

**The Enigma of the Anelloviridae**

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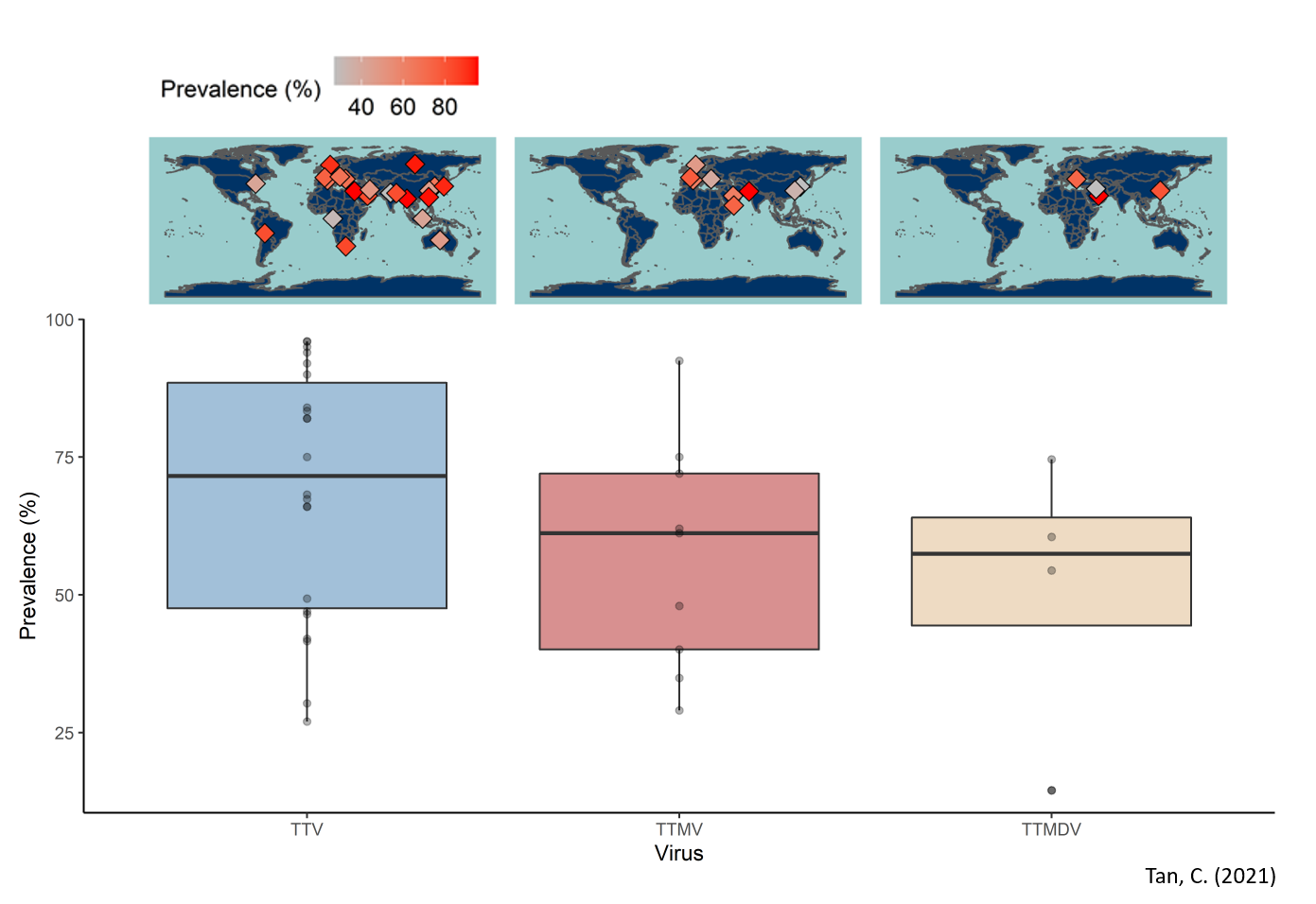
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# Abstract (200 words)

