

**Understanding the Enigmatic Anelloviridae**

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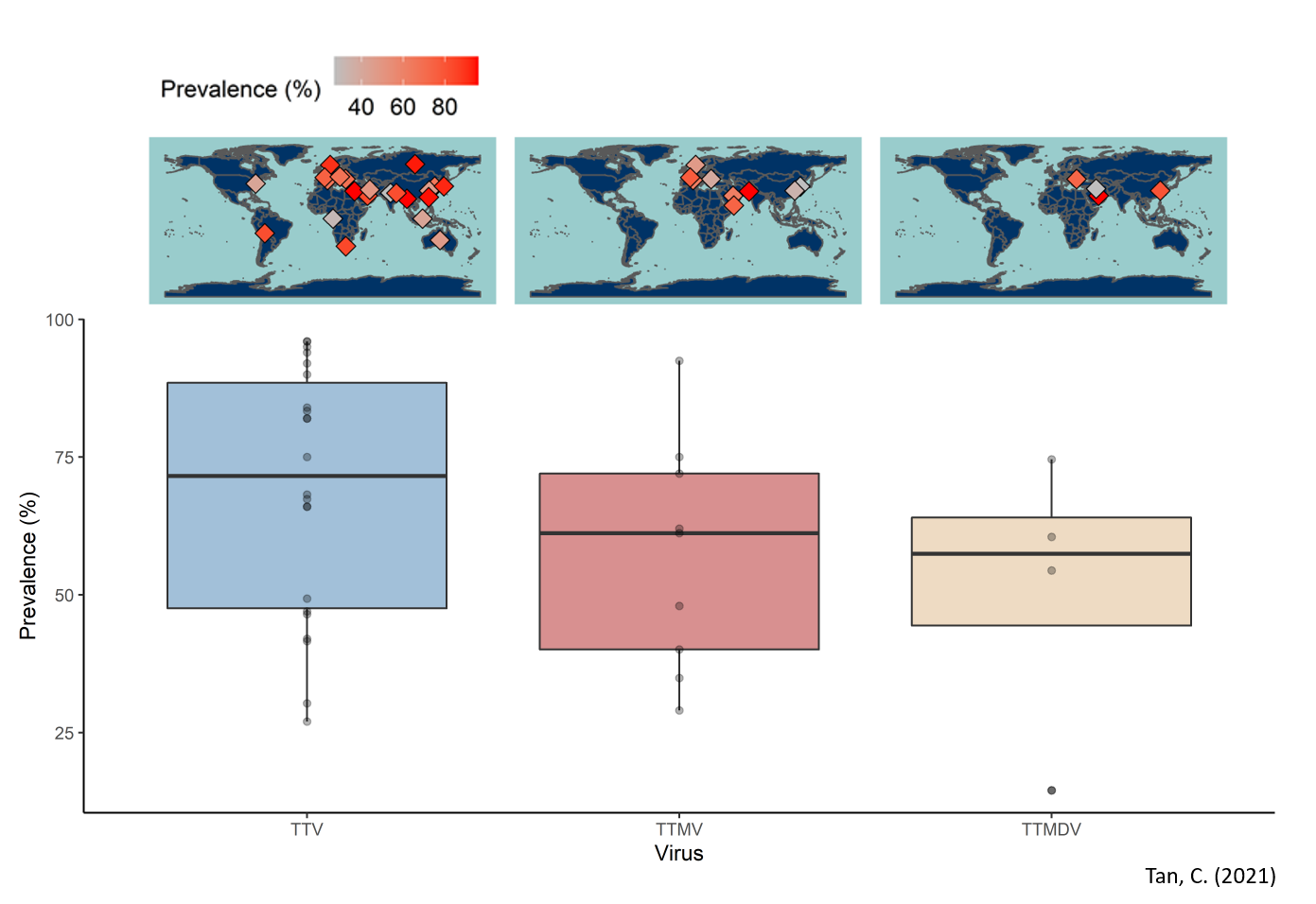
# Abstract (200 words)

The *Anelloviridae* is a diverse family of circular, negative-sense, single-stranded DNA viruses. These viruses are known to cause lifelong but asymptomatic infections in a wide range of mammals and birds and can infect multiple cell and host types. Anelloviruses are ubiquitous in the hosts they infect, with some species reaching near 100% prevalence globally. Despite this, little is known about these viruses. Indeed, their life cycle, the proteins they encode, or the interactions with their hosts remain poorly characterised. This review provides an overview of these enigmatic viruses, exploring the anelloviral genome, a potential replication mechanism, and key interactions with host immunity. Additionally, the extant genomic diversity of the family, how it may be studied, and potential mechanisms for the accrual of such diversity will be discussed. Finally, important themes on viral evolution such as how anelloviruses may have acquired its broad host range, how this relates to the risk of zoonotic spillover, and potential animal reservoirs will be explored.

# Introduction

The family *Anelloviridae* comprises 14 genera and 76 species as of the 2019 release of the International Committee on Taxonomy of Viruses (ICTV) virus taxonomy [1]. The word ‘anello’ is Italian for ‘ring’, which refers to their circular, negative-sense, single-stranded DNA (ssDNA) genomes. As of 20 Oct 2020, there were 1143 complete anelloviruses genomes of length ranging from 1.5-4 kilobases (kb) deposited in the *NCBI Virus* database.

Anelloviruses infect a diverse range of hosts, including humans [2], rodents [3], bats [4], and livestock [5,6]. The first and most well-studied anellovirus is the torque teno virus (TTV), which was discovered in the plasma of a hepatitis patient in 1997 [7]. The torque teno mini virus (TTMV) and torque teno midi virus (TTMDV) are also known to infect humans. Human anelloviruses are highly prevalent in the human population. Indeed, consolidation of the results from 23 studies [2,8–29] found that the prevalence of TTV, TTMV and TTMDV were in the range of 27-96%, 29-92.5%, 14.5-74.6%, respectively (**Figure 1**). Despite this, there has been no convincing evidence thus far implicating human anellovirus infection in symptomatic infection. The torque teno sus virus (TTSuV) in pigs and chicken anemia virus (CAV) in chickens are also anelloviruses in widespread circulation globally. The former has been associated with postweaning multisystemic wasting syndrome (PWMS) in pigs [30] while the latter has been found to cause immunosuppression and anemia [5].



**Figure 1. Global distribution of human anelloviruses**. Prevalence estimates of human-associated anelloviruses in healthy individuals were obtained from 26 independent studies and summarised in this figure. The world maps were generated using the *ne\_countries* function from the *rnaturalearth* package [31] in *R*.

The high prevalence and transmissibility of anelloviruses around the world warrants further surveillance. Indeed, it is possible that an animal anellovirus may acquire the ability to symptomatically infect and transmit efficiently amongst humans, resulting in the emergence of a new human infectious disease that significantly affects public health. While current global health efforts mainly involve post-emergence outbreak control [32], it is prudent to pre-empt and mitigate the emergence of the next novel disease-causing pathogen. However, doing so for members of the *Anelloviridae* is difficult since our understanding of anelloviruses is limited relative to other more prominent pathogens. In fact, there are currently no stable cell culture systems or animal models available for anelloviruses [33–35]. This review will explore the limits of our current knowledge about the anelloviral genome, its replication, and how anelloviruses persistently infect their hosts in the face of immune pressure. Key considerations for phylogenetic investigations into the vast genomic diversity of anelloviruses, and potential mechanisms for the accumulation of such diversity will be discussed. Finally, an assessment of the potential for anelloviruses to emerge as pathogens responsible for human infectious diseases will be provided.

# **The anelloviral genome**

## Structure

Anelloviral genomes can be transcribed to form a single polycistronic precursor messenger RNA (mRNA) via transcription initiation at a TATA-box promoter. Though small (1 - 4.5kb), anelloviral genomes encode multiple proteins via alternative splicing of the precursor mRNA, and alternative translation initiation[[1]](#footnote-1) on the spliced mRNA species. For example, transfection experiments of human TTV found at least six proteins translated from three alternatively spliced mRNA species [36,37]. All anelloviruses possess an untranslated region (UTR) containing a GC-rich region [38]. Additionally, anelloviruses can have up to four overlapping open reading frames (ORFs), named ORF1-4, but only ORF1 and ORF2 are conserved across all known anelloviruses [33]. The functions of the proteins encoded by anelloviral ORFs are poorly characterised with most insights based on the study of CAV and TTV. Based on the TTV genome structure and gene nomenclature (**Figure 2**), ORF1 is thought to encode the anellovirus capsid protein that contains an arginine-rich N-terminus [35], and a Rep-like protein. ORF2 encodes a protein with a WX7HX3CXCX5H sequence motif that is highly conserved across most anelloviruses, and resembles the consensus signature motif of protein tyrosine phosphatases (PTPases) [39]. In particular, expression of CAV and TTMV ORF2[[2]](#footnote-2) yielded proteins with the ability to dephosphorylate serine, threonine and tyrosine residues and are hence dual-specificity phosphatases [40]. TTV ORF3 encodes the TTV-derived apoptosis-inducing protein (TAIP), which was found to induce apoptosis in human hepatocellular carcinoma cells [41]. A similar protein named apoptin is encoded by the VP3 ORF in CAV. Apoptin induces apoptosis of thymocytes and erythroblasts[[3]](#footnote-3), resulting in anemia and immunodeficiency in chickens [42]. Although TAIP and apoptin share low sequence homology, they have similar apoptotic activities, are both proline-rich, and have stretches of hydrophobic residues [41]. Further, CAV genome replication is apoptin-dependent and TAIP can rescue replication in apoptin-deficient CAV [43]. These findings suggest potential structural homology between the two proteins, but no protein solution structures are currently available to verify this.



