 1997). Using an RT-PCR-based approach, the authors were able to amplify 932-base pairs (bp) of the viral genome, covering almost the complete *protease* gene (*pro*) and part of the *polymerase* (*pol*) domain corresponding to the reverse transcriptase (RT). The high sequence similarity between different isolates and the conservation of the open reading frames (ORFs) of *pro* and *pol*, together with the low viral levels detected in tissues, led the authors to believe that this was a human exogenous retrovirus and to name it HRV-5 (Griffiths et al., 1997). Further sequencing of the viral genome and its integration sites revealed that this virus was in fact an endogenous retrovirus (ERV) from European rabbits (Griffiths et al., 2002). The first hypothesis was that the virus, then called RERV-H, was present in human tissues due to zoonotic infections from rabbits. However, the detection of integration sites of rabbit origin and rabbit mitochondrial sequences in the human samples, raised the possibility of contamination (Griffiths et al., 2002), although no clear explanation was presented for the presence of rabbit DNA in human tissues (Forsman et al., 2003a; Forsman et al., 2003b).

RERV-H belongs to the genus of betaretroviruses, being also closely related to rodent intracisternal A-particle retrotransposons (IAPs) (Griffiths et al., 1997). Consistent with other members of this genus, RERV-H's genome is composed of long terminal repeats (LTRs), *gag*, *pol* and *pro* domains, while the *env* domain remained unidentified. This could have been due to its loss, which is common to ERVs, or its

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https://doi.org/10.1016/j.virusres.2017.12.001
Received 23 February 2017; Received in revised form 1 December 2017; Accepted 1 December 2017
Available online 05 December 2017
0168-1702/

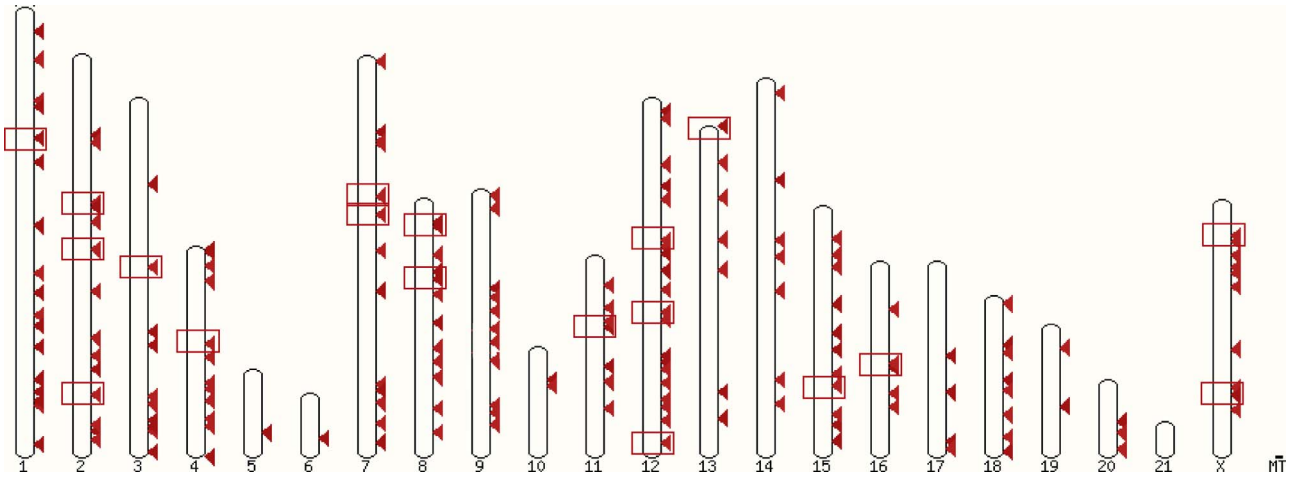

Fig. 1. Approximate chromosomal location of the hits found in the European rabbit genome using as a query the reference clone identified in human samples (GenBank accession number AF480924). The complete viral genome copies are identified by red boxes. This figure was adapted from the Ensembl database.

detection was hampered by a high degree of polymorphism (Boeke and Stoye, 1997; Griffiths et al., 2002). The estimated proviral copy number per haploid rabbit genome ranges from 700 (Forsman et al., 2003b) to 5000 copies (Griffiths et al., 2002), depending on the method used. Besides the predicted high proviral copy numbers, RERV-H show a low expression level in several rabbit tissues (Griffiths et al., 2002).

