Identification and evolution of avian endogenous foamy viruses

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Abstract

A history of long-term co-divergence means that foamy viruses (family *Retroviridae*) provide an ideal framework to understanding virus-host evolution over extended time-periods. Endogenous foamy viruses (EFVs) are rare, and to date have only been found in a limited number of genomes from mammals, amphibians, reptiles and fish. By screening 510 avian genomes we identified endogenous foamy viruses in avian species - from the Maguari Stork (*Ciconia maguari*) and Oriental Stork (*C. boyciana*). Phylogenetic analysis and analysis of the genome structures and flanking sequences indicated a single origin of EFVs into *Ciconia* during evolutionary history, and the marked incongruence with the host phylogeny suggested that this integration event occurred independently in birds. In sum, this study provides evidence that birds can be infected with foamy viruses, filling the last major gap in the taxonomic distribution of foamy viruses and their animal hosts.

Key words: endogenous foamy viruses; birds; incongruence; vertical transmission

Introduction

Retroviruses (family *Retroviridae*) are viruses of substantial medical and economic significance as some are associated with severe infectious disease or are oncogenic (Hayward, et al. 2015; Aiewsakun and Katzourakis 2017; Xu, et al. 2018). Retroviruses are also of evolutionary importance as they have occasionally invaded the host germ line, leading to the generation of endogenous retroviruses (ERVs) and hence genomic 'fossils' (Stoye 2012; Johnson 2015, 2019). ERVs are widely distributed in vertebrates (Hayward, et al. 2013; Cui, et al. 2014; Hayward, et al. 2015; Xu, et al. 2018) and provide important insights into the origin and long-term evolution of retroviruses. However, some complex retroviruses such as lenti-, delta- and spuma viruses, only relatively rarely appear as endogenized forms.

As well leaving a litany of endogenous copies in host genomes, foamy viruses are of particular importance because they exhibit a history of long-term co-divergence with their vertebrate hosts (Switzer, et al. 2005). Endogenous foamy viruses (EFVs) were first discovered in sloths (Katzourakis, et al. 2009), and then found in several primate genomes (Han and Worobey 2012b, 2014; Katzourakis, et al. 2014). The subsequent discovery of EFV and EFV-like copies in fish genomes indicated that foamy viruses may have a deep evolutionary history within the vertebrates in general (Han and Worobey 2012a; Ruboyianes and Worobey 2016; Aiewsakun and Katzourakis 2017). Recently, a novel endogenous virus - denoted ERV-Spuma-Spu - was identified in genome of the reptile *Sphenodon punctatus* (the tuatara), suggesting that foamy viruses have co-diverged with their vertebrate hosts for hundreds of million years (Aiewsakun, et al. 2019; Wei, et al. 2019). However, although

57 endogenous foamy viruses have been found in the genomes of fish, reptiles, amphibians and
58 mammals, to date they have not been identified in avian genomes.
59
60 **Materials and Methods**
61 **Genome screening and viral genome structure identification**
62 All 147 avian genomes available in GenBank as of June 2019 (Supplementary Table S1) and
63 363 genomes from the 'Bird 10K' program were screened for endogenous foamy viruses
64 using the TBLASTN algorithm (Altschul, et al. 1990) and the protein sequences of
65 representative exogenous foamy viruses, EFVs and endogenous foamy-like viruses
66 (Supplementary Table S2). A 35% sequence identity over a 30% region with an e-value set to
67 0.0001 was used to filter significant hits (Supplementary Table S3). Viral hits within large
68 scaffold (>20 kb) were assumed to represent *bona fide* ERVs. We then extended the flanking
69 sequence of these hits to identify the viral long terminal repeats (LTRs) using BLASTN
70 (Altschul, et al. 1990), LTR Finder (Xu and Wang 2007) and LTRharvest (Ellinghaus, et al.
71 2008). In accordance with the nomenclature proposed for ERVs (Gifford, et al. 2018),
72 endogenous foamy viruses were identified in the genomes of the Maguari Stork (*Ciconia*
73 *maguari*) and the Oriental stork (*C. boyciana*), were termed 'ERV-Spuma.n-Cma'
74 and 'ERV-Spuma.n-Cbo', respectively (in which n represents the number of the viral
75 sequences extracted from host genome) (Supplementary Table S4). Putative genome
76 structures and conserved EFV domains were identified using BLASTP, CD-search
77 (Marchler-Bauer and Bryant 2004; Marchler-Bauer, et al. 2017) and ORFfinder
78 (https://www.ncbi.nlm.nih.gov/orffinder/) in NCBI.