# Introduction (truncated)

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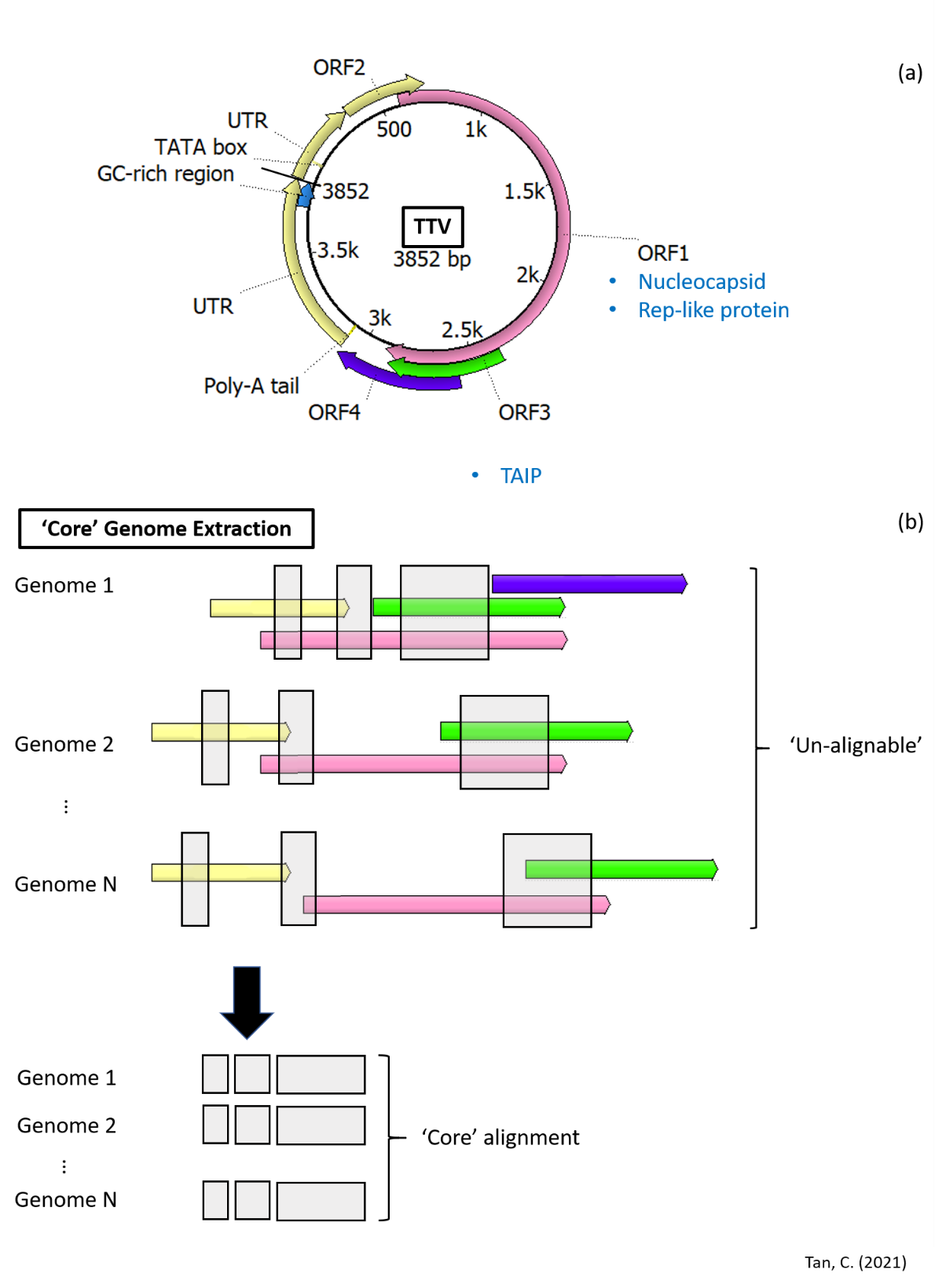
**Figure 1. Global distribution of human anelloviruses**. Prevalence estimates of human anelloviruses in healthy individuals were obtained from 26 independent studies and used to generate this figure. The world maps were generated using the *ne\_countries* function from the *rnaturalearth* package in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