**Figure 2. Genome structure.** The complete genome of TTV (*GenBank*: NC\_002076.2) was visualised and annotated with the putative genomic elements, protein functions and conserved motifs using UGene [44].

## Replication

Currently, the genome replication mechanism of anelloviruses is poorly understood relative to other families of circular ssDNA viruses such as *Geminiviridae* [45] or *Circoviridae* [46]. Fundamentally, anelloviruses are dependent on their hosts replication machinery since TTV replication is inhibited by administering the DNA polymerase inhibitor, aphidicolin [47]. It is believed that anelloviruses replicate via a rolling circle replication (RCR) mechanism. This is because anelloviral genomes contain a conserved sequence motif that is part of a stem-loop structure, and encode Rep-like proteins, both of which are found conserved in other circular ssDNA replicons including plasmids and viruses. A potential mechanism for anelloviral replication modelled off that for porcine circovirus (PCV; family *Circoviridae*) [46] is shown in **Figure 3a**.



**Figure 3. Genome replication.** (a) Potential model of RCR for anelloviruses. The negative-sense ssDNA anellovirus genome is converted into double-stranded DNA (dsDNA) intermediate by host enzymes (A). Subsequently, a Rep-like protein nicks one of the two DNA strands at the origin of replication (*ori*) to generate a 3’OH, which acts as a primer for host DNA polymerase (DNAP) to incorporate new nucleotides using the other DNA strand as a template (B). As the former DNA strand is elongated, the parental strand is displaced (C). After a round of replication (D), the displaced parental strand is cleaved at the ori, the resultant 5’ and 3’ ends covalently linked, and the molecule released as circular DNA (E). The newly synthesised strand can then proceed for another of replication. (b) Manual alignment of the octanucleotide and nonanucleotide motifs conserved in anelloviruses and other ssDNA families, respectively (right). Abbreviations: chicken anemia virus (CAV); torque teno virus (TTV); porcine circovirus (PCV); coconut foliar decay virus (CDFV); subterranean clover stunt virus (SCSV); banana bunchy top virus (BBTV). (c) Proposed stem-loop structure containing the nonanucleotide motif in PCV (*GenBank*: U49186.1). Predicted secondary structures of the region surrounding the octanucleotide and nonanucleotide motif in TTV (d; *RefSeq*: NC\_002076.2) and CAV (e; *GenBank*: AY843527.2), respectively. Structure prediction for TTV and CAV was performed with RNAStructure [48] using default parameters. (f) During RCR, HUH endonucleases recognise dsDNA via a dsDNA-binding domain and catalyse the breaking or joining of ssDNA via a series of reversible steps A catalytic tyrosine performs nucleophilic attack on the phosphorus of the substrate, resulting in the formation of a negatively charged intermediate. This intermediate is stabilised by a divalent metal cation (denoted M2+; *e.g.* Mg2+, Mn2+) coordinated by the HUH motif, which contains an invariant histidine, an invariably polar residue (denoted U), and a second histidine or a glutamine [63].

Anelloviruses have conserved sequence motifs within a stem-loop structure, reminiscent of other circular ssDNA replicons such as plasmids and viruses that replicate via RCR. For example, CAVs and TTVs contain the conserved nona- and octanucleotide motifs, 5‘-TACTATTCC-3’ and 5’-AGTTTAAA-3’, respectively. The latter motif was proposed to contain the TTV origin of replication at the +5 position [49]. These motifs resemble the origin-containing nonanucleotide motifs in multiple other circular ssDNA replicons [49–51] (**Figure 3b**). Additionally, the nonanucleotide motifs in circular ssDNA replicons tend to be found within stem-loops, which are a type of DNA secondary structure. The proposed stem-loop structure for porcine circoviruses (PCVs; family *Circoviridae*) [51] is shown in **Figure 3c**. Stem-loop structure have been shown to be essential for RCR. For instance, mutations in PCVs [52] and tomato golden mosaic virus (family *Geminiviridae*) [53] that significantly affected these stem-loops disrupted RCR. Conversely, mutations that did not affect the stem-loop structures allowed RCR, suggesting that structural conservation is more important than sequence conservation. RNAStructure [48], a tool for predicting nucleic acid secondary structure, predicts that both the CAV nonanucleotide and TTV octanucleotide motifs are within similar stem-loop structures (**Figure 3d** and **e**), which further supports an RCR mechanism for anelloviruses.

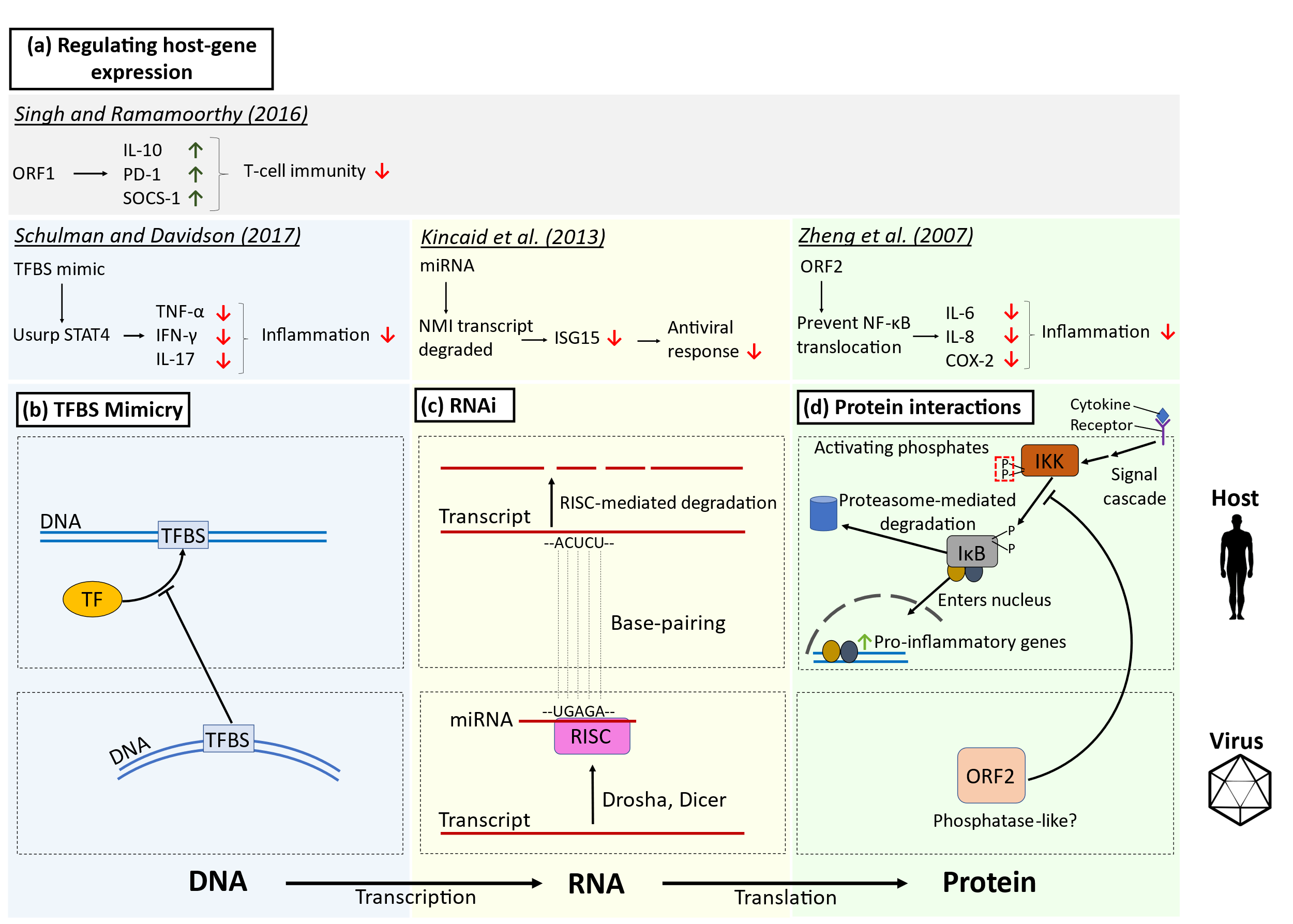
Anelloviral genomes also encode proteins [54–58] that resemble the replication (Rep) proteins crucial for RCR in other circular DNA replicons [59]. In particular, anelloviruses possess the conserved FTL and FXXK motifs [60] found in *Circoviridae* and *Geminiviridae* Rep proteins [61]. Of the nine known families of eukaryotic DNA viruses other than the *Anelloviridae*[[4]](#footnote-4), all except *Bidnaviridae* are believed to possess Rep proteins of the HUH endonuclease superfamily [62]. As such, it is tempting to speculate that anelloviral Rep-like proteins are also of this superfamily. If true, a potential catalytic mechanism for these proteins can be modelled of that for HUH endonucleases (**Figure 3f**). Given the essential role of Rep proteins in RCR, a possible first step to elucidating the replication mechanism of anelloviruses could involve the prediction of protein domains in anelloviral Rep-like proteins via sequence alignment. However, this is difficult since anelloviral Rep-like proteins have a low sequence homology with other Rep proteins [63]. Moreover, *InterPro* [64], a bioinformatics tool for functional characterisation of protein sequences, could not identify any protein functions, domains or structural motifs based on the TTV ORF1 protein sequence (*RefSeq*: YP\_009505723.1). In contrast, *InterPro* identified a P-loop motif and a helicase domain, both of which were previously described [65,66], on the PCV Rep protein sequence (*GenBank*: QOC60271.1). An alternative approach is to identify regions that share structural homology with other Rep proteins. However, this requires a protein structure solution for anelloviral Rep-like proteins, which is currently unavailable.