So far, the clones retrieved from rabbit samples represented defective proviruses. One of the clones had a frameshift mutation and an in-frame nonsense mutation in *gag*, whereas the second clone had two frameshift mutations in *gag* and *pol* and two other in-frame nonsense mutations in *pol* (Griffiths et al., 2002). Despite the deleterious mutations found in these two clones, other domains revealed complete ORFs, having the potential to be functionally expressed. It was already demonstrated that RERV-H protease encode a functional enzyme, that, acting like a retroviral aspartic protease, is able to catalyze its own cleavage from the precursor protein and cleave the RERV-H Gag polyprotein precursor *in vitro* (Voisset et al., 2003).

In order to understand the evolutionary history of RERV-H, Griffiths et al. studied several lagomorphs. They screened for the presence of this virus in the Afghan pika (*Ochotona rufescens*) that belongs to the family *Ochotonidae*, genus *Chonotoa*, which separated from the European rabbit ~35 million years ago (Melo-Ferreira et al., 2015), and in two *Lepus* species, the European hare (*Lepus europaeus*) and the black-tailed jackrabbit (*Lepus californicus*) which diverged from the European rabbit ~12 million years ago (Matthee et al., 2004). RERV-H could not be amplified for the tested species, which was an indication that this virus was introduced after the divergence of European rabbits and hares (Griffiths et al., 2002). However, no data were available for other leporid species to more accurately determine when the endogenization of RERV-H occurred. Here we show that the RERV-H is additionally present in two other leporids, *Bunolagus* and *Pentalagus*, that are evolutionarily closely related to the European rabbit. This suggests that the virus was endogenized in the ancestor of the three species, which diverged about 9 million years ago.

2. Material and methods

Genomic DNA was extracted from tissue samples provided by the CIBIO Lagomorpha tissue collection using the EasySpin Genomic DNA Minipreps Tissue Kit (Citomed, Torun, Poland) according to manufacturer's instructions: European rabbit (*Oryctolagus cuniculus*), riverine rabbit (*Bunolagus monticularis*), amami rabbit (*Pentalagus furnessi*), brush rabbit (*Sylvilagus bachmani*), pygmy rabbit (*Brachylagus idahoensis*), European brown hare (*Lepus europaeus*) and American pika (*Ochotona princeps*).

The sequences obtained from human (GenBank accession number AF480924) and from the European rabbit genome (GenBank accession AF480925) were considered for primer design. To detect the presence of RERV-H in the samples mentioned above, several primer pairs previously described (Griffiths et al., 2002) were used to amplify different regions of the viral genome. The PCR was performed using the PCR Master Mix (Promega) with different annealing temperatures and elongation times for each primer pair (PCR amplification conditions are available upon request). The primer pair that amplified the RNaseH fragment was the only combination producing clear PCR results for additional species besides the European rabbit, and was therefore chosen for subsequent analyses. PCR products were purified (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel, Germany) and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI, USA). Sixteen clones were selected for each sample. Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) using primers designed on the vector (pUC/M13 sequencing primers from Promega). Sequences were submitted to GenBank under the following accession numbers: MG603347-MG603391. BLASTN searches of the European rabbit genome (OryCun2.0, INSDC assembly GCA_000003625.1, Nov 2009) and pika (Ochotona princeps) genome (OchPri2.0, Jun 2007) were conducted using the Ensembl database. BLASTP searches for viral domains were performed in Gypsy Database 2.0 (Llorens et al., 2011). Genomic and protein alignments were done using ClustalW Multiple Alignment (Thompson et al., 1994) within BioEdit 7.0.9.0 (Hall, 1999) and Geneious (Kearse et al., 2012). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al., 2016).

3. Results

Using the reference clone identified in human samples as a query sequence (GenBank accession number AF480924), it was possible to retrieve at least 19 copies of the entire viral genome from different European rabbit chromosomes and more than 400 fragments with more than 90% identity distributed over several chromosomes (Fig. 1). Most of the smaller fragments were around 400 bp in length and aligned either with the end or the beginning of the query, most likely representing single LTRs. The complete copies are highly conserved and several ones do not show any deleterious mutation within the main viral components (Table S1 and Fig. S1), confirming that the endogenization process did not lead to a loss of function and consequent inactivation of viral replication within the European rabbit. The same BLASTN search was performed for the pika genome but, as expected, no hits were found.