While no anelloviruses have been found to cause disease in humans, the high prevalence and transmissibility of anelloviruses around the world warrants further surveillance. The extensive contact between farm animals or pests and humans facilitates zoonoses or anthroponoses that might result in the emergence of new infectious diseases, which could significantly affect human health or livestock productivity. Indeed, pathogens capable of human-animal transmission are twice as likely to be associated with emerging infectious diseases than those that are not (8). While global health efforts mainly involve post-emergence outbreak control (9), it is prudent to pre-empt and mitigate the emergence of the next infectious disease. This review therefore focuses on the potential of anelloviruses as a pathogen for human and animal hosts. Possible mechanisms for the accumulation of genomic diversity in the Anelloviridae will be explored. Additionally, transmission routes, potential animal reservoirs and evidence for cross-species transmission will be discussed. Lastly, an overview of host-virus interactions and pathogenicity will be provided.

# **Accumulation of Genomic diversity**

## Genome structure and replication (310 words)

Anelloviruses have a largely conserved genome organisation, containing three to four overlapping open reading frames (ORFs) (10) within a coding region demarcated by a TATA-box and poly-A tail sequence. There is also an untranslated region (UTR) containing a GC-rich region (11) (**Figure 2a**). Transfection experiments of human TTV found that at least six proteins are generated by alternative translation initiation (12) on three mRNA species, which were produced by alternative splicing (13). ORF1 putatively encodes the nucleocapsid (14) and a Rep-like protein that are conserved in circular DNA replicons like plasmids (15). Additionally, ORF2 potentially encodes a phosphatase (16) and ORF3, the TTV-derived apoptosis-inducing protein (TAIP) (17,18). Currently, the genome replication mechanism of anelloviruses is poorly understood relative other families of circular ssDNA viruses such as *Geminiviridae* (19) or *Circoviridae* (20). This is largely due to the lack of a stable cell culture system for anelloviruses to date (21,22). Kakkola et al. (23) showed that TTV replication is inhibited by administering the DNA polymerase inhibitor, Aphidicolin, suggesting dependency on the host’s replication machinery. In addition to this, the presence of Rep-like motifs and putative stem-loop structures formed by the GC-rich region (24) alludes to a rolling circle mechanism, which is typical of ssDNA viruses (25). 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 (26–28). A similar strategy could be explored to reduce the impact of anellovirus-related mortality in livestock.



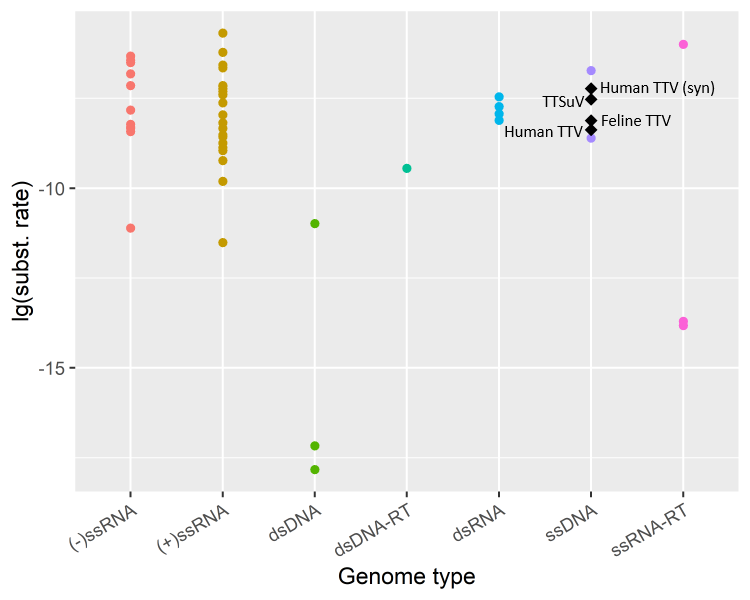
**Figure 2. Genome structure of TTV and ‘core’ genome extraction**. (a) The complete genome of TTV retrieved from NCBI GenBank (NC\_002076.2) was visualised and annotated using UGene (29). (b) Illustration of ‘core’ gene extraction to obtain alignable conserved genomic regions for phylogenetic analysis.