Further investigation into the genome replication of anelloviruses may prove to be useful in the future, especially when developing antiviral therapeutics and strategies. For example, transgenic expression of non-functional viral Rep proteins in plants was shown to interfere with Rep-mediated recruitment of host polymerases for genome replication, inducing resistance to tomato yellow leaf curl geminivirus (family: *Geminiviridae*) [67–69]. A similar strategy could be explored to reduce the impact of anellovirus-related mortality in livestock.

# Host-virus interactions: evading host immunity

There is an intimate relationship between anelloviruses and their hosts’ immune systems. Upon successful viral infection, the host’s immune system must recognise the pathogen to mount an antiviral response. Anelloviral infections result in the production of anellovirus-specific antibodies as part of antibody-mediated (or humoral) immunity [70–75], suggesting that these viruses can be detected by their hosts. However, many anelloviral infections are asymptomatic and can persist over long periods (possibly life-long) [76–78]. This suggests that anelloviruses are adept at evading or suppressing host immunity. Their different strategies for immune evasion and the underlying host-virus interactions will be discussed.

During infection, anelloviruses can regulate host gene expression to suppress host immunity (summarised in **Figure 4a**). For example, after transfection of TTSuV ORF1 expression vectors into swine macrophages, the expression of the immunity-associated genes IL-10, PD-1 and SOCS-1 was upregulated [79]. Upregulation of these genes is believed to suppress T cell immunity and is also observed in the establishment of other chronic viral infections [80–82]. Notably, macrophages are but one of the many key players in host immunity. As such, a cell culture model involving only macrophages in isolation precludes complex interactions between the different immune cells. For example, macrophages can present processed antigens to T-cells, resulting in T-cell activation. Activated T-cells, when in direct cell-cell contact with macrophages, in turn trigger the production of TNF-α[[5]](#footnote-5) in macrophages [83]. As such, models involving co-culture of multiple immune cells may more accurately reflect the immunogenic effects of anelloviruses *in vivo*, providing deeper insights into the host-virus interactions of anelloviruses.

 **Figure 4. Host-virus interactions.** (a) Summary of key anellovirus studies discussed in this section. (b) Regions on the viral genome mimic host TFBS binding sites (TFBSs), competitively bind host transcription factors, which would typically bind to regulatory elements on the host genome to activate or suppress gene transcription. (c) Anelloviral transcripts hijack the host RNA interference (RNAi) pathway. Regions of viral transcripts are processed by the host enzymes, Drosha and Dicer, to form short single-stranded RNAs (ssRNAs) of about 22 nucleotides called miRNAs. The miRNAs are loaded on the RNA-induced silencing complex (RISC) and base-pair with complementary sequences on host transcripts, hence acting as guides to target host transcripts for RISC-mediated degradation [89]. (d) Anelloviral ORF2 disrupts NF-κB transcriptional activation. In this pathway, NF-κB complexes are typically sequestered in the cytoplasm by inhibitory proteins, called inhibitors of κB (IκBs). When a cell’s receptors bind pro-inflammatory cytokines, a series of downstream signalling events results in the phosphorylation and subsequent activation of IκB kinases (IKK). These activated kinases phosphorylate the inhibitory proteins, resulting in their proteasome-mediated degradation, releasing the NF-κβ complexes to enter the nucleus and upregulate pro-inflammatory gene transcription. TTV ORF2 interacts with IKKs to prevent the nuclear translocation of NF-κB complexes [92].

Studies summarised in (a) have their panels color-coded according to the interaction mechanisms shown in (b)-(d).

Anelloviruses may modulate host gene expression pre-transcriptionally by host transcription factor binding site (TFBS) mimicry (**Figure 4b**). Using PROMO [84], a TFBS detection tool, Schulman and Davidson [85] searched for regions on the TTV genome (*GenBank*: AB017610) with at least 99% sequence homology to human TFBSs. They found four to eleven copies of 44 TFBS mimics on the TTV genome, some of which were targets for host transcription factors that regulate immunity-associated genes. For example, one of the TFBSs detected is a target for the transcription factor, STAT4. STAT4 upregulates the expression of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-17 [86], which are chemical signals that are critical in coordinating the host’s antiviral response. Indeed, STAT4-deficient mice infected with herpes simplex virus 2 had higher viral loads and took longer to resolve infection than controls [87]. As such, usurping of transcription factors like STAT4 by TFBS mimics on the TTV genome may suppress the host’s immune response to TTV infection. This suggests that TFBS mimicry might be an immune evasion strategy for anelloviruses. Anelloviruses may also modulate host gene expression post-transcriptionally by hijacking the host RNA interference (RNAi) pathway (**Figure 4c**). The TTV genome was found to encode an miRNA that targets NMI (N-myc and STAT interactor) transcripts for degradation via this mechanism [88]. Depletion of NMI transcripts resulted in the downregulation of ISG15 [88], which is a key player in triggering host antiviral responses [89]. This may be another potential strategy that anelloviruses use to evade host immunity and establish chronic infections. Finally, anelloviruses can modulate host gene expression post-translationally via protein-protein interactions. For example, a protein encoded by TTV ORF2 (henceforth ORF2 protein) was shown to physically interact with IκB kinases (IKKs) to disrupt the NF-κB pathway, which is a key pathway for upregulating pro-inflammatory gene expression [90] (**Figure 4d**). Since ORF2 proteins resemble phosphatases, which dephosphorylate proteins, it is possible that ORF2 proteins disrupt this pathway by removing the activating phosphates on IKKs. However, this has not been experimentally demonstrated. Further investigations to verify this must show that TTV ORF2 proteins indeed have phosphatase activity, and that they specifically target the activating phosphorylation sites on IKKs.

While evidence for pathogenicity has only been found for CAV in chickens, the ability of other anelloviruses to alter the susceptibility or severity of other diseases cannot be ruled out. Considering that anelloviruses persistently infect their hosts, their immunosuppressive nature may alter the host’s immune response to other pathogens, affecting the progression or severity of other infectious diseases. This may explain the observation that piglets infected with both TTSuV and porcine circovirus (PCV; family Circoviridae) developed acute PWMS, but piglets infected with either virus alone showed no signs of disease [30]. Further investigation into whether anelloviruses impair host defences against other pathogens may open new paths to understanding the pathogenesis of current infectious diseases.