A PCR-based strategy was used to verify the presence of RERV-H genome in other leporid species, using different primer pairs for the amplification of different RERV-H regions (according to Griffiths et al., 2002). Although all of the primer pairs were able to amplify these regions in the European rabbit, only the primer pair that amplifies the RNase H fragment led to amplification in other species. From the tested samples (*Oryctolagus cuniculus*, *Bunolagus monticularis*, *Pentalagus furnessi*, *Sylvilagus bachmani*, *Brachylagus idahoensis*, *Lepus europaeus* and *Ochotona princeps*) this region from RERV-H was only amplified in *Oryctolagus cuniculus*, *Pentalagus furnessi* and *Bunolagus monticularis*. Several PCR conditions were tested to ensure that the unsuccessful amplification in other species was not PCR-related but due to the absence of viral elements in these species. To eliminate the possibility of non-amplification due to poor quality of DNA, we used samples that have been previously amplified for genes of the immune system (Neves et al., 2015a; Neves et al., 2015b; Neves et al., 2016; van der Loo et al., 2016).

Since several copies of this viral region were expected to be present in the genome of *Oryctolagus*, *Pentalagus* and *Bunolagus*, the PCR amplicons were cloned and 16 clones were randomly selected for sequencing. All the sequences found in *Oryctolagus* represented a possible functional enzyme, whereas for *Pentalagus* several sequences showed mutations, including the presence of stop codons that truncated the predicted translation product, and for *Bunolagus* only one sequence was found without any deleterious mutation (Fig. 2).

The evolutionary analysis showed that the amplified sequences were grouped and included in the betaretrovirus genus (Fig. 3 and Fig. S2). This analysis was complemented by estimating the average evolutionary divergence between and within species using the Maximum Composite Likelihood model (Tamura et al., 2004) (Table 1). The average divergence within species is lower in *Oryctolagus* when compared to *Bunolagus* or *Pentalagus*. Among species, a higher divergence was observed between *Bunolagus* and *Pentalagus* (0.138 ± 0.015) than between *Bunolagus* and *Oryctoagus* (0.121 ± 0.017).

4. Discussion

Since the discovery of RERV-H as an endogenous virus of the European rabbit, little has been done to elucidate the time of integration of this virus in the leporid genome. Previous results only suggested that the endogenization had occurred after the divergence of European rabbit and hares (Griffiths et al., 2002). It remained to be determined when the virus was endogenized within leporids. In the present study, more leporid species were included in the analysis and the PCR strategy used allowed the identification of viral components in the genome of *Oryctolagus cuniculus*, *Bunolagus monticularis* and *Pentalagus furnessi*. As expected, the virus was absent in *Lepus europaeus* and *Ochotona princeps* samples, but also in *Sylvilagus bachmani* and *Brachylagus idahoensis*. Therefore, this virus was identified in the genome of three genera that inhabit distant geographical regions. Indeed, *Oryctolagus* is native from the Iberian Peninsula, France and North Africa, whereas *Pentalagus* is native from Japan and *Bunolagus* is an endemic species of South Africa (Robinson and Matthee, 2005). According to these results, RERV-H most likely became endogenized in the common ancestor of *Pentalagus*, *Bunolagus* and *Oryctolagus* in a single endogenization event that must have occurred at ~9 million years ago (Fig. 4) (Matthee et al., 2004). An alternative scenario of three independent endogenization events of the same or very similar betaretrovirus seems very unlikely.

While for *Oryctolagus* other regions were successfully amplified by PCR, RNaseH was the only viral part amplified from the *Bunolagus* and *Pentalagus* samples, either due the loss of other viral domains in these species or due to a high polymorphism level. Either way, the rabbit RERV-H RNaseH ortholog sequences were identified in the genome of two other related leporid species. Few sequences from *Pentalagus* (38%) and a unique sequence from *Bunolagus* (7%) showed a complete ORF for RNaseH (Fig. 2). These data indicate a higher mutation rate and loss of function of this viral component in the genome of *Bunolagus* and *Pentalagus*. However, it remains to be determined whether this extends to other viral domains and if RERV-H can be expressed in these species. Consistent with the evolutionary history of these species, the average divergence between the RNaseH sequences show that the sequences retrieved from *Bunolagus* are more closely related to the ones from *Oryctolagus* than to the sequences from *Pentalagus* (Table 1). The evolutionary analysis confirmed RERV-H being part of the *betaretrovirus* genus and grouped the sequences retrieved by PCR with the original HRV5/RERV-H sequence (AF480924.1) (Fig. 3).