## Genomic diversity (255)

Even though anelloviruses have similar genome structures, there is great sequence divergence within the family. Indeed, the mean genetic distance of aligned amino acid sequences for human TTVs alone 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 (21). This diversity poses a major challenge for full-genome alignments during the reconstruction of phylogenetic relationships. As such, the ICTV recommends restricting family-wide phylogenetic reconstruction analyses to ORF1 and a 35% nucleotide sequence identity threshold to demarcate viral species (1). Several studies have presented a family-wide phylogeny (10,30–33) in accordance with this. However, doing so does not consider the full-length genomic diversity of the family. Additionally, sequence alignment algorithms are known to have limited accuracy when used for aligning highly divergent sequences (34). As such, alignment-free methods may be more appropriate for future studies investigating the family-wide diversity of the *Anelloviridae*. Notably, many sophisticated methods in population genetics such as those for phylogenetic dating (35) or inferring past population dynamics (36,37) are alignment-dependent. As such, future studies seeking to employ such methods could consider extracting conserved ‘core’ genes or genomic regions from the ‘un-alignable’ viral sequences (**Figure 2b**), which has been previously used on highly divergent members of microbial communities (38) and recently for the family *Coronaviridae* (39). This can be done using gene annotation tools such as *Prokka* (40), *Roary* (41)or *Mauve* (42) and would allow the construction of a sequence alignment that retains a larger proportion of the viral genomes.

## Mutation rates (325)

Understanding how viruses mutate and accumulate genomic diversity has profound practical implications. For example, the emergence of SARS-CoV-2 mutations that putatively aid in immune escape has been of great concern with regards to vaccine efficacy and development (43) in the present COVID-19 pandemic. Additionally, elevating short-term viral mutation rates via mutagen treatment, termed lethal mutagenesis, has been shown to enhance the effectiveness of antiviral treatments (44). Only a few studies to date have provided estimates of the mutation rate of anelloviruses (45–47), which ranges from 2.3-7.3 × 10-4 substitutions/site/year (s/s/y). Cadar et al. (45) reported a substitution rate of 5.4 × 10-4 s/s/y for the TTSuV genomic region containing the UTR, ORF2 and 5’ end of ORF1 sequences sampled from wild boars over six years. Umemura et al. (46) reported a synonymous substitution rate of 7.3 × 10-4 s/s/y for TTV-positive sera over 12 years. Lastly, Bedarida et al. (47) reported a value of ~2 × 10-4 s/s/y for anellovirus sequences obtained from human sera and cat saliva reported over 16 and 6.5 years, respectively. These rates were compared to that of other viruses, which were collated from eight other studies (48–55) (**Figure 3**). The mutation rates of anelloviruses are similar to that of RNA viruses. Additionally, they are discernibly higher than that of double-stranded DNA (dsDNA) viruses, which echoes previous findings that single-stranded viruses mutate faster than their double-stranded counterparts (44). This phenomenon has been suggested to be due to single-stranded viruses being more prone to chemical damage such as oxidative deamination (56).



