

An Intact Retroviral Gene Conserved in Spiny-Rayed Fishes for over 100 My

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Abstract

We have identified a retroviral envelope gene with a complete, intact open reading frame (ORF) in 20 species of spiny-rayed fishes (Acanthomorpha). The taxonomic distribution of the gene, “*percomORF*”, indicates insertion into the ancestral lineage >110 Ma, making it the oldest known conserved gene of viral origin in a vertebrate genome. Underscoring its ancient provenience, *percomORF* exists as an isolated ORF within the intron of a widely conserved host gene, with no discernible proviral sequence nearby. Despite its remarkable age, *percomORF* retains canonical features of a retroviral glycoprotein, and tests for selection strongly suggest cooption for a host function. Retroviral envelope genes have been coopted for a role in placentogenesis by numerous lineages of mammals, including eutherians and marsupials, representing a variety of placental structures. Therefore *percomORF*'s presence within the group Percomorpha—unique among spiny-finned fishes in having evolved placentation and live birth—is especially intriguing.

Key words: endogenous retrovirus, viral gene cooption, evolution.

Vertebrate genomes include vast numbers of sequences derived from the infection of germ cells by retroviruses, which integrate into host DNA as a normal part of their replication cycle (Jern and Coffin 2008; Johnson 2015). Most of these endogenous retrovirus (ERV) sequences are overwritten by mutations over time, but occasionally an ERV gene is coopted for a function that benefits the host, and becomes fixed in the population. While investigating the origins of a sequence that had been published as potentially representing a remnant of a filovirus in the genome of the stickleback (*Gasterosteus aculeatus*) (Belyi et al. 2010), we found that the sequence is actually part of an ORF encoding a complete and intact retroviral envelope glycoprotein. Retroviral glycoproteins stud the surface of the virion and mediate receptor binding and membrane fusion, allowing entry of the viral cargo into the target cell.

The UCSC Genome Browser (Speir et al. 2016) indicates orthologs of the stickleback sequence in two species of pufferfishes, *Takifugu rubripes* and *Tetraodon nigroviridis*, and BLAST searches of the NCBI databases with the full-length translated sequence recovered the ORF from an additional 17 species of spiny-rayed fishes (supplementary table S1, Supplementary Material online). Although no matches were found within the EST database, a search of the NCBI Sequence Read Archive (SRA) revealed an exact match to the stickleback sequence among RNA transcripts expressed in embryonic tissue at 3 days post-fertilization (supplementary methods S1, Supplementary Material online). All species belong to the crown group known as the Percomorpha, thus we named the ORF “*percomORF*”.

Analysis of the flanking regions revealed that *percomORF* is positioned within an intron of a gene encoding an auxilin-like

protein, *DNAJC6*, that is widely and highly conserved among vertebrates. We were able to trace the *DNAJC6* coding region across 18 exons in *Xiphophorus maculatus*, and *percomORF* lies within the last intron, between exons 17 and 18, in opposite orientation to *DNAJC6* (fig. 1). This distinctive location allowed us to establish that *percomORF* is in a syntenic position in all 20 species and thus arose from a single event in a common ancestor. We also examined the corresponding regions in other teleost lineages to generate a presence/absence profile (fig. 2). Its presence in multiple lineages of Percomorphaceae indicates that *percomORF* originated no more recently than 109–140 Ma, the estimated range for the Percomorphaceae divergence (Near et al. 2012; Betancur-R et al. 2013).