Molecular dating

ERV integration time can be calculated using the simple relation T = (D/R)/2, in which T is the integration time (million years, MY), D is the number of nucleotide differences per site between the pairwise LTRs, and R is the genomic substitution rate (nucleotide substitutions per site, per year). We used the previously estimated neutral nucleotide substitution rate for birds (1.9×10^{-9} nucleotide substitutions per site, per year) (Zhang, et al. 2014). Two full-length ERVs-Spuma-Cma containing a pairwise intact LTRs were used to estimate integration time in this manner (Supplementary Table S5). We excluded ERV-Spum.1-Cbo from this dating exercise due to its defective 5' LTR.

Phylogenetic analysis

To describe the evolutionary relationship of EFVs in relation to other representative retroviruses, sequences of the Pol (Supplementary data set S1) and concatenated Gag-Pol-Env proteins (Supplementary data set S2) were aligned using MAFFT 7.222 (Katoh and Standley 2013) and confirmed manually in MEGA X (Kumar, et al. 2018). A phylogenetic tree of these data was then inferred using the maximum likelihood (ML) method in PhyML 3.1 (Guindon, et al. 2010), incorporating 100 bootstrap replicates to assess node robustness. The best-fit LG+Γ+I+F was selected for both Pol and concatenated Gag-Pol-Env protein data sets using ProtTest (Abascal, et al. 2005).

Results and Discussion

Discovery and characterization of endogenous foamy viral elements in avian genomes

To identify potential foamy (-like) viral elements in birds, we collected all available bird genomes from GenBank (Supplementary Table 1) and the 'Bird 10K' project (Zhang, et al. 2015) and performed *in silico* TBLASTN, using the amino acid sequences of representative retroviruses (Supplementary Table 2). This genomic mining identified 16 significant hits in the Maguari Stork and the 12 in Oriental Stork (Supplementary Table 3). We designated these ERV-Spuma.n-Cma and Spuma.n-Cbo, respectively (Gifford, et al. 2018) (Supplementary Table S3, Table S4). We considered hits within large scaffolds (>20 kilobases in length) to represent *bona fide* ERVs. We then extended the flanking sequences of these EFVs on both sides to search for LTRs as these define the boundary of the viral elements. Through this analysis we were able to discover two full-length EFVs in the Maguari stork genome and one in the Oriental stork genome. The low copy number of EFVs found in both two bird species accords with the observation that avian genomes generally harbor small numbers of endogenous viruses (Cui, et al. 2014).

To further elucidate the relationship between these novel avian EFVs and other retroviruses, the long Pol (>800 amino acid) gene sequences were used in a phylogenetic analysis (Fig. 1). Accordingly, our maximum likelihood phylogenetic tree revealed that the EFVs discovered in birds formed a close and well supported monophyletic group within the foamy virus clade compatible with the idea that these avian EFVs might have a single origin. Notably, however, because they were most closely related to the endogenous foamy viruses found in mammals rather than to those found in reptiles, the phylogenetic position of the avian EFVs described

here was incongruent with that of the host phylogeny (although the node associated with the tuatara EFV was relatively poorly supported) (Fig. 2). This, and the overall rarity of EFVs in birds, suggests that these avian EFVs have an independent origin in birds and were not acquired through virus-host co-divergence.

Genomic structure characterization

The genomes of the new EFVs documented here contained a pair of LTRs, although with no sequence similarity to other EFV LTRs, and exhibited a typical spuma virus structure, with three main open reading frames (ORF) - gag, pol, and env - and one putative additional accessory gene, ORF 1 (Fig. 3, Supplementary Fig. S1). Similar to the LTRs, this accessory ORF 1 exhibits no sequence similarity to any known accessory genes from foamy viruses. In addition, by searching for conserved domains against the Conserved Domains Database (www.ncbi.nlm.nih.gov/Structure/cdd), we identified three typical foamy conserved domains in the three full-length avian EFVs: (1) the Spuma virus Gag domain (pfam03276) (Winkler, et al. 1997), (2) the Spuma aspartic protease (A9) domain (pfam03539) that is present in all mammalian foamy virus Pol proteins (Aiewsakun and Katzourakis 2017), and the (3) foamy virus envelope protein domain (pfam03408) (Han and Worobey 2012a; Wei, et al. 2019) (Supplementary Fig. S2).