**Figure 3. 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 human and animal 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 (530)

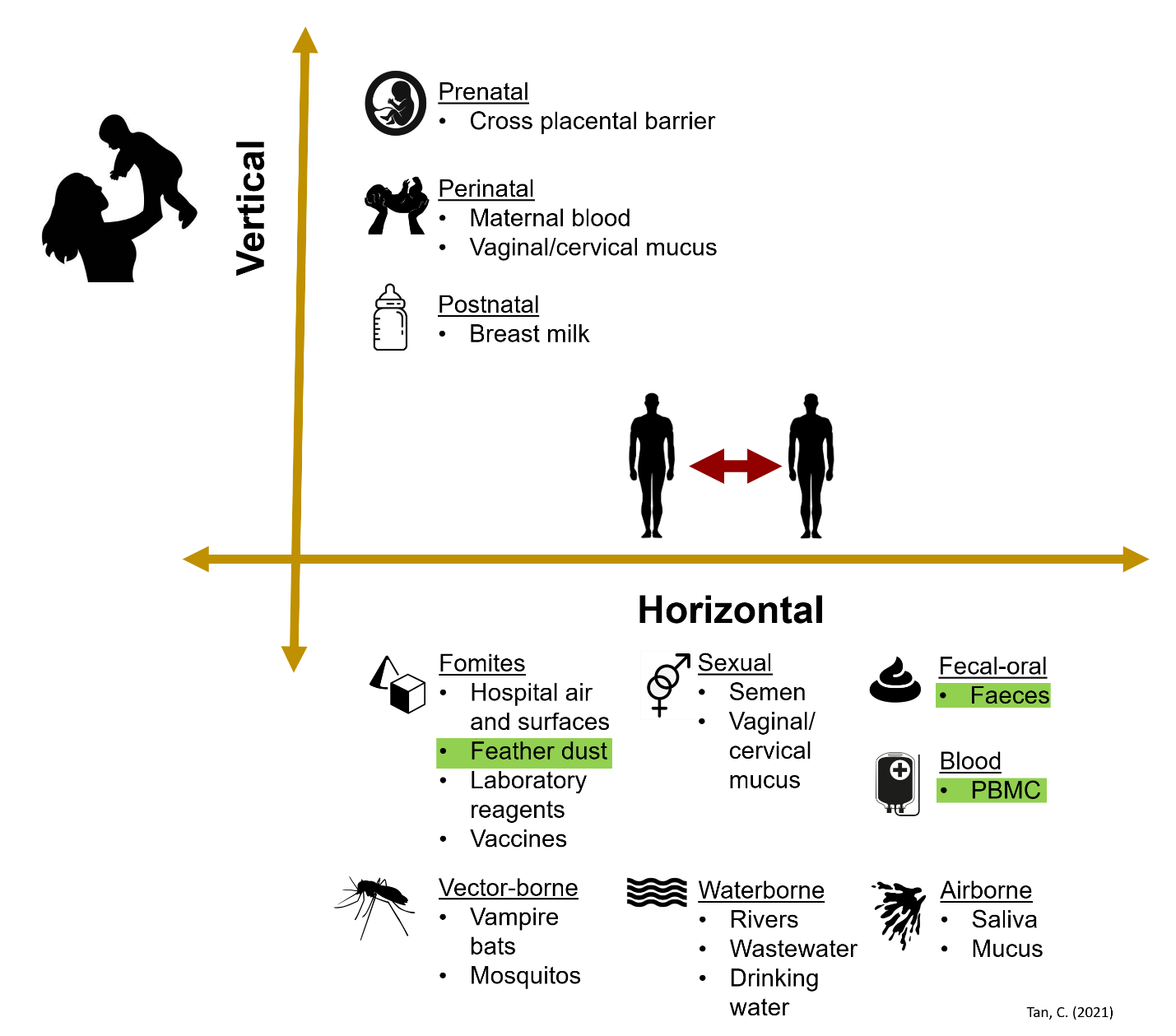
There has been limited studies to date investigating potential sources of genomic diversity. Given that genome replication is performed by host polymerases (23), which are highly accurate, nucleotide misincorporation may play only a minor role in the accumulation of mutations. Alternatively, host innate antiviral responses may contribute to the genomic diversity. For example, host APOBEC3 proteins are cytidine deaminases that have been found to cause C->T hypermutations in other DNA viruses like human papillomavirus (57,58). While such host-editing by APOBEC3 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 (59). 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.