Despite its ancient age, *percomORF* encodes all of the typical features of a functional retroviral envelope glycoprotein, with no stop codons interrupting the predicted reading frame in any of the species, implying maintenance of function for the benefit of the host, or exaptation. For example, the maintenance of receptor-binding function is suggested by 14 cysteines positionally conserved in the region corresponding to the surface (SU) subunit (fig. 1). Cysteines in SU typically help the protein fold into a globular conformation that functions in receptor binding (Chiou et al. 1992; van Anken et al. 2008). Likewise, *percomORF* retains motifs associated with membrane fusion such as: (1) a run of basic residues typical of a canonical furin cleavage site by which SU and the fusion subunit (TM) are cleaved to generate a fusion-ready trimer of heterodimers and (2) a stretch of hydrophobic residues at the N-terminus of TM, consistent with the fusion peptide. The CX₆CC motif in the region coding for TM is typical of retroviruses whose subunits are joined by a covalent disulfide

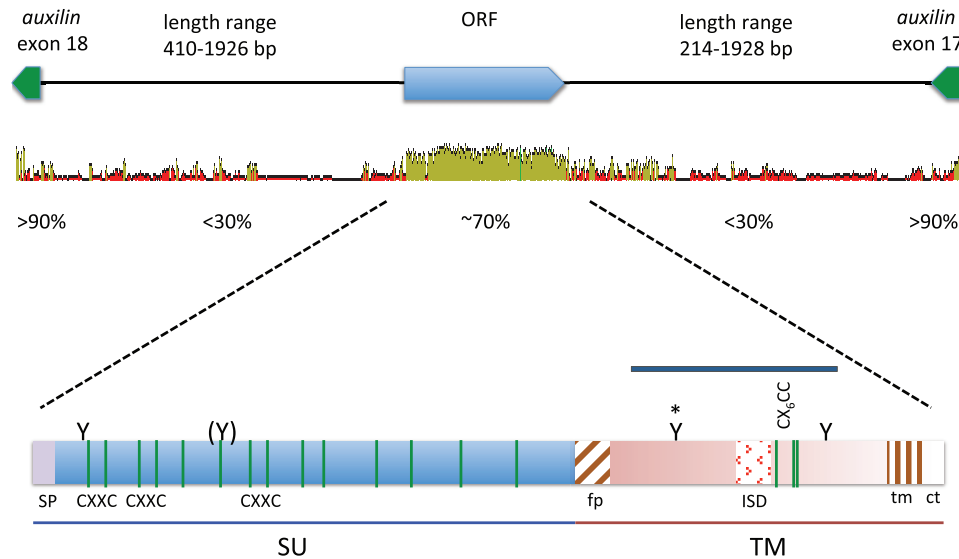


Fig. 1. Top, genomic position of *percomORF*. The orientation of the coding regions for auxilin exons and *percomORF*, and the varying lengths of the ORF-flanking intron sequences are shown along the top. Below is a graph representing the average pairwise percent nucleotide identity of each region, generated using GENEIOUS v. 6.0.4, available from <http://www.geneious.com>. Dark green indicates 100% identity across all sequences at a given site; green, >30% identity; red, <30% identity. Height of graph at each position conveys the fraction of sequences with an identical nucleotide at that position. Bottom, conserved features of *PercomORF*. SU and TM subunits are indicated at the bottom of the figure; green vertical lines represent cysteines, with approximate positions of the CXXC motifs in SU, and the CX₆CC motif in TM indicated; fusion peptide (fp), immunosuppressive domain (ISD), and transmembrane region (tm) are shown as patterned boxes; SP, signal peptide; Y, predicted N-glycan site, with parentheses at partially conserved site, and asterisk marking the “heptad stutter”; ct, cytoplasmic tail. The blue bar above TM represents the partial sequence from the stickleback that was included in a study on filoviruses in mammalian genomes (Belyi et al. 2010).

bond (Pinter et al. 1997; Henzy and Coffin 2013), and these motifs are usually found in conjunction with an isomerase domain (CXXC) in SU, which has been shown to mediate bond dissociation upon receptor binding (Wallin et al. 2004). Interestingly, *percomORF* encodes three such motifs (fig. 1). In addition to the conservation of motifs associated with function, exaptation is further supported by dN/dS analysis (supplementary table S2, Supplementary Material online). Under various evolutionary models, we found that between 189 and 232 of the 422 codons analyzed have dN/dS values that are consistent with a regime of strong purifying selection (average dN/dS: 0.25), while only a handful of positions (0–5) have dN/dS ratios suggesting positive selection.