Vertical transmission of bird EFVs

Surprisingly, ERV-Spuma.2-Cma and ERV-Spuma.1-Cbo shared 98% nucleotide sequence identity and contained the same deletion in the pol gene (Supplementary Fig. S3). By

145 comparing the flanking sequences of these two EFVs using BLASTN, we discovered that two
146 scaffolds containing EFV (BDFF02011124.1 in the Oriental stork and scaffold3222 in the
147 Maguari Stork) shared 99% coverage with 98.66% sequence identity (e-value = 0.0).
148 Furthermore, upon the ERV insertion, the target DNA fragment is also duplicated, resulting
149 in target site duplication (TSDs) that differs among ERV insertions (Hughes and Coffin
150 2001). We were able to identify the same TSD flanking these two avian EFVs
151 (Supplementary Fig. S3), confirming that they have been vertically inherited. However,
152 neither EFVs nor any solo LTR were present in other bird species from same order
153 (Pelecaniformes), including little egret (*Egretta garzetta)*, crested ibis (*Nipponia nippon*) and
154 Yellow-throated sandgrouse (*Pterocles gutturalis)*. Clearly, the study of additional genomes
155 sampled across the avian phylogeny is merited.
156
157 **Estimation of ERV insertion times**
158 To approximately estimate the insertion time of endogenous foamy viruses in two birds, we
159 utilized the LTR-divergence based on the degree of sequence divergence between the 5' and
160 3' LTRs with a known host nucleotide substitution rate (Dangel, et al. 1995; Johnson and
161 Coffin 1999). Only two intact pairwise LTRs of ERVs-Spuma-Cma were selected for time
162 estimation (Supplementary Table S5). This analysis revealed insertion times of 3.15 and
163 13.95 MYA (million years ago). Although, the insertion time is surprisingly young compared
164 to the age of birds, the presence of multiple premature stop codon suggests the invasion was
165 ancient, and all estimates of integration times should be treated with caution (Kijima and
166 Innan 2010).

In sum, we describe the presence and evolution of two novel avian endogenous foamy viruses. This discovery fills the last major gap in our understanding of the taxonomic distribution of the foamy viruses and will help us to understand the evolutionary interactions between retroviruses and their hosts over extended time-periods

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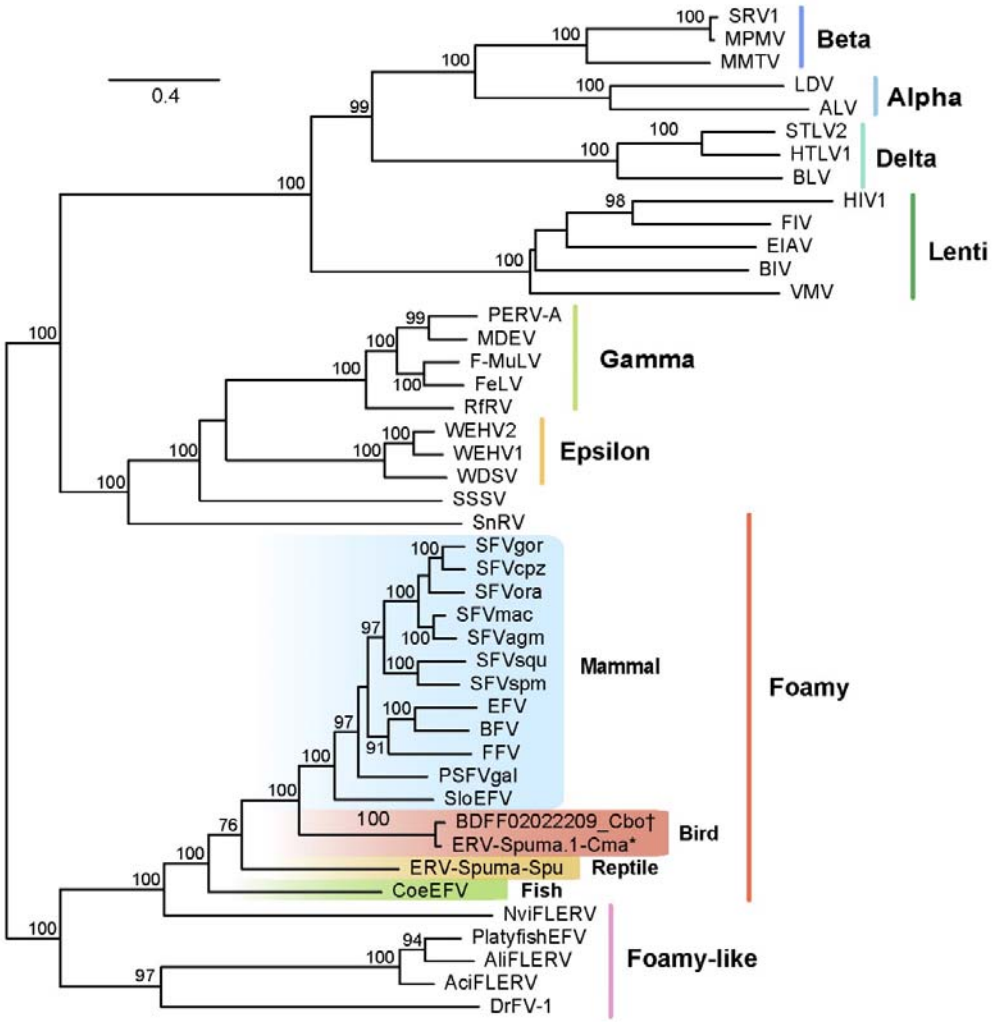