Recombination also plays a substantial role in shaping the extant genomic diversity of the *Anelloviridae* by allowing viruses to sample a larger region of the sequence space than by mutation (60). Indeed, a single recombination event between two viral genomes could generate new combinations of their existing mutations, introducing several nucleotide polymorphisms at a time, which putatively prevents the accumulation of deleterious mutations (61–63). Using the popular recombination detection tool RDP4 (64), which identifies recombination using an ensemble of different detection methods, Fahsbender et al. (65) 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 putative recombination breakpoints in TTLwV-1 tend to fall within the UTR region. This agrees with a previous study (60), 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 (66,67) and within TTSuVs isolated from wild boars and domestic pigs (45). 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 (68–72), one even reporting a 48% frequency of dual or triple infection of TTV, TTMV and TTMDV in infants. 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. This has significant implications for phylogenetic inference since recombination results in portions of genomes representing different evolutionary histories. This violates the assumption of most phylogeny estimation methods – that a single evolutionary history underlies the sequences analysed (73). In such cases, the resultant phylogeny topologies obtained often differs from the true phylogeny (74), especially when recombination occurs between divergent sequences (73), as in the case of anelloviruses. Future studies attempting phylogenetic estimations should opt for alignment-free methods, which are minimally affected by recombination (34). Alternatively, they may remove putative recombinant segments using breakpoints identified by tools such as GARD (75) or RDP (64,76), which was demonstrated by Cadar et al. (45).

# The potential for cross-species transmission

## Transmission routes: anelloviruses are everywhere (761)

In line with its high prevalence, anelloviruses may 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 3**). Most studies probed the transmission of anelloviruses in humans or animals via the PCR-based detection of viral DNA. However, only a few studies demonstrated the viability of detected anelloviruses (highlighted in green; Figure 1). Anelloviruses have been detected in bodily fluids like blood, bile, saliva, mucus, semen and faeces has been described repeatedly in many studies (77–83). This suggests that anelloviruses can be transmitted via sexual, faecal-oral and airborne routes. However, without confirming the viability of virions in the samples, the presence of viral DNA in samples could have been due to transient shedding of inactivated virus from a distal source. For faecal-oral transmission, stronger evidence was provided by visualising the mature virions extracted using electron microscopy (79). Additionally, anellovirus-positive blood could be shown to infect cells and proliferate (78,83). Notably, Okamoto et al. (84) detected human TTV in nine different tissue samples. The ability of anelloviruses to infect multiple tissues and cell types suggests that they are ubiquitously distributed inside their hosts, which potentiates multiple transmission routes.