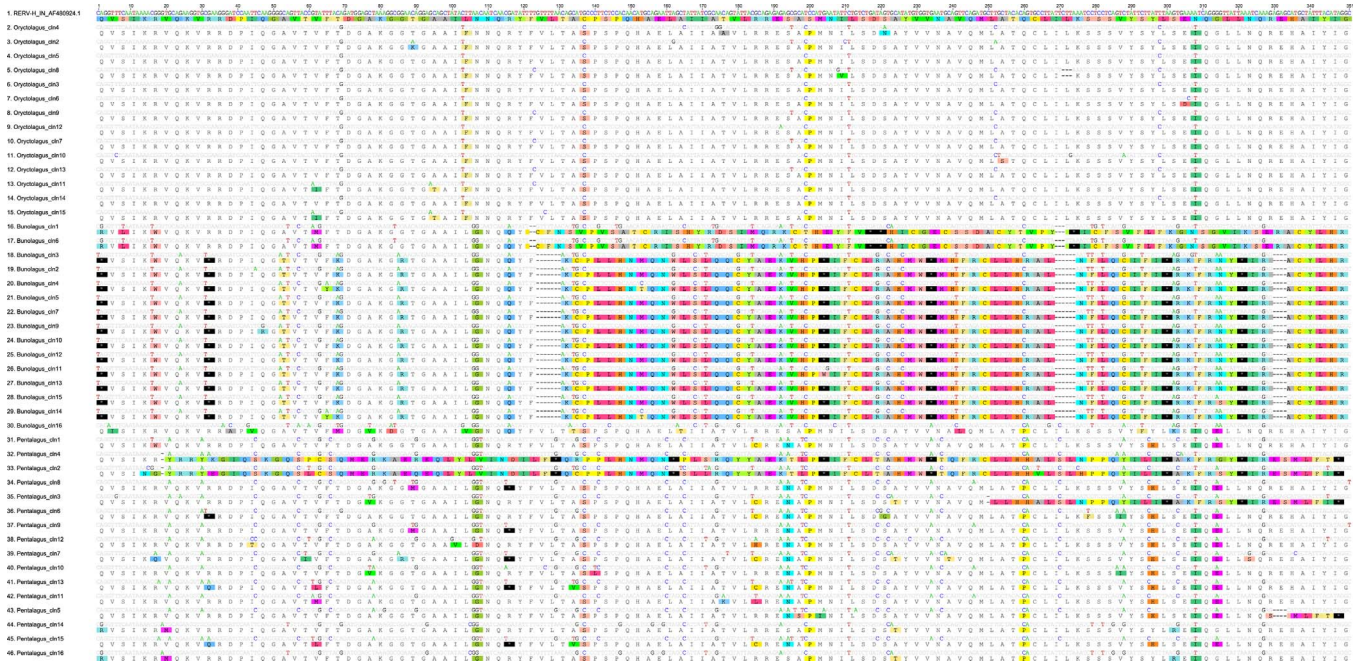

Fig. 2. Alignment of the region amplified by IN primers, corresponding to the viral RNaseH. The deduced amino acid sequences are depicted on top of the genomic alignment. Ambiguous residues are highlighted and asterisks indicate STOP codons.