Fig. 1. Phylogenetic tree of retroviruses and endogenous retroviruses, including the endogenous foamy viruses found in avian genomes. The tree was inferred using amino acid sequences of the Pol gene, and is midpoint rooted for clarity only. The newly identified viral elements are marked with a red-shaded box. * indicates the EFV found in the Maguari Stork genome, while † indicates the EFV found in the Oriental Stork genome. The scale bar indicates the number of amino acid changes per site. Bootstrap values >70 % are shown.

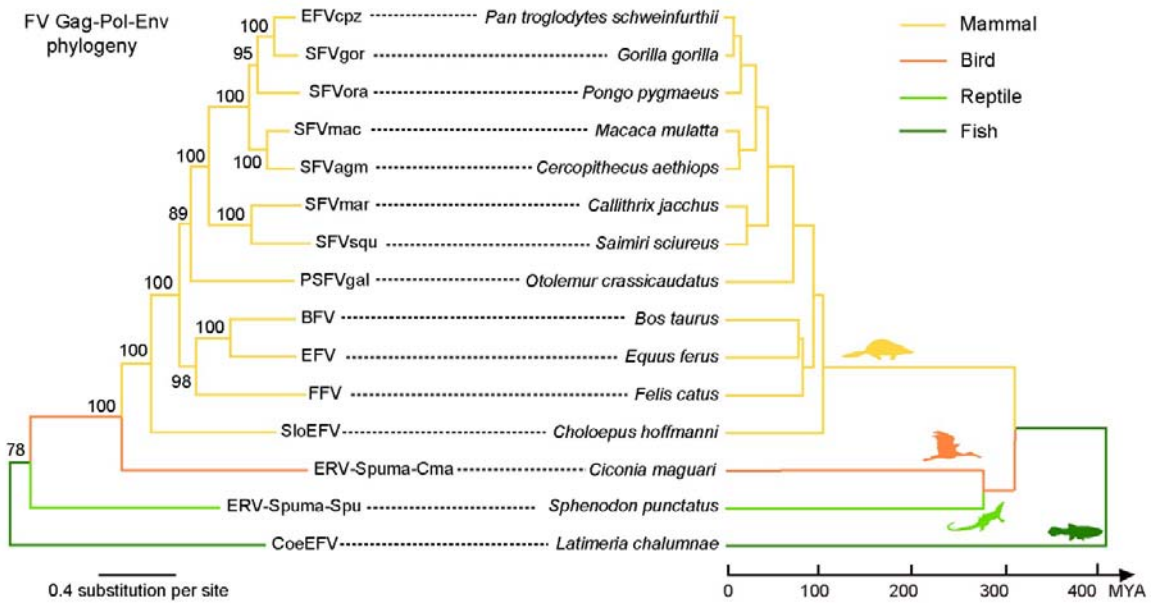


Fig. 2. Evolutionary history of foamy viruses (left) and their vertebrate hosts (right). Associations between foamy viruses and their hosts are indicated by connecting lines, although note the avian EFVs are more closely related to mammalian foamy viruses than the reptilian EFV. The scale bars indicate the number of amino acid changes per site in the viruses and the host divergence times (million years ago, MYA).