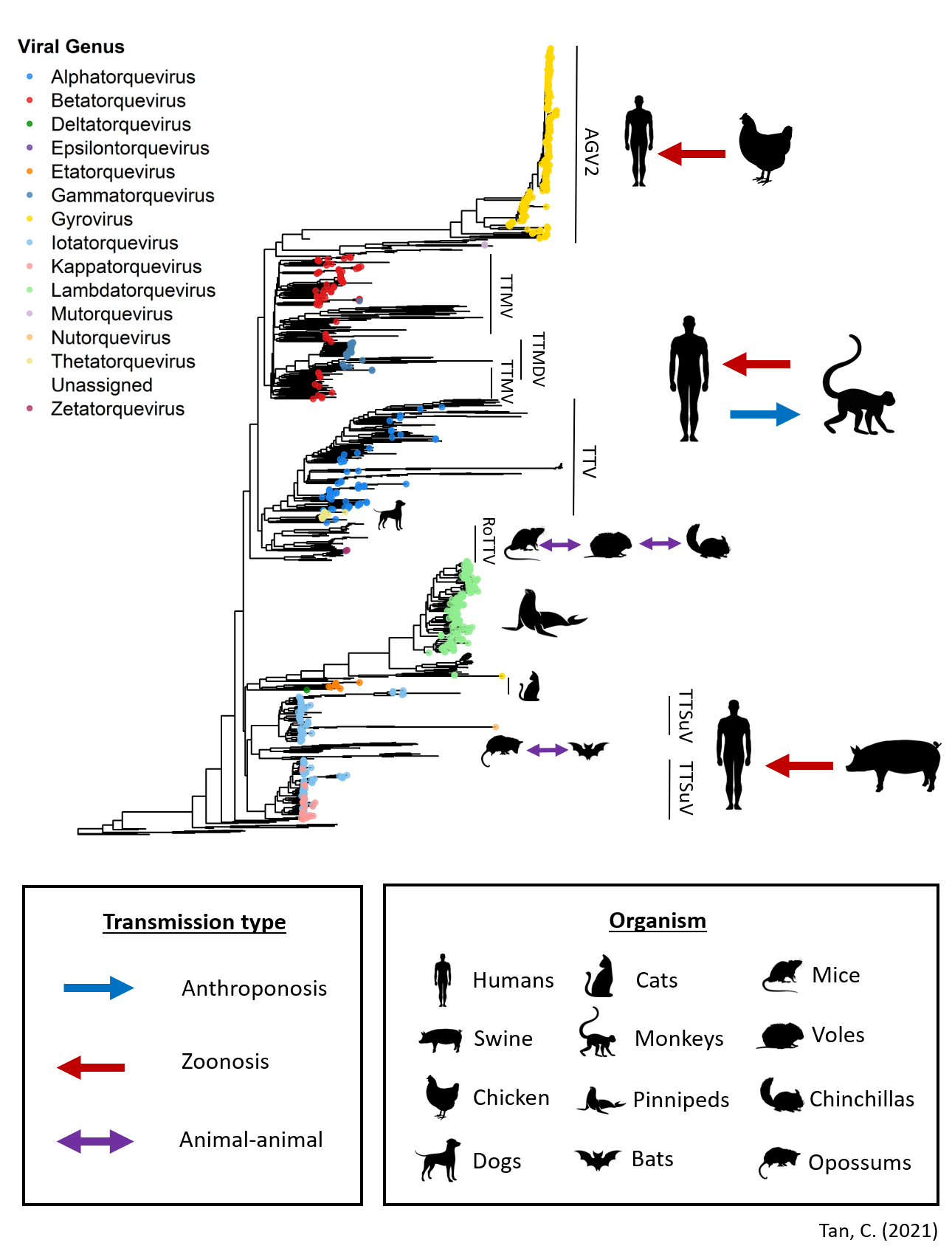
**Figure 3. Summary of potential transmission routes for anelloviruses**. Transmission routes where the infectious vehicle has been shown to contain viable virions are highlighted in green.

Anelloviruses may also be transmitted horizontally via environmental sources such as water or fomites, which are inanimate objects or surfaces that can become contaminated with pathogens (85). Studies have demonstrated their persistence in rivers (86–88), wastewaters (89), drinking water (90,91), feather dust (92), air and surfaces in hospitals (93) or even laboratory reagents (94) and vaccines (95). Alternatively, anelloviruses may be transmitted via vectors. Anellovirus sequences extracted from the liver of a vampire bat (*Carollia perspicillata*) and serum of an opossum (*Didelphis albiventris*) were phylogenetically clustered relative to other species of the Anelloviridae (30). Additionally, Shi et al. found viral sequences extracted from mosquitos and swine of pig farms were phylogenetically interspersed (96) with previously reported TTSuV sequences. These studies suggest potential bat-borne and mosquito-borne transmission via blood-feeding (*i.e.* hematophagy).