One feature of the stickleback sequence that suggested it may be a filoviral glycoprotein is 32% amino acid sequence identity with Reston ebolavirus glyco-protein over a region in the TM subunit encoding 89 residues (fig. 1, blue bar) (Belyi et al. 2010). However, although filovirus glycoproteins are known to share structural features with retroviral glycoproteins (Gallaher 1996; Weissenhorn et al. 1998; Jeffers et al. 2002), several motifs encoded by *percomORF* are specific to retroviruses. These are (1) isomerase motifs in SU, which are common in retroviruses with covalently-bonded subunits (Pinter et al. 1997; Wallin et al. 2004; Henzy and Coffin 2013) but have not been found in filoviruses; (2) a fusion peptide that is N-terminal, whereas filoviral fusion peptides are internal and flanked by cysteines and (3) a motif (QNRAALD) in the immunosuppressive domain (ISD) that is typical of nonmammalian retroviruses rather than

filoviruses (Henzy and Johnson 2013). A consensus sequence generated from an alignment of all 20 orthologs retains these motifs (supplementary fig. S1, Supplementary Material online), consistent with their presence in the ancestral retroviral protein.

Another feature that suggested that the stickleback sequence may be a filoviral gene is its existence in isolation from other viral genes (Belyi et al. 2010). Indeed, all of the *percomORF* loci we identified contained no discernible retroviral genes within a 10-kb flanking window, consistent with previous findings (Belyi et al. 2010). Whereas retroviruses insert a DNA copy of the entire viral genome (known as a “provirus”) into host DNA as a normal part of replication, other endogenous virus sequences are thought to arise through the interaction of viral mRNA with a retrotransposon such as a LINE-1 element (Horie et al. 2010; Katzourakis and Gifford 2010; Taylor et al. 2010), and thus exist as isolated genes. However, at a different locus we found a *bona fide* retroviral provirus in the Japanese eel (GenBank: K1305800.1) with a predicted Env protein that has 50% amino acid identity with the original partial sequence (compared with only 32% identity seen with Reston ebolavirus), serving as additional evidence for the retroviral origin of *percomORF*. Notably, we did not find any degenerate sequences more closely related to *percomORF* than that of the Japanese eel, likely reflecting divergence of *percomORF*'s evolutionary trajectory as a host gene from that of the originating retrovirus.

The presence of *percomORF* as an isolated gene may reflect its age. Notably, the *Fv-1* gene of mice, like *percomORF*, is