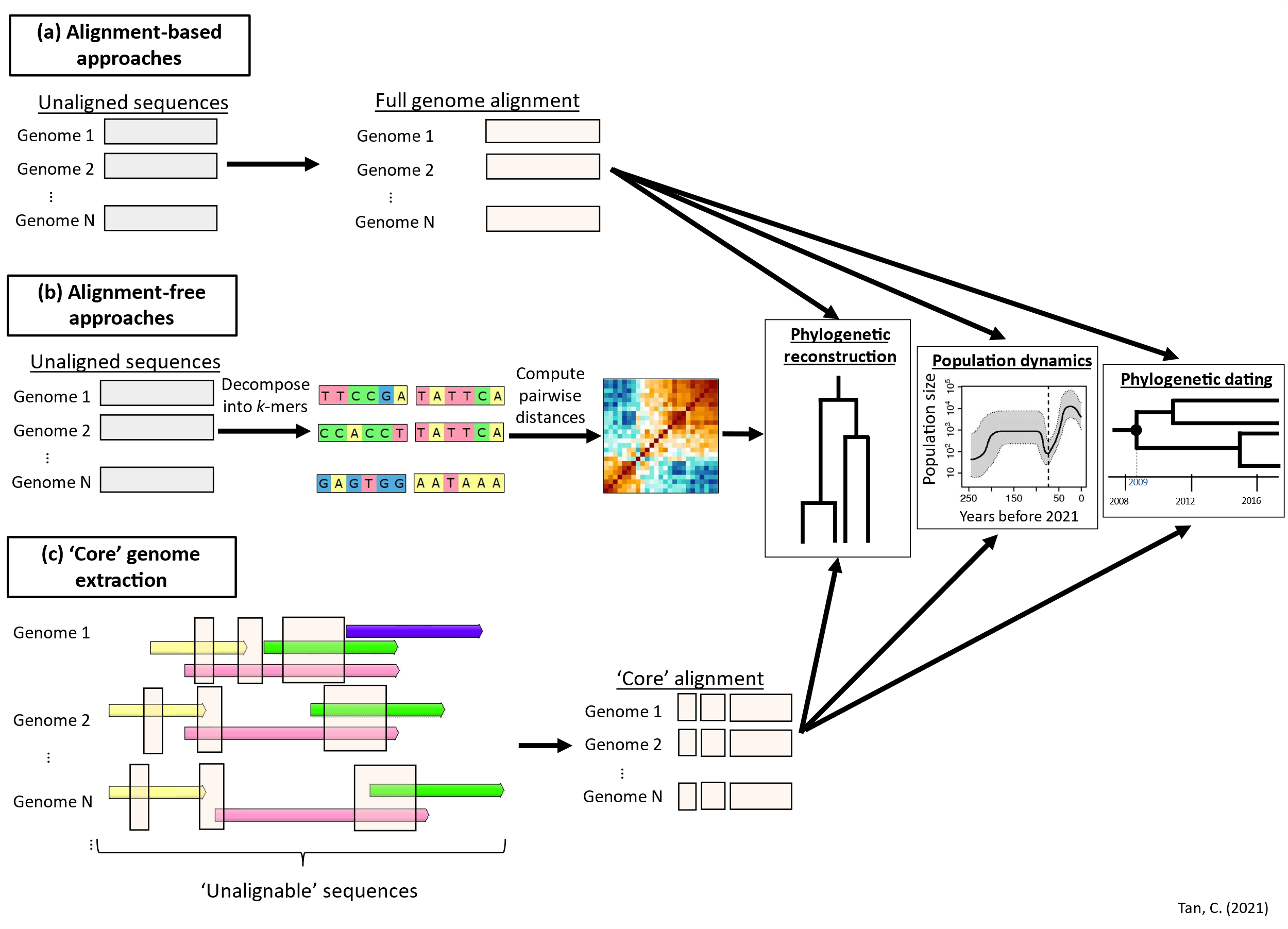
# **Anelloviral evolution**

## Investigating the genomic diversity of anelloviruses

Phylogenetic studies probing the genomic diversity of can yield valuable information. Such studies reconstruct evolutionary trees or phylogenies that represent the evolutionary relationships between genomes. Using a phylogeny, it is possible to reconstruct the transmission chain of infectious disease outbreaks. This provides insight as to whether transmission is driven by local or imported cases, thus informing outbreak management [91]. Additionally, phylogenies can be probed to test if particular mutations are associated with viral transmissibility, as was demonstrated with SARS-CoV-2, the agent responsible for the present COVID-19 pandemic [92]. In this section, potential considerations for leveraging phylogenetic methods to study the genomic diversity of anelloviruses will be discussed.

There is high sequence divergence within the *Anelloviridae*, which is observed even at the species level. Indeed, the amino acid sequence diversity within human TTVs is approximately 7.6, 2.2 and 2.1 fold greater than that for hepatitis B virus, human immunodeficient virus-1 and hepatitis C virus, respectively [34]. This diversity poses a major challenge for generating full-length genome alignments, which is a critical step in phylogenetic reconstruction (**Figure 5a**). This is because sequence alignment algorithms are known to have limited accuracy when used for aligning highly divergent sequences [93]. As such, the ICTV recommends restricting phylogenetic reconstruction analyses to ORF1 [1]. Most studies probing the diversity of different anelloviral species, or of the family, reconstructed phylogenies [33,38,94–96] in accordance with this. However, these phylogenies do not capture the full-length genomic diversity of the sequences analysed. Given these limitations, alignment-free approaches may be more suitable for phylogenetic reconstruction. As the name suggests, alignment-free approaches quantify the similarity between sequences without the use of an alignment [96]. One common alignment-free approach is illustrated in **Figure 5b**. Since alignment-free approaches are relatively robust to high sequence divergence [93], they may be more appropriate for future studies investigating the family-wide diversity of the *Anelloviridae*. Moreover, alignment-free approaches are less affected by the effects of recombination [93], which occurs in anelloviruses and will be discussed below. When viruses undergo recombination, portions of their recombinant genomes represent different evolutionary histories and therefore cannot be expressed by a single phylogeny [97] (**Figure 7b** in section, ‘*Host-editing and recombination*’). In such cases, the resultant phylogeny cannot be confidently reconstructed and often differs from the true phylogeny [98]. This effect is more prominent when recombination occurs between divergent sequences [97], as in the case of anelloviruses.

Though alignment-free approaches can circumvent some limitations of alignment-based approaches, many sophisticated methods in population genetics such as those for phylogenetic dating [99] or inferring past population dynamics [100,101] are alignment-dependent. As such, future studies seeking to employ such methods could consider generating a concatenated genome alignment from conserved (or ‘core’) genome segments or genes (**Figure 5c**). Notably, the identification and annotation of ‘core’ genomic regions can be challenging in viruses with multiple overlapping reading frames. Nonetheless, concatenated alignments capture a larger region of the viral genomes and hence their genomic diversity while allowing downstream alignment-based analyses. Such approaches have been previously used on highly divergent members of microbial communities [102] and recently for the family *Coronaviridae* [103]. This can be done and would allow the construction of a sequence alignment that retains a larger proportion of the viral genomes. A potential ‘core’ gene alignment for anelloviruses may include regions within ORF1 and ORF2, which are found in all anelloviruses [33]. Additionally, to minimise the impact of recombination, genomic sites identified by recombination detection tools such as *PhiPack* [104], can be removed.

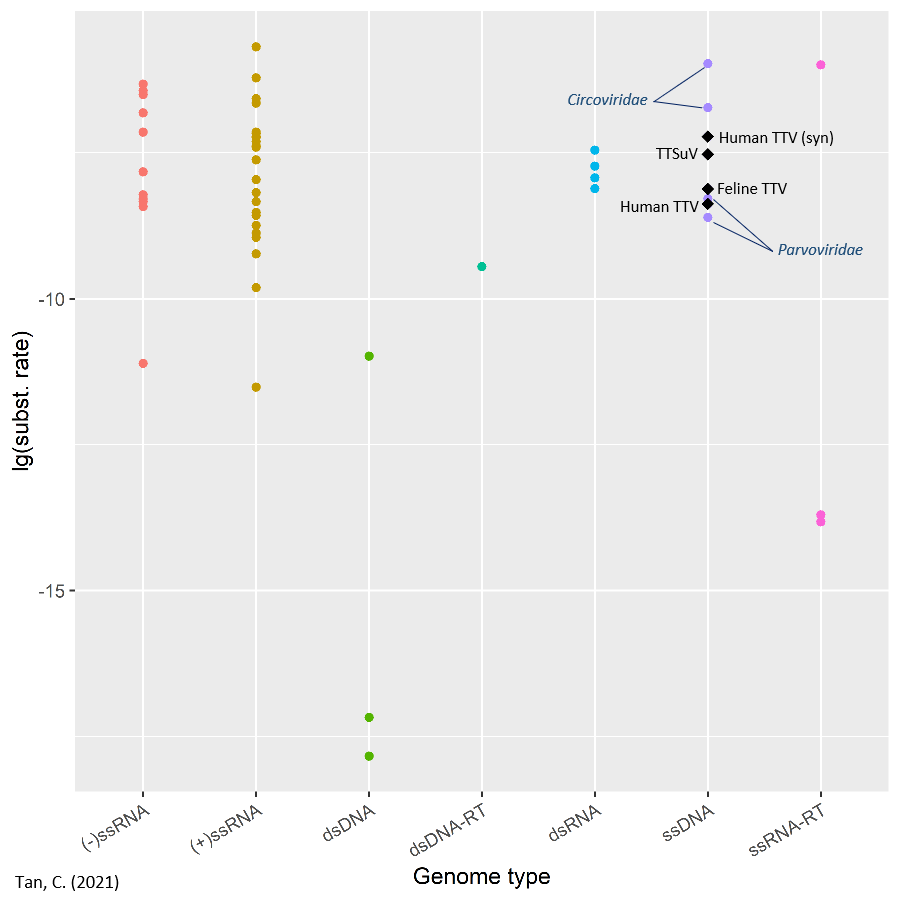


**Figure 5. Phylogenetic approaches.** (a) Simplified workflow of typical phylogenetic reconstruction. A sequence alignment of all genomes in question must first be generated. This alignment is then used as input for tree-building tools such as RAxML [91] or IQ-TREE [92] to reconstruct the evolutionary relationships of the sequences in the form of a phylogeny. The alignment can also be used for phylogenetic dating or inferring past population dynamics. (b) Each full-length sequence is split into short sequence blocks called *k*-mers. A summary statistic representing the sequence dissimilarity (*i.e.* distance) between a pair of sequences can derived from how many of these k-mers are shared between said sequences. This distance is calculated for every possible pairwise combination of sequences and subsequently used to reconstruct a phylogeny via algorithms such as the neighbour-joining method [99]. (c) ‘Core’ genes or genomic regions from ‘un-alignable’ viral sequences are extracted using gene annotation tools such as Prokka [103], Roary [104] or Mauve [105]. These ‘core’ genomic regions can then be combined to produce a concatenated genome alignment, which can be used for downstream alignment-based phylogenetic analyses.