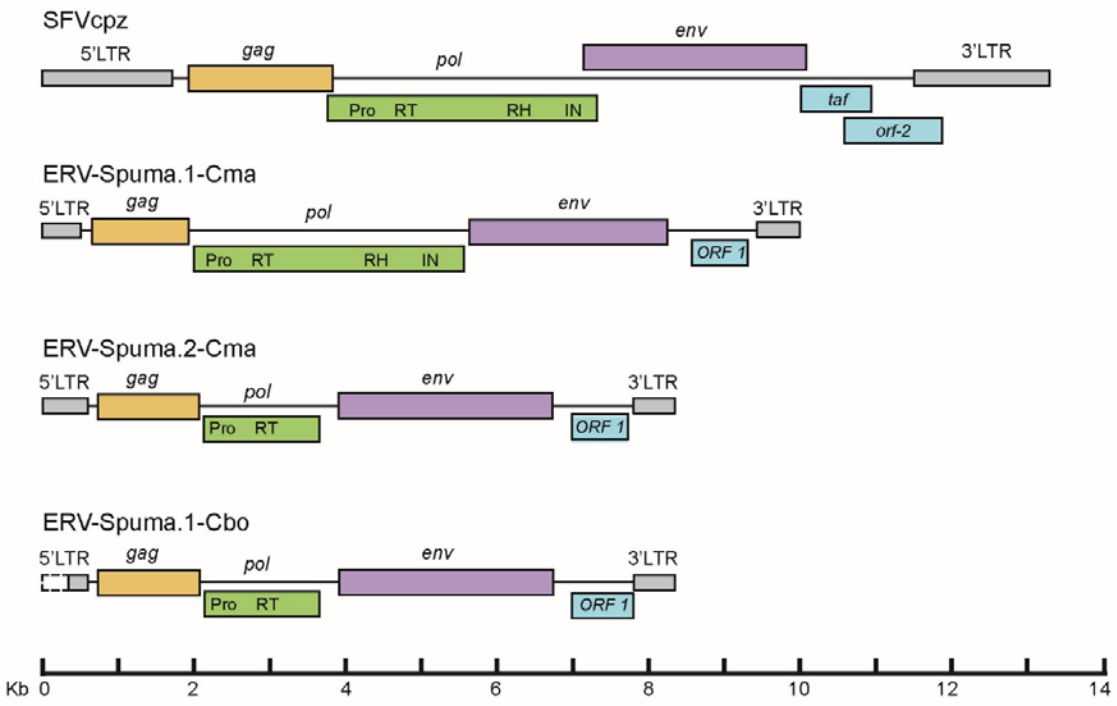