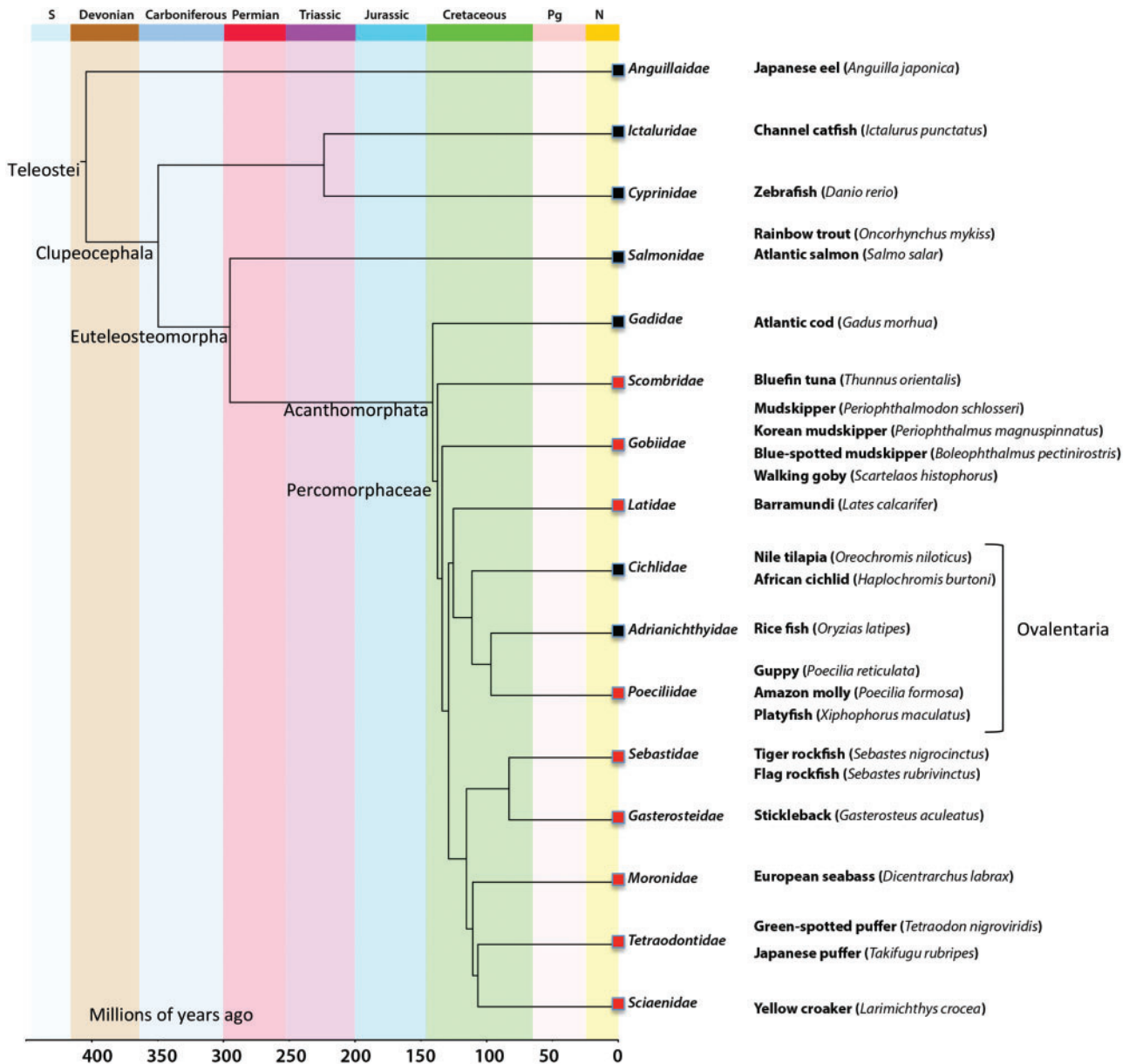


Fig. 2. Estimated age of *percomORF*. Time-calibrated phylogeny scaled to the geological time scale, adapted from Near et al. (2012) and based on 9 nuclear genes and 36 fossil age constraints. Orders in which *percomORF* or an empty site was found are indicated by red boxes and black boxes, respectively. Lineages that are part of the Ovalentaria clade are indicated. The S, Pg, and N (top) signify the Silurian, Paleogene, and Neogene geological periods.

present in some species as an isolated gene with no discernible proviral sequence flanking it (Schlecht-Louf et al. 2010). An analysis of *Fv-1* orthologous sites suggests that the locus has undergone multiple insertions, deletions, and rearrangements in various lineages, which may have caused the loss of proviral sequence in mice while the gene itself was maintained under selection (GR Young and JP Stoye, personal communication). The location of *percomORF* within the intron of *DNAJC6* allowed us to compare the flanking sequence among the 20 orthologs. Similar to *Fv-1*, the regions flanking *percomORF* are very poorly conserved both in sequence and in length (fig. 1), consistent with loss of proviral sequence due to degeneration of neutral sites over a long period of evolution. By the same reasoning it is likely that any markings of

noncanonical integration, such as the inverted repeats that accompany LINE-1-mediated insertion, would be erased by mutations. Notably, the much “younger” ERVWE1 locus—a co-opted *env* gene originating from a retroviral insertion in the genome of a hominoid ancestor 25–40 Ma—is part of a provirus in which the *gag* and *pol* genes have acquired multiple inactivating mutations (Mallet et al., 2004).