## Mutation rates

Understanding how viruses mutate and accumulate genomic diversity has profound practical implications. For example, the emergence of SARS-CoV-2 mutations that are associated with immune escape has been of great concern with regards to vaccine efficacy and development in the present COVID-19 pandemic [105]. Additionally, elevating short-term viral mutation rates via mutagen treatment, termed lethal mutagenesis, has been shown to enhance the effectiveness of antiviral treatments [106]. Only a few studies to date have provided estimates of the mutation rate of anelloviruses [76,107,108], which ranges from 2.3-7.3 × 10-4 substitutions/site/year (s/s/y). The estimated substitution rate for the TTSuV genomic region containing the UTR, ORF2 and 5’ end of ORF1 sequences sampled from wild boars over six years was 5.4 × 10-4 s/s/y [107]. Additionally, the substitution rates of anellovirus sequences obtained from human sera and cat saliva reported over 16 and 6.5 years, respectively, were approximately 2 × 10-4 s/s/y [76]. Lastly, a synonymous substitution rate of 7.3 × 10-4 s/s/y was estimated from TTV-positive human sera over 12 years [108].

Rates estimated in anelloviruses were compared to that of other vertebrate viruses, which were collated from ten other studies [8,109–117] (**Figure 6**). Within ssDNA viruses, anelloviruses have mutation rates that are considerably lower than that of circoviruses (family *Circoviridae*), but in the same order of magnitude as that of parvoviruses (family *Parvoviridae*). Additionally, anelloviruses mutate much faster than that of double-stranded DNA (dsDNA) viruses, which echoes previous findings that single-stranded viruses mutate faster than their double-stranded counterparts [106]. This phenomenon has been suggested to be due to single-stranded viruses being more prone to chemical damage such as oxidative deamination [118]. Most importantly, the mutation rates of anelloviruses are comparable to that of RNA viruses. This is intriguing given that anelloviral genome replication is performed by DNA polymerase [47], which is generally more accurate than RNA polymerases used by RNA viruses [118]. This suggests that nucleotide misincorporation by host polymerases may play only a minor role in the accumulation of mutations in the *Anelloviridae*.



**Figure 6. Mutation rates of Anelloviridae in the context of other viruses**. The substitution rates of all genomic sites, obtained from the literature and expressed as substitutions/site/year, was visualised in log10 scale for the different viruses, stratified by their genome types. Anelloviruses are indicated by black diamonds and annotated with their corresponding names. The substitution rate where only synonymous mutations were considered is denoted by “syn”.

## Host-editing and recombination

The mechanisms underlying the accumulation of genomic diversity in anelloviruses has not been explored. However, knowledge of potentially relevant mechanisms may inform our understanding of the selective pressures that shape the genomic diversity of the *Anelloviridae*.

Host innate antiviral responses may contribute to the genomic diversity. For example, cytidine deaminases such host APOBEC3 proteins have been found to cause C🡪T and G🡪A mutations in other DNA viruses like human papillomavirus via deamination [119,120] (**Figure 7a**). While host-editing by APOBEC3 proteins has not been demonstrated for anelloviruses, *in vitro* experiments found that these proteins preferentially bind ssDNA over dsDNA and may account for higher C🡪T mutations on the negative-sense strand of HIV reverse transcripts [121]. This suggests that negative-sense ssDNA anelloviruses are likely to be subject to host-editing. Investigations of how anelloviruses evade or overcome APOBEC3-mediated editing may prove to be invaluable in our understanding of how viruses evolve.



**Figure 7. Potential mechanisms of accumulating genomic diversity**. (a) Reaction equation catalysed by host cytidine deaminases. Cytidine on ssDNA is deaminated to form uradine, which is subsequently base-paired with adenosine during DNA replication, resulting in C 🡪 T and G 🡪 A mutations. (b) Recombination is the process where two co-infecting viral genomes exchange regions of their genomes. A single recombination event between these genomes could generate new combinations of their existing mutations, introducing several nucleotide polymorphisms at a time. Different portions of the recombinant genome demarcated by the recombination breakpoint represent different evolutionary histories, which confounds phylogenetic reconstruction.

Recombination plays a substantial role in shaping the extant genomic diversity of virusesby generating new genetic combinations that could not have been attained via point mutations alone [122] (**Figure 7b**). Frequent recombination may therefore explain, in part, the high mutation rates of anelloviruses. Using the popular recombination detection tool RDP4 [123], which identifies recombination using an ensemble of different detection methods, Fahsbender et al. [124] found that 89% of torque teno Leptonychotes weddellii virus (TTLwV)-1 and 26% of TTLwV-2 had at least one and one putative recombination event, respectively. They also found that the recombination breakpoints detected in TTLwV-1 tend to fall within the UTR region. This agrees with a previous study [122], which found a significantly higher density of recombination breakpoints outside coding regions and at the edges of genes for almost all of 14 ssDNA viruses spanning 6 families (including TTV and TTMV). Other studies have similarly detected recombination within the different human TTV species [125,126] and within TTSuVs isolated from wild boars and domestic pigs [107]. While no studies to date have formally estimated the rate of recombination in anelloviruses, multiple studies have found a high frequency of co-infections of different anellovirus species [11,20,127–129], one even reporting a 93% frequency of dual or triple infection of TTV, TTMV and TTMDV in human subjects [130]. This high frequency of co-infection, together with the strong recombination signals detected by the abovementioned studies, suggest that anelloviruses recombine frequently and that recombination may be a major contributor to the accumulation of genomic diversity.

Moreover, recombination has even suggested to result in viral lineages gaining the ability to infect new host species. For example, human adenoviruses may have emerged from the recombination of chimpanzee and bonobo adenoviruses [131], while turkey coronaviruses may have emerged from chicken infectious bronchitis virus and another unknown coronavirus [132]. As such, it is

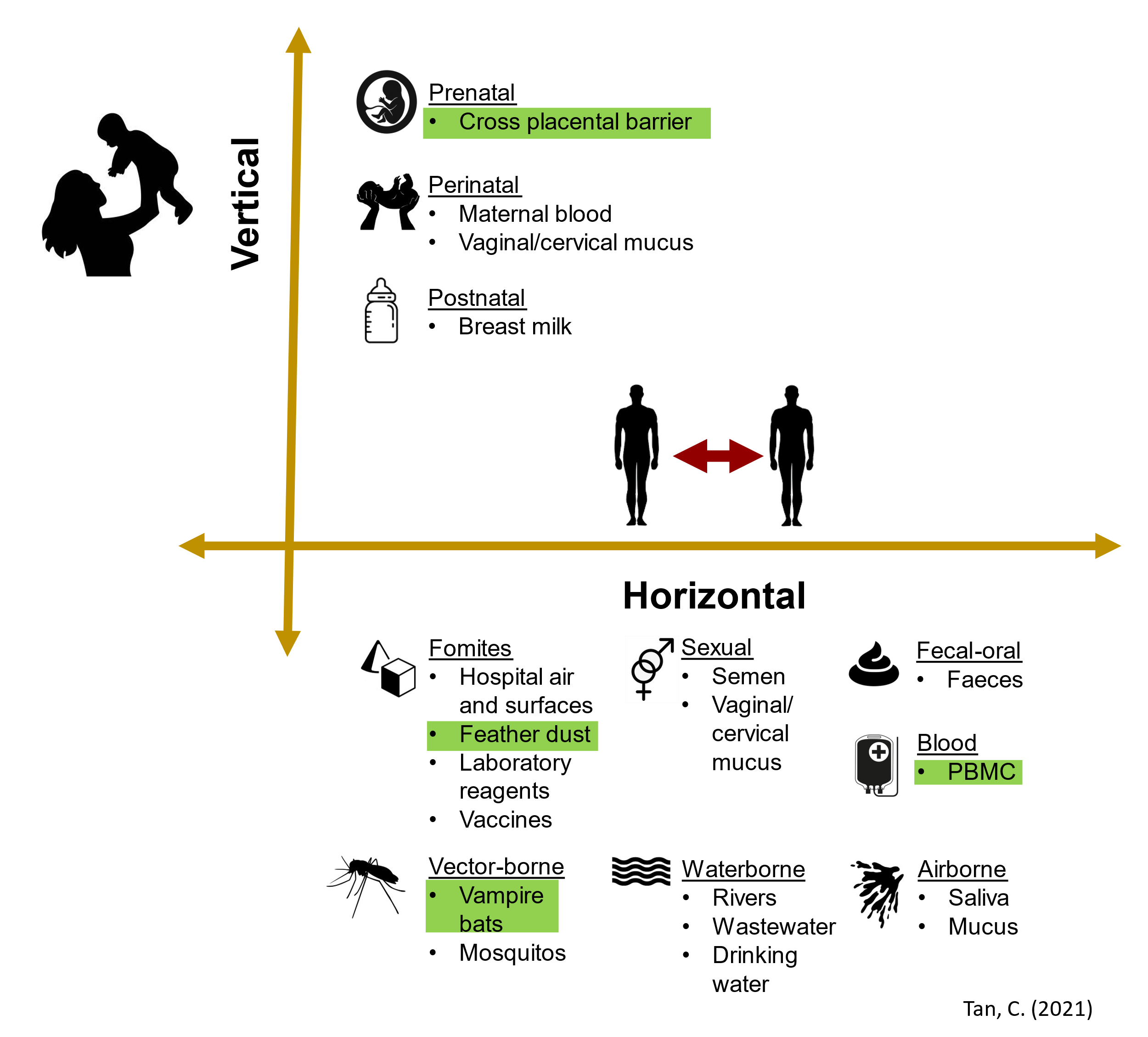
# Potential for the emergence of infectious diseases