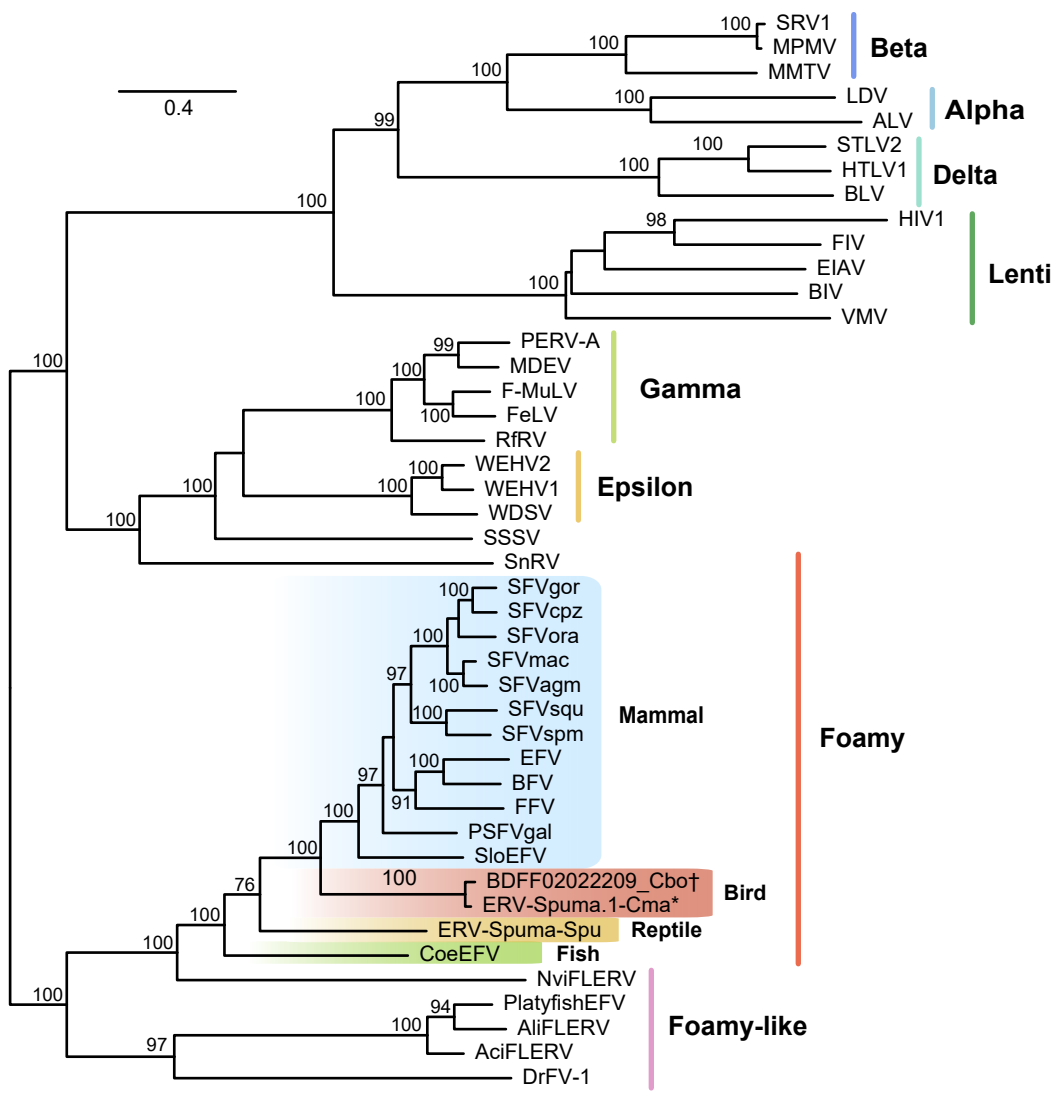