Among the features that *percomORF* does share with filovirus glycoproteins is a rigidly conserved predicted N-glycan site in the N-terminal portion of TM (fig. 1 and supplementary fig. S1, Supplementary Material online). N-glycans at this position have been found to disrupt the register of the heptad repeat of the fusion subunit, causing a loosening of the coiled-coil region, or “stutter” (Igonet et al. 2011; Higgins et al. 2014),

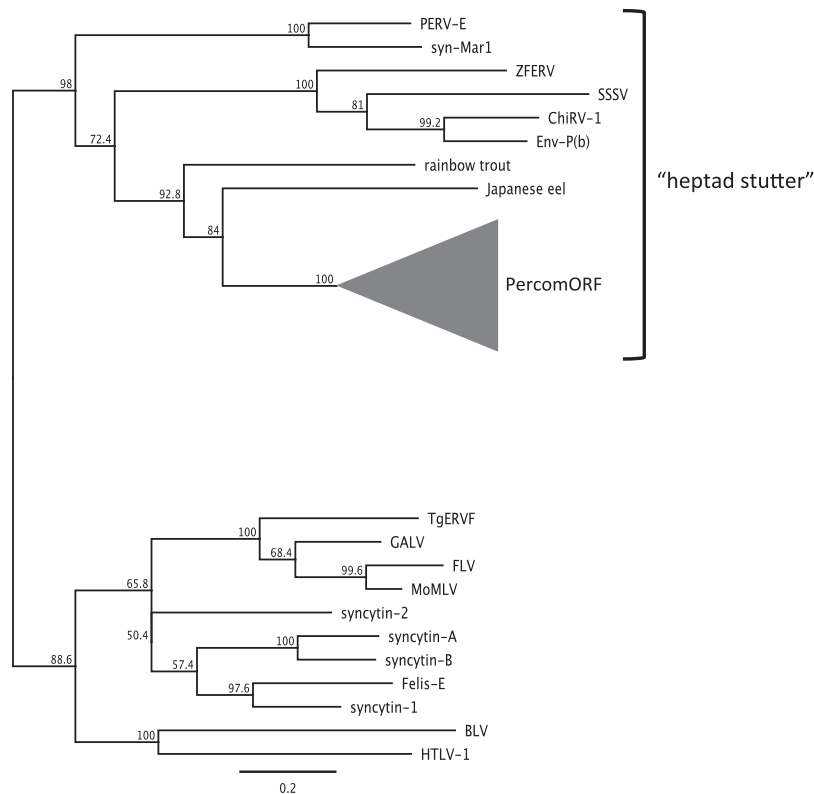


Fig. 3. Relationship of PercomORF to other gammaretroviral-like Env proteins. A neighbor-joining tree was generated (midpoint-rooted) using GENEIOUS v. 6.0.4, based on an amino acid alignment spanning the TM coding region, excluding the cytoplasmic tail. PercomORF sequences (collapsed into a clade represented by the gray triangle) cluster with the group of gammaretroviral-like Env proteins that carry a “heptad stutter” in the N-terminal heptad repeat. The lower clade consists of gammaretroviral sequences that do not include the stutter. All of the sequences besides PercomORF occur in the context of proviruses.

which has been associated with viruses that infect via an endocytic pathway (Igonet et al. 2011). However this feature is typical of nonmammalian retroviruses (Henzy and Johnson 2013), as well as filoviruses (Koellhoffer et al. 2012), snake arenaviruses (Higgins et al. 2014; Koellhoffer et al. 2014), influenza virus, and SARS coronavirus (Supekar et al. 2004). On phylogenetic trees, gammaretrovirus-like *env* sequences form two large clades that reflect the presence or absence of the predicted heptad stutter. The PercomORF TM forms a well-supported branch within the heptad stutter superclade (fig. 3), which also includes a syncytin from squirrel-related rodents, MAR-1 (Redelsperger et al. 2014). Basal to the PercomORF clade is the TM sequence from the Japanese eel, possibly representing the original viral lineage from which *percomORF* arose.