## Transmission routes: anelloviruses can go anywhere (761)

In line with its high prevalence, anelloviruses may potentially be transmitted via multiple routes, both vertically (*i.e.* from mother to offspring) and horizontally (*i.e.* between organisms not in a mother-offspring relationship) (**Figure 8**). In general, most studies of anellovirus transmission thus far have employed PCR-based methods of detecting anelloviral DNA. In such methods, PCR with anellovirus-specific primers is used to amplify viral genomic fragments, which are then visualised via agarose gel electrophoresis. Suitably sized gel bands corresponding to the length of the PCR target are extracted and subsequently sequenced to confirm that the correct genomic sequences were amplified. Successful amplification of the PCR target is generally regarded as a positive result.

Using PCR-based methods, anelloviral DNA has been detected in human bodily fluids like blood, bile, saliva, mucus, semen, faeces [133–139]. This suggests that anelloviruses may potentially be transmitted horizontally via sexual, faecal-oral and airborne routes, or via direct contact with bodily fluids. Additionally, the detection of anelloviral DNA in multiple environmental sources suggest that they may be transmitted via fomites, which are inanimate objects or surfaces that can become contaminated with pathogens [140]. For example, studies have found anelloviral DNA in rivers [141–143], wastewaters [144], drinking water [145,146], shed feathers [74], air and surfaces in hospitals [147] or even laboratory reagents [148] and vaccines [149]. Anelloviruses can also be transmitted horizontally via vectors. Two studies found that anelloviral sequences extracted from mosquitos in pig farms were not restricted to a mosquito-associated lineage, but shared common ancestry with multiple lineages of pig-associated TTSuVs [150,151]. These phylogenetic relationships may reflect the random acquisition of TTSuVs from porcine hosts during mosquito blood-feeding (*i.e.* hematophagy). Finally, there is evidence that anellovirus transmission can occur vertically either before (prenatal; through the placental barrier), during and immediately after (perinatal), or after birth (postnatal). Indeed, TTV and TTMV DNA has previously detected in human breast milk, umbilical cord blood and amniotic fluid [152,153]. TTSuV DNA was also detected in sows, their breast milk, and their aborted foetuses or stillborns [154,155].

Notably, even if anelloviral DNA is detected via PCR-based methods, this does not necessarily entail that infectious virions are present. Of all the studies, only a few provide evidence for the viability of anelloviral DNA and its ability to infect naïve hosts (highlighted in green; **Figure 8**). For example, persistent viremia of TTV and TTMDV from blood donors was found in blood transfusion recipients [156], providing confirmatory evidence for blood as a vehicle of transmission. Additionally, dripping feather shaft extracts obtained from the feathers of CAV-infected chicks into the eyes and mouths of unexposed chicks resulted in infection [74], confirming that feathers can act as fomites to transmit CAV. Separately, anellovirus sequences extracted from the liver of a vampire bat (*Desmodus rotundus*) and serum of an opossum (*Didelphis albiventris*) were of the same species at approximately 79% nucleotide sequence identity [94]. Phylogenetic analysis suggests that these anelloviruses are likely to share a common ancestor that was transmitted between the two host types [94]. This is considerable evidence that vampire bats may be a vector of transmission for anelloviruses between the bats’ mammalian hosts. Lastly, TTSuV sequences in mother-stillborn pairs had nucleotide sequence identities of 91-98% [155]. The presence of anelloviral DNA with such high sequence identity was unlikely to be due to cross-contamination during sample collection, since such cross-contamination would result in identical sequences. This provides reasonable evidence that infections of the aborted foetuses were acquired prenatally even without proving the viability of detected TTSuV DNA.

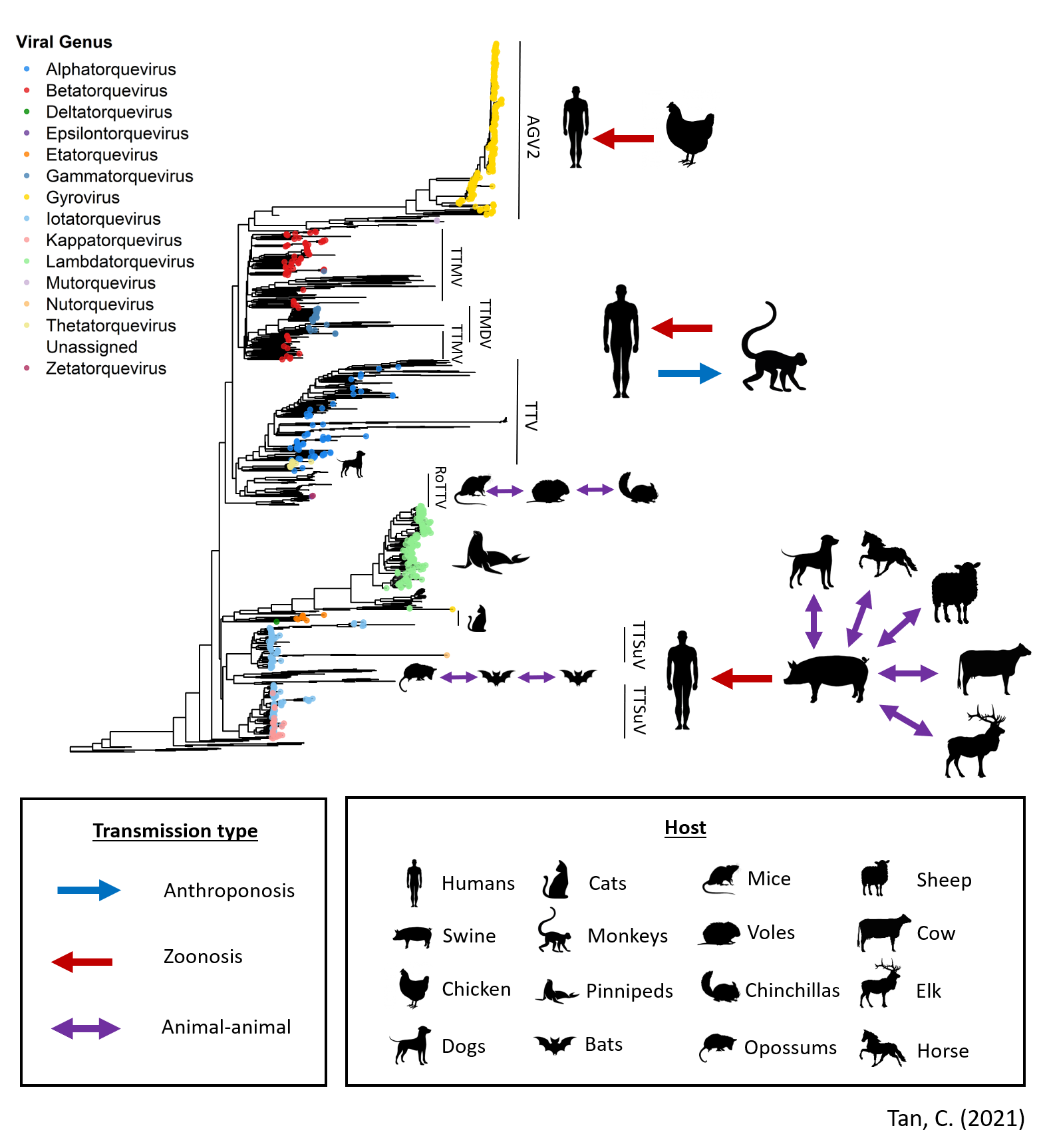


**Figure 8. Summary of potential transmission routes for anelloviruses**. Transmission routes that are supported by substantial evidence in the literature are highlighted in green.

## Anelloviruses are everywhere

After the discovery of the first anellovirus, TTV, in 1997 [7], multiple studies have detected a diverse range of anelloviruses in humans and animals, including rodents [3], primates, birds, marine mammals [124,157,158], bats [4] and other wild or domesticated animals [94,159–162]. An alignment-free phylogeny of 1143 complete anellovirus genomes isolated from at least 43 annotated host species (NCBI Virus; accessed 20 Oct 2020) is presented here (**Figure 9**). The phylogeny demonstrates significant clustering by viral genus and a broad diversity of host types that are segregated by phylogenetically distinct clades (**Figure 9**). Given the extensive sampling of the broad diversity of anelloviruses from a variety of hosts, it may be feasible to investigate the selective pressures involved in viral speciation events (e.g. the emergence of TTMDV from a TTMV lineage; **Figure 9**) or changes to host-specificity. While unexplored in the literature, probing these ideas may yield valuable insights as to how new pathogens can emerge.