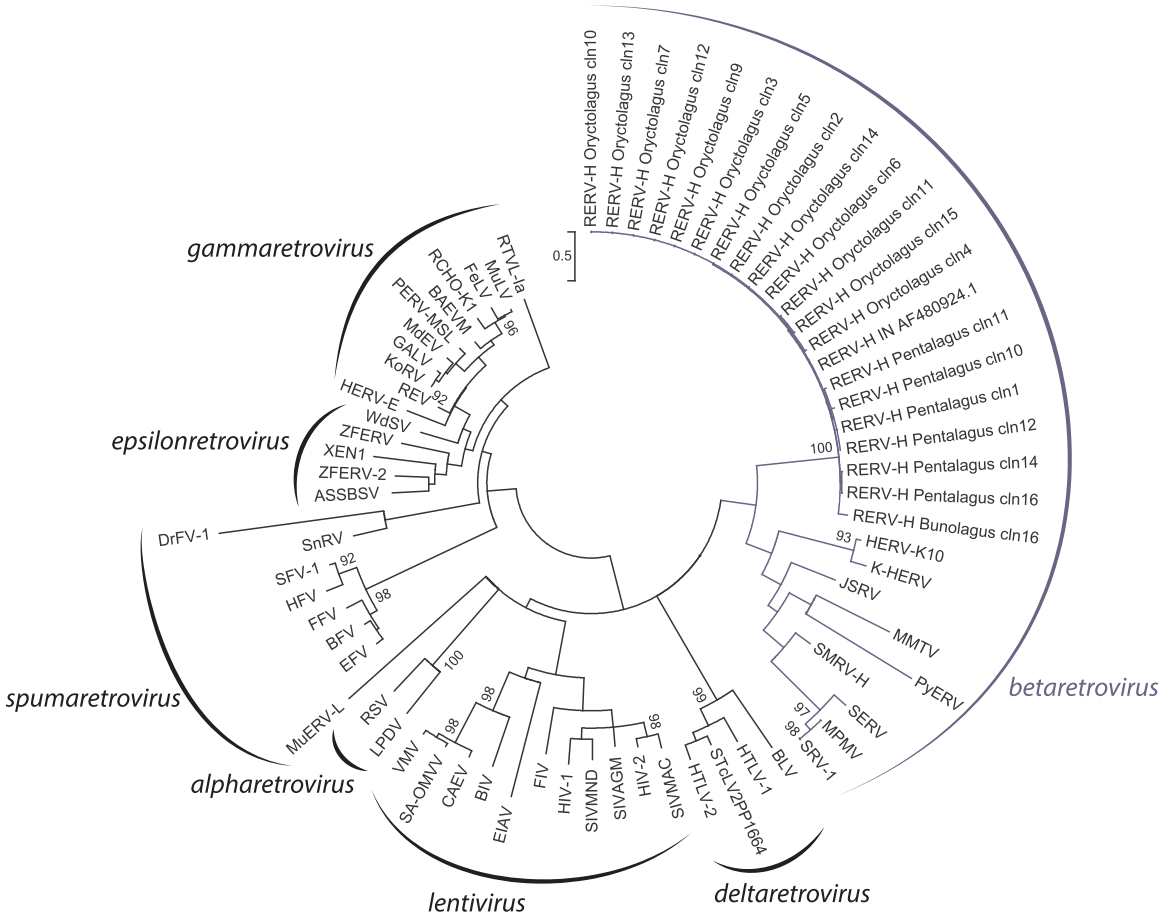

Fig. 3. Evolutionary analysis of the amplified viral RNaseH and of other retroviruses obtained from the Gypsy database 2.0. Maximum Likelihood method based on the General Reverse Transcriptase model was used (Dimmic et al., 2002) with a discrete Gamma distribution and Invariable sites. Bootstrap values over 90% are depicted in the tree (100 bootstrap replicates). Evolutionary analyses were conducted in MEGA7. Retrovirus subfamilies are indicated based on (Mourier et al., 2015).

Table 1
Estimates of Average Evolutionary Divergence within and between different RNaseH sequences (sequences depicted in Fig. 2). The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Standard error estimates were obtained by a bootstrap procedure (150 replicates). Analyses were conducted in MEGA7 using the *p*-distance model.

	Oryctolagus cuniculus	*Bunolagus monticularis*	*Pentalagus furnessi*
Oryctolagus cuniculus	0.013 ± 0.003		
Bunolagus monticularis	0.121 ± 0.017	0.040 ± 0.005	
Pentalagus furnessi	0.114 ± 0.015	0.138 ± 0.015	0.056 ± 0.006

For *Oryctolagus*, besides the PCR approach, the sequences retrieved from the Ensembl database allowed confirmation of the existence of all viral components in the rabbit genome. At least 19 complete viral genomes were retrieved from the available rabbit genome assembly (Fig. 1), with some of the copies showing complete *gag*, *pro* and *pol* genes with no deleterious mutations (Table S1 and Fig. S1).

The Pika-BERV (pika endogenous betaretrovirus) represents another example of an endogenous virus in the lagomorph family (Lemos de Matos et al., 2015). This betaretrovirus is closely related to the MMTV (mouse mammary tumor virus) and the endogenization event was calculated to have occurred at ~3-7 million years ago, being present in several *Ochotona* species but not in leporids (Lemos de Matos et al., 2015). Although both Pika-BERV and RERV-H belong to the *betaretrovirus* subfamily, these represent distinct viruses that seem to have been introduced in their respective host independently and at different times (Fig. S2).