Fig. 3. Genomic organization of exogenous foamy viruses (acc: NC_001364) and avian endogenous foamy viruses. The dotted box marks the LTR deletion. LTR, long terminal repeat; Pro, protease; RT, reverse transcriptase; RH, ribonuclease H; IN, integrase.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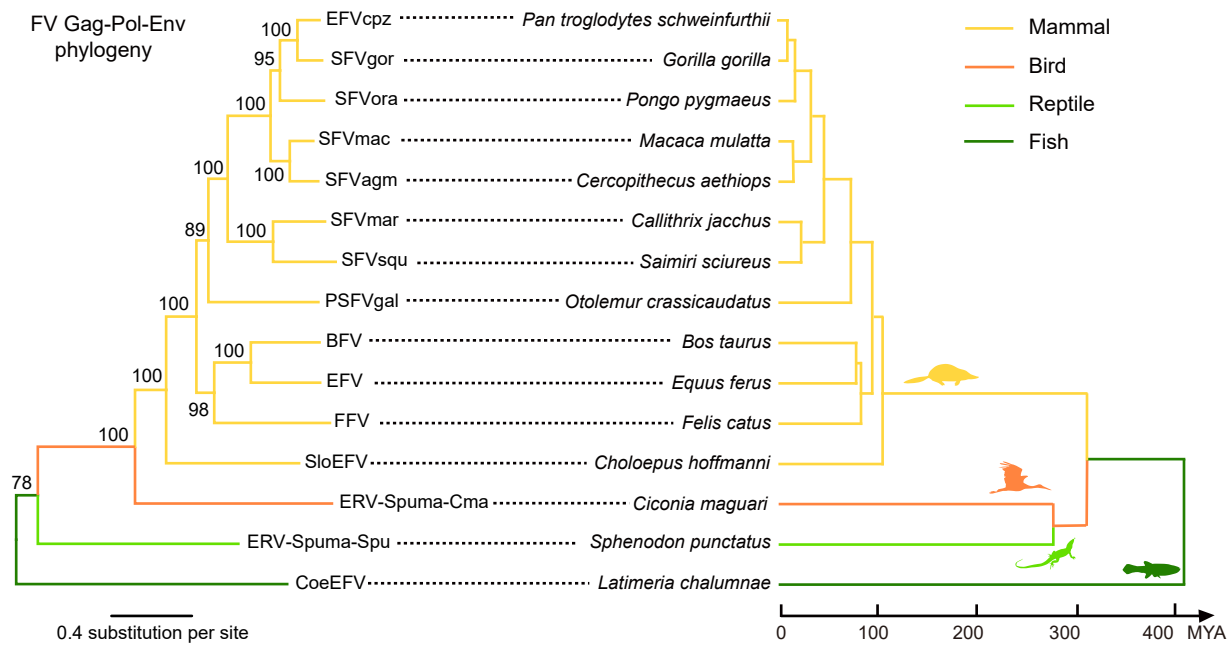
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FFV
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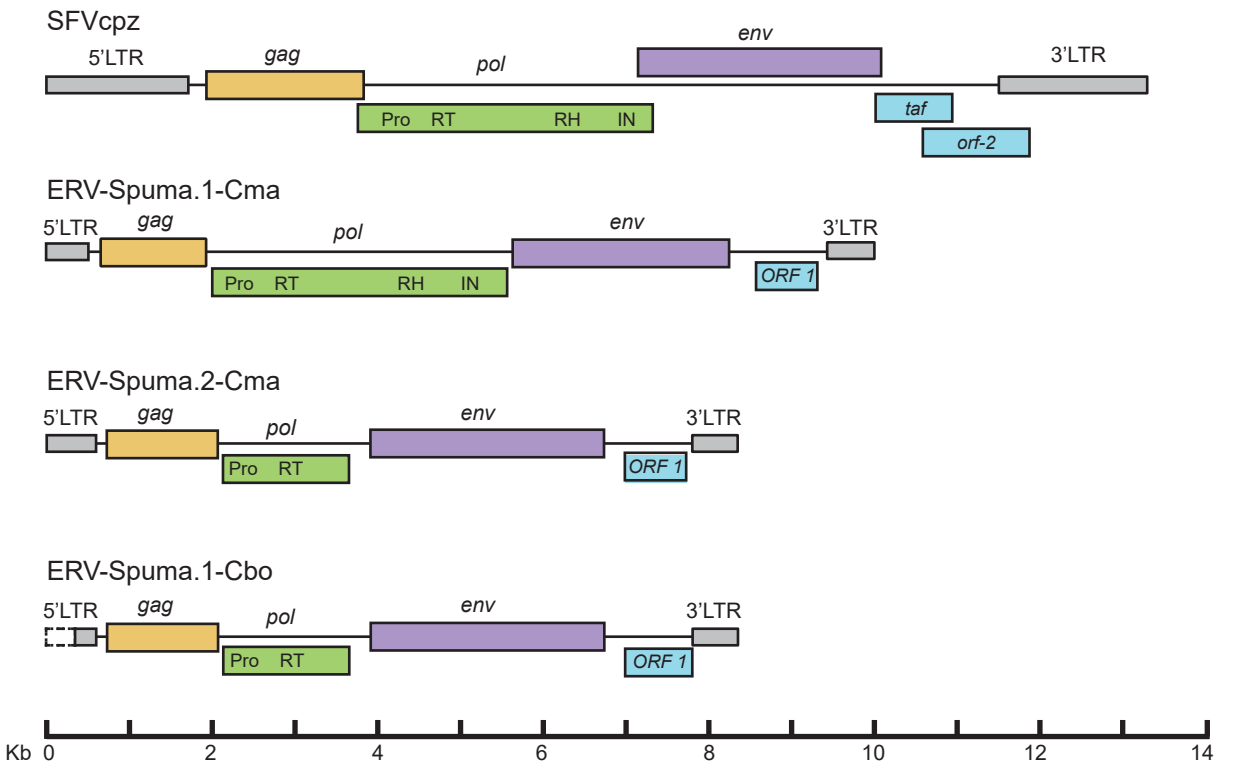
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