Studies that have explored the biodiversity of anelloviruses in humans and animals can be non-exhaustively categorised into virus-specific and metagenomic efforts. The former category refers to studies that performed PCR using anellovirus sequence-specific primers, followed by sequencing of the PCR amplicons. For example, Ye et al. amplified and sequenced a 346bp region of avian gyrovirus 2 (AGV2) in chickens from live poultry markets and healthy human blood [159]. Similarly, Singh et al. used TTSuV-specific primers to amplify and subsequently characterise anellovirus sequences in bovine, equine, ovine, canine and elk hosts [160]. The latter category refers to studies that perform unbiased sequencing of all DNA sequences in samples (*i.e.* ‘shotgun’ metagenomic sequencing), followed by *de novo* assembly[[6]](#footnote-6). For example, studies have employed this approach for samples obtained from mosquitos [150,151,157], rodents [3,163] and pinnipeds [158,164]. To explore the host range and biodiversity of anelloviruses in different animal reservoirs, metagenomic-based approaches appear to yield much more information. Indeed, *a priori* knowledge of viral sequences is required for virus-specific approaches. Given the large sequence divergence within the family, species-specific PCR primers are unlikely to be able to amplify novel anellovirus sequences. In contrast, metagenomic-based approaches do not require *a priori* sequence information and therefore can detect novel anelloviruses. Furthermore, because anellovirus genomes are small, fewer sequencing reads are required for sufficient genomic coverage to produce reliable genome assemblies. This entails that while the genomic fragments retrieved via virus-specific PCR is restricted by the amplicon length, longer or even full-length genomic sequences can be assembled via metagenomics-based approaches. Further, there may be many anelloviral sequences already present in published metagenomic datasets which could be screened. In short, metagenomics may capture more genomic information from a larger proportion of the anelloviral diversity, which will be vital for understanding biodiversity of anelloviruses in different host and environmental contexts.

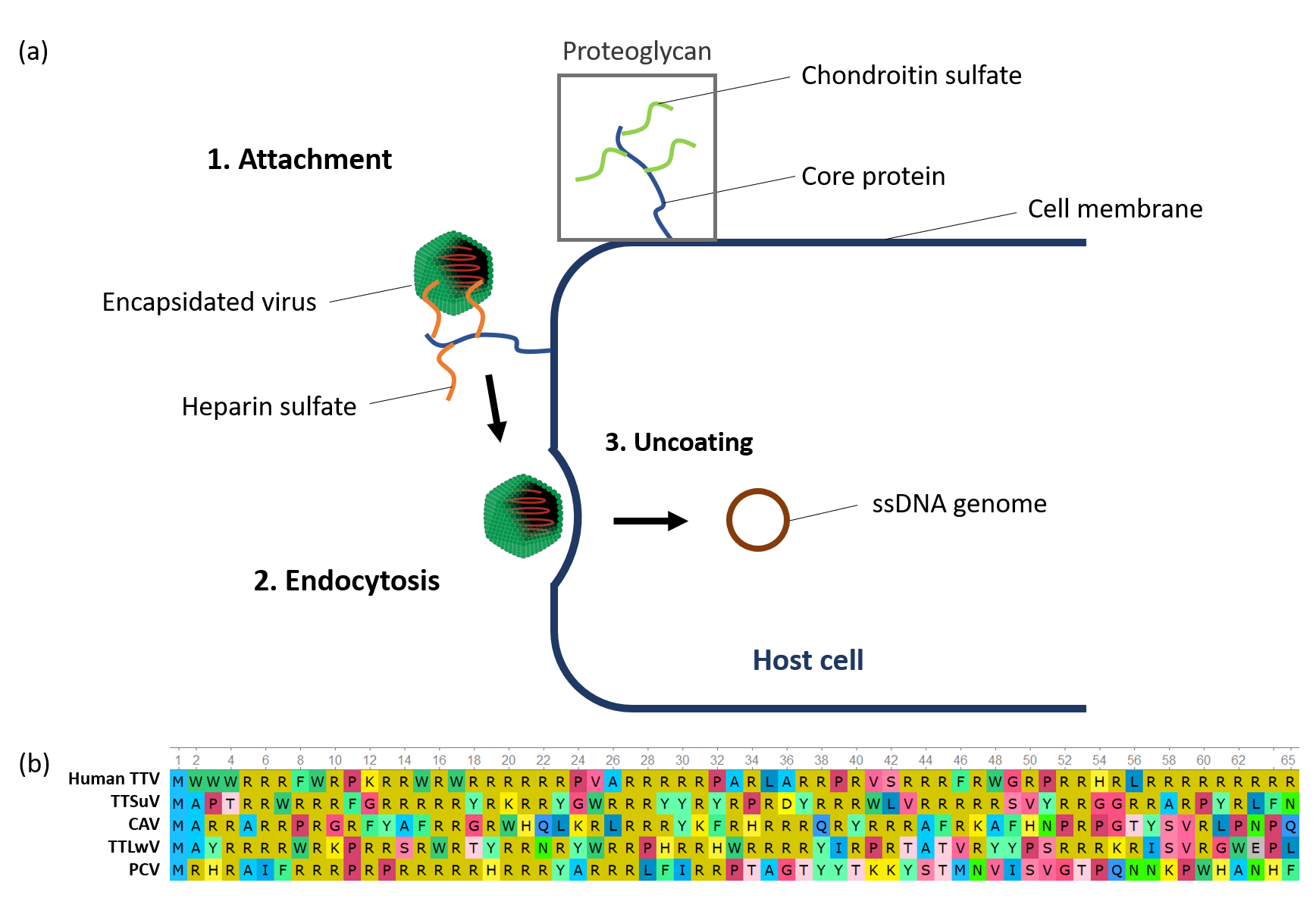


**Figure 9. Genomic diversity of the Anelloviridae and summary of evidence for cross-species transmission**. The genomic diversity of 1143 Anelloviridae genome sequences is represented here as a whole-genome neighbour-joining phylogeny, which was reconstructed using alignment-free Mash distances [165] and rooted with the genome accession MK012481. Tip colours provdie the viral genus as given by the legend at top right. This phylogeny is annotated with symbols illustrating the host range and cross-species transmission of various viral species obtained following literature review.

## Evidence for cross-species transmission

There has been considerable evidence supporting the ability of anelloviruses to transmit between host species. Singh and Ramamoorthy [160] detected both TTSuV and human TTV sequences in canine, bovine, ovine, equine, and elk sera using species-specific PCR primers. They further detected TTSuV-specific antibodies in bovine, ovine and equine samples using enzyme-linked immunoabsorbant assays (ELISA). Since a virus must sustain a sufficiently high viral load for a sufficiently long duration to trigger this adaptive immune response, the results confirm the presence of productive TTSuV infection in these animals. Notably, the authors did not find TTSuV-specific antibodies in elk and canine samples despite the presence of TTSuV DNA, suggesting that either the magnitudes of antibody responses were below the limit of detection for the ELISA assay used, or that viral replication was insufficient to trigger the production of TTSuV-specific antibodies. The former possibility implies considerable variability in the strength of adaptive immune responses to anellovirus infection while the latter suggests that TTSuV may not be adapted for efficient replication in said hosts. Nonetheless, these findings provide strong evidence for the ability of TTSuV to infect multiple animal hosts and possible cross-species transmission of both TTSuV and human TTV. Separately, rodent TTVs have also been shown to infect a broad range of hosts. A large metagenomic surveillance study on rodent samples collected from 20 provinces in China found that rodent TTV lineages were found in multiple hosts from the same family, or even hosts from three different families (*Circitidae*, *Muridae* and *Chinchillidae*) [163]. Additionally, as mentioned previously, de Souza et al. [94] found two phylogenetically related anelloviruses in a vampire bat and an opossum. A third anellovirus sequence obtained from the frugivorous (i.e. fruit-eating) bat, *Carollia perspicillata*, was also estimated to share a common ancestor with the other two sequences [94]. Moreover, the node where the three sequences diverged from the other anelloviruses had a high bootstrap support[[7]](#footnote-7) value, suggesting that their evolutionary relationships can be confidently inferred. Given that *D. rotundus* and *C. perspicillata* are known to share roosts in caves [166,167], horizontal transmission of anelloviruses between the two hosts may have occurred during roosting. Collectively, these studies suggest that anelloviruses, or at least those associated with mammalian hosts, tend to be ‘generalists’ that have retained the ability to infect a broad range of hosts.

Understanding how anelloviruses are internalised by cells during infection may help to account for the broad host range of anelloviruses. However, the exact mechanism for viral entry is currently unknown. A potential mechanism for anellovirus viral entry may be modelled by that observed in PCV. This mechanism involves the binding of arginine-rich regions at the N-termini of PCV capsid proteins to the extracellular receptors, heparin sulfate (HS) and chondroitin sulfate-B (CSB) proteoglycans [169](**Figure 10a**). Comparison of the PCV and anellovirus capsid protein sequences found similar arginine-rich N-termini in anellovirus capsid proteins (**Figure 10b**). This suggests that proteoglycans are possible candidates for receptors used by anelloviruses during viral entry.