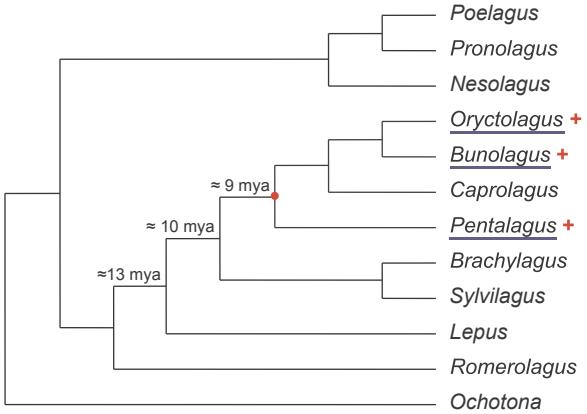

Fig. 4. Evolutionary relationships among 11 Leporid genera. The approximate divergence time is shown for relevant branches for this study. The underlined species have the recombinant CCR5/CCR2 and the pseudogenized CCL8. The red cross indicate species where RERV-H elements were identified and a red dot mark the common ancestor where the endogenization of RERV-H occurred. This image is based on Matthee et al., 2004 and Abrantes et al., 2011.

Interestingly, RERV-H is present only in the leporid species that also harbor the CCR5/CCR2 recombinant receptor and its MPC2/CCL8 pseudogene ligand (Abrantes et al., 2011; Carmo et al., 2006; Pinheiro et al., 2016; van der Loo et al., 2012; van der Loo et al., 2016) (Fig. 4). It remains to be determined whether both events are linked, but the

possibility of a link between the endogenization of a virus in the past and the shape of the host genome is attractive and appealing. Nevertheless, it is likely that RERV-H, like other endogenous viruses, may have shaped the evolution of rabbits from the ancestral *Oryctolagus-Pentalagus*.

The process of viral endogenization can greatly affect host gene expression with the effect of several ERVs already shown in mammalian transcriptomes (Chuong et al., 2016; Feschotte and Gilbert, 2012; Jern and Coffin, 2008). The insertion of ERVs is also linked to mechanisms of recombination that shape the host genome and confer genomic plasticity. One example is the high density of human endogenous retrovirus and other repetitive elements in the human *loci* for MHC classes I and II, conferring a great genomic flexibility to this region (Andersson et al., 1998; Jern and Coffin, 2008). The effect of ERVs is thus extended to innate immunity, including interferon responses, representing enhancers of this immune defense (Chuong et al., 2016). Similar to RERV-H, RELIK (Rabbit Endogenous Lentivirus type K) is an endogenous virus identified in several rabbits but also in hare genomes, suggesting a previous endogenization event ~12 million years ago (Katzourakis et al., 2007; Keckesova et al., 2009; van der Loo et al., 2009). This endogenous virus was also suggested to be a driving force for the conservation of the antiretroviral activity of TRIM5 in leporids (de Matos et al., 2011; Fletcher et al., 2010; Yap and Stoye, 2013), and documents the impact of endogenous viruses in the leporids genome.

Consent for publication

This paper has been reviewed and approved by all the authors, and is not under consideration for publication elsewhere.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: PSP, PJE. Performed the experiments: PSP. Analyzed the data: PSP. Wrote the paper: PSP, PJE. Revised the paper: PJE, JA, HMB.

Acknowledgments

We thank Rose Mage for critically reading the manuscript and for all the helpful advices.

The financial disclosure is the follow: Patrícia de Sousa-Pereira received support from the Portuguese Ministry of Education and Science and the European Social Fund, through the Portuguese Foundation of Science and Technology (FCT), under PhD grant PD/BD/52602/2014. within the Doctoral Programme in Biodiversity, Genetics & Evolution (BIODIV). The Portuguese Foundation for Science and Technology also supported the FCT Investigator grant of Joana Abrantes (ref.: IF/01396/2013) and Pedro J. Esteves (ref.: IF/00376/2015).

This work was funded by FEDER funds through the Operational Programme for Competitiveness Factors—COMPETE, and by National Funds through Foundation for Science and Technology (FCT) under projects PTDC/BIA-ANM/3963/2012 and FCOMP-01-0124-FEDER-028286. 'Genomics Applied To Genetic Resources', co-financed by North Portugal Regional Operational Programme 2007/2013 (ON.2–O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF), also supported this work. This research project was also funded by LMU Munich's Institutional Strategy LMU excellent within the framework of the German Excellence Initiative.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2017.12.001.

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