Exapted retroviral glycoproteins described in the literature have been associated with several functions (Jern and Coffin 2008; Lavialle et al. 2013; Johnson 2015; Malfavon-Borja and Feschotte 2015). *Fv-4*, for example, is a partial retroviral glycoprotein sequence in mice that expresses a defective envelope protein (Ikeda and Sugimura 1989) that binds to its cognate receptor, downregulating or blocking its expression (Kai et al. 1986) and thereby protecting the host from infection by related retroviruses (Limjoco et al. 1993). Other examples exploit the fusogenicity of the glycoprotein. Mammalian “syncytins”, for example, are maintained in various lineages

for their ability to fuse syncytiotrophoblast cells during formation of the placenta (Lavialle et al. 2013), and appear to contribute to myoblast fusion in mice (Redelsperger et al. 2016). Syncytin-mediated cell–cell fusion is mechanistically analogous to membrane fusion during entry of the virus into the target cell. Thus, syncytins must maintain the fusion-mediating domains of retroviral Env proteins in addition to receptor-binding regions. The maintenance of fusion-related motifs in *percomORF* suggests that it could provide a fusogenic function. Moreover, the predicted cytoplasmic tail (CT) of *percomORF* is unusually short (supplementary fig. S1, Supplementary Material online) compared with that of related retroviral glycoproteins. The C-terminal 16 amino acids of the CT of murine leukemia viruses (MLVs) is cleaved by a viral protease to activate fusion (Yang and Compans 1997), and in HIV-1, truncation of the CT has been shown to increase cell–cell fusion in vitro (Kolchinsky et al. 2001). Therefore, a truncated CT in *percomORF* could represent selection for fusogenicity.

The divergence of the Percomorpha was associated with an expansion in the diversity of reproductive modes. Although the majority of its members are lecithotrophic (deriving nutrients from the yolk), the Percomorpha are the only group within the Acanthomorpha that includes taxa that have evolved placentation and viviparity (Wourms 1981). Specifically, most of the taxa with these traits belong

to the clade “Ovalentaria”, which means “sticky eggs”, referring to the demersal eggs with adhesive filaments that are produced by taxa of this group (Wainwright et al. 2012). Ovalentaria are known to have very complex and highly derived reproductive strategies involving peculiar chorionic morphology (Reznick et al. 2007), and have experienced the evolution and loss of placentas on numerous occasions (Reznick et al. 2002; Pollux et al. 2009). While the majority of *percomORF*-positive species from this limited study are not viviparous, reflecting the composition of the Percomorpha group as a whole, it is intriguing to speculate on a role for *percomORF* in the dynamic innovation in reproductive strategies that characterizes this group, possibly contributing to syncytia formation in placenta, “sticky eggs”, or another chorionic feature.

In conclusion, *percomORF* is to our knowledge the oldest intact gene of viral—older by as much as 50 My than *syncytin-Car1*, which entered the germline of carnivores an estimated 60–85 Ma (Cornelis et al. 2012). The maintenance of an intact ORF and canonical functional motifs, its expression in stickleback embryos, together with dN/dS analyses strongly suggest that *percomORF* has been coopted by the host species. The role of similar retroviral *env* genes in chorionic structures and placenta formation in mammals, combined with *percomORF*'s association with the only group of fish in which placentation evolved leads naturally to speculation on a syncytin-like role for *percomORF*. However, follow-up studies involving a wider range of taxa and morphological data; expression profiles in a variety of tissue types and developmental stages; and assays for fusogenicity of the protein, are needed to assess its role in the evolution of ray-finned fishes.

Supplementary Material

Supplementary methods S1, tables S1 and S2 and figure S1 are available at *Molecular Biology and Evolution* online.

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