**Figure 10. Potential mechanism of viral entry** (a) In general, non-enveloped viruses, which include anelloviruses, generally enter host cells by the binding of capsid proteins to cell surface receptors and inducing uptake via endocytosis [168]. Subsequently, the viral particles penetrate the cell membrane and release their genomes into the cell where it proliferates by shedding its capsid protein coat. PCV was shown to use proteoglycans, which consist of a membrane-bound core protein that is covalently attached to several glycosaminoglycan chains such as heparin sulfate (HS) and chondroitin sulfate-B (CSB), as their receptor for viral entry [169]. The PCV genome encodes an arginine-rich region at the N-terminus of the capsid protein, which can act as a cell-penetrating peptide (CPP) that triggers endocytosis and hence endocytosis of its cargo into the cell [170]. While a direct binding interaction between the N-terminal sequence of the PCV capsid and proteoglycan receptors has not been described, most CPPs are understood to allow the translocation of macromolecules into cells by binding proteoglycan receptors [171,172]. (b) *UGene* [44] visualisation of the first 65 amino acid residues in the capsid protein of Human TTV (QHS01920.1), TTSuV (YP\_003587901.1), CAV (AYN79688.1), TTLwV (YP\_009388631.1) and PCV (YP\_009315911.1). A meaningful alignment of these sequences could not be produced.

Interestingly, PCVs were found to be able to infect cells lacking both HS and CS proteoglycans [169]. Additionally, inhibition of major endocytotic pathways such as clathrin-mediated endocytosis or macropinocytosis was found to reduce but not abolish uptake of PCV-derived N-terminal sequences [170]. These findings suggest that multiple cellular pathways are involved in PCV viral entry. This, in addition to the fact that HS proteoglycans are expressed on the surface of most animal cells [173], may explain why PCV can infect multiple cell types and host species [174]. Conversely, the promiscuous cell- [175] and host-specificity of anelloviruses points to the involvement of common host cell receptors during viral entry. Arguably, with a better understanding of viral entry mechanisms, it may be possible to estimate the full host range of anelloviruses and their potential for cross-species transmission.

## The risk of zoonoses

The ‘generalist’ nature of anelloviruses is a risk factor for the emergence of zoonotic infectious diseases. Statistical modelling of 1415 human-associated pathogens, which includes viruses, bacteria and other types of pathogens, found that pathogens capable of infecting multiple hosts were at least 1.29 times more likely to emerge as zoonotic infectious diseases [176]. This echoes another study of 162 zoonotic viruses, which found that 63% of zoonotic viruses can be found in hosts from at least two different taxonomic orders [177]. These findings may be explained, in part, by the importance of virus-receptor interactions during viral entry that was highlighted in the previous section. In alpharetroviruses, a single amino acid substitution was sufficient for the extension of host range from avian cell types to include human, canine, feline, and rodent cells [178]. If receptor binding is the first barrier to viral entry, pathogens that already possess a promiscuous host-specificity may require the accumulation of fewer mutations to overcome this barrier. Since anelloviruses have a broad host range, there is a higher risk that they can emerge as novel pathogens responsible for infectious diseases that affect humans and livestock. Further, the biodiversity of anelloviruses within host species increases the risk of zoonotic spillover. A study of 415 viruses found that the number of zoonotic viruses is proportional to the number of viral species maintained by each host type [179]. That is, the richer the diversity of viruses in a particular host type, the higher the likelihood of observing viruses that cause zoonotic infectious diseases. This is the case for anelloviruses, where metagenomic-based studies often detect different species of anelloviruses, sometimes from different viral genera. For instance, a survey of anelloviruses in civets (*Paguma larvata*) found viral species from at least four genera [180], while that in rodents spanned at least two viral genera [163].

In fact, several studies have already demonstrated the potential for zoonotic transmission of anelloviruses. For example, AGV2 was detected in the blood of 2/178 (1.1%) individuals and shared at least a 90% nucleotide sequence identity with AGV2 isolated from chickens [159]. Additionally, simian-associated TTVs (SiTTV) were detected in 6.8% of 487 human serum samples [181]. Another study detected CAV and AGV2 in 8.6% and 8.7%, respectively, out of 748 human faecal samples from three separate cohorts [182]. However, in contrast to the extremely high prevalence of TTV, TTMV or TTMDV detected in humans, the proportions of samples that tested positive for these anelloviruses are low. This suggests that SiTTV, CAV or AGV2 may not yet be adapted for efficient replication and transmission in humans. There is stronger evidence for zoonosis of TTSuV. Ssemadaali et al [183] showed that out of 40 human serum samples, TTSuV DNA was detected in 67.5% and 80% of samples in two separate PCR assays targeting different regions of the TTSuV genome. Additionally, PBMCs transfected with TTSuV DNA could be used to sustain infection in unexposed PBMCs for three serial passages, suggesting that TTSuV is capable of proliferating in human cells. Most importantly, TTSuV-specific antibodies could be found in 40% of the human samples, confirming that TTSuV can productively infect human hosts. These results highlight the zoonotic potential of animal anelloviruses.

## Animal reservoirs

Animal reservoirs are groups of animals that harbour viral pathogens, some of which may be transmitted to humans (*i.e.* zoonoses), resulting in the emergence of novel human infectious diseases. The rich biodiversity of anelloviruses across host species suggest that there may be multiple animal reservoirs where zoonotic spillover may occur. Animal reservoirs that co-exist with humans such as livestock, rodents, pigeons, cats and dogs are of particular concern. As discussed above, anelloviruses seem to be ‘generalists’ that have the ability to infect multiple host species. However, even if an anellovirus manages to infect humans, it must be able to proliferate and transmit efficiently in humans for the emergence of a zoonotic disease in the human population. In fact, upon acquiring the ability to jump into a host species, there is a period of viral adaptation to its new host, where the virus accumulates mutations that potentially improves its transmissibility [184]. Animal reservoirs that are in close contact with humans may provide ample opportunities for such adaptation to occur. This is in line with the suggestions that a 20% geographical overlap is required for viral sharing between host species [185] and that the probability of sharing increases with increasing overlap up till a 50% overlap [186]. Furthermore, a network analysis of the number of viruses shared between different hosts showed that wild rodents and domestic animals such as cattle, pigs, cats and dogs were centrally located and highly connected [177]. That is, viruses in said animals are shared with the greatest number of host types. This reflects the potential of such animals to act as vectors that facilitate the movement of viruses between humans and wild-animal reservoirs that we may not be in direct contact with. Given the vast amount of metagenomic sequencing data from bats [187–189], rodents [190,191] and livestock [192–194], a meta-analysis of these datasets may shed light on the current diversity of anelloviruses, the novel anelloviruses that are emerging in these animal reservoirs and their potential for zoonotic emergence.

# Conclusion

The *Anelloviridae* is a family of ubiquitous viruses that is still poorly understood. There is an overrepresentation of studies investigating what bodily sites, environmental sources, or hosts anelloviruses can be found in, and at what prevalence. In contrast, the mechanisms underlying their persistence in the face of host immune pressures, high mutation rate, and broad host range are poorly understood. These seemingly ‘perfect’ pathogens can infect a wide range of host and cell types, transmit via multiple routes, and elude host immunity. Understanding the impact of these immunosuppressive viruses on host responses to other pathogens may unveil new paradigms in the pathogenesis of infectious diseases. Additionally, anelloviruses present as a good model system to explore important questions about pathogen evolution such as how viruses can acquire the ability to infect more host species, or whether ‘generalist’ viruses have a higher potential for zoonotic emergence. The answers to such questions will be invaluable for pre-emptive global health frameworks that may one day be able to prevent the emergence of novel infectious diseases.

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# Checklist

1. good use of figures and appropriate use of legends and references
2. “general-to-specific” structure
3. Understandable to the non-specialist
4. Introduction with relevance of the review topic, aims and roadmap present
5. Logical transitions between topics
6. Conclusions and perspectives present
7. Highlights limitations / challenges / questions in field.
8. Provides (few) directions for future experimental work based on the limits and challenges mentioned
9. Clear and relevant figures

1. Translation occurs at multiple translation initiation sites on a transcript. [↑](#footnote-ref-1)
2. Also known as VP2 in CAV. [↑](#footnote-ref-2)
3. Key progenitors of T-cells and red blood cells, respectively. [↑](#footnote-ref-3)
4. *Bidnaviridae*, *Parvoviridae*, *Bacilladnaviridae*, *Circoviridae*, *Geminiviridae*, *Genomoviridae*, *Nanoviridae*, *Redondoviridae*, *Smacoviridae*. [↑](#footnote-ref-4)
5. A ‘master-regulator’ of inflammatory cytokine production [195]. [↑](#footnote-ref-5)
6. Joining sequencing reads into longer sequences without a reference sequence with the aim of producing full genomes. [↑](#footnote-ref-6)
7. A statistic assigned to each node in a phylogeny to assess the robustness and accuracy of the observed tree topology. [↑](#footnote-ref-7)