There is strong evidence for vertical transmission of anelloviruses, although the exact route(s) of infection – whether infection occurs before (prenatal), during and immediately after (perinatal), or after birth (postnatal) – has not been conclusively identified. Gerner et al. (97) detected human TTV in breast milk, and cord blood. Separately, Matsubara et al. (98) found TTV and TTMV DNA in breast milk, cord blood and amniotic fluid. Both studies employed polymerase chain reaction (PCR) followed by agarose gel electrophoresis to test the fluid samples. PCR amplicons were also sequenced via Sanger sequencing to rule out non-specific amplification. These studies suggest that breastfeeding might be a potential transmission route. However, cross-sample contamination during collection of cord blood or amniotic fluid is difficult to rule out and so these studies may not be sufficient to prove transplacental transmission. In fact, Tyschik et al. (99) did not find TTV in the cord blood from TTV-positive mothers using a highly sensitive quantitative PCR (qPCR) assay that could detect viral loads as low as 1000 viral DNA copies/ml of blood. The authors also detailed stringent measures to prevent cross-contamination, which the previous groups did not do. This suggests that the previous findings could have been due to contamination, which must be accounted for in future studies. Nevertheless, Gerner et al. (97) provided evidence for vertical transmission by showing that follow-up samples taken from infants with TTV-positive cord blood had persistent viremia.

Vertical transmission has also been demonstrated in animals. In particular, Martinez-Guino et al. showed in separate studies that sows, their breast milk and their aborted foetuses (100) or stillborns (101) were PCR positive for TTSuV. They also showed that TTSuV sequences in mother-offspring pairs had nucleotide sequence identities of 91-98%, implying that sample cross-contamination is unlikely. Indeed, it is likely that samples that were cross-contaminated at the point of collection should share identical sequences. As such, these findings may suggest that infections of the foetuses were acquired prenatally and that TTSuV may be transmitted through breast feeding. Vertical transmission of TTSuV through breast milk can be further confirmed in future studies by feeding of TTSuV-positive milk to TTSuV-negative offspring. Separately, human TTMDV was detected in domestic hens and the yolks of their eggs (102), providing evidence for vertical transmission. Confirmation that chicks that developed from TTMDV-positive eggs were also persistently infected would have provided stronger evidence for vertical transmission. Collectively, the studies discussed above provide substantial evidence for vertical transmission of anelloviruses.

## Host range and animal reservoirs

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**Figure 4. 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 (110) and rooted with the genome accession MK012481 (see **Appendix**). This phylogeny is annotated with symbols illustrating the cross-species transmission of various viral species.

## Animal-animal transmission, zoonoses and anthroponoses

# Host-virus interactions (1000 words)

# Pathogenicity (1000 words)

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# Appendix

**Table 1. Summary of evidence for cross-species transmission of anelloviruses**.

|  |  |  |  |
| --- | --- | --- | --- |
| **Study** | **Information about host range** | **Transmission type** | **Notes** |
| De Souza et al. (30) | * Anelloviruses found in: * rodents (Criticidae) * bats (Molossidae, Phyllostomidae) * opossums (Didelphidae) * Bat and opossum viruses clustered phylogenetically with strong bootstrap support. Possible transmission via hematophagy. | Animal-animal | * Bat virus found in kidney and liver samples. Likely to be viable. |
| Nishiyama et al. (3) | * RoTTV1 and RoTTV2 found in: * wood mice (Apodemus sylvaticus) * field voles (Microtus agrestis) * bank voles (Myodes glareolus) | Animal-animal | * Absent in mus musculus |
| Du et al. (111) | RoTTV lineages found to infect hosts from one to three families (Circritidae, Muridae, Chinchillidae). | Animal-animal |  |
| Ng et al. (103) | * Mosquito TTV sequences phylogenetically clustered with TTSuVs. | Animal-animal | * Low bootstrap support * Viability of virions not confirmed |
| Shi et al. (96) | * TTSuV1-like viruses detected in mosquitos using TTSuV1-specific PCR primers. * Phylogenetically interspersed with TTSuV1 viruses isolated from pigs. | Animal-animal | * Viability of virions not confirmed |
| Singh et al. (106) | * TTSuV detected in: * Bovine * Equine * Ovine * Canine * Elk | Animal-animal |  |
| Ssemadaali et al. (112) | * Transfection of TTSuV1 genomes into human PBMCs resulted in viral proliferation. * TTSuV DNA detected in both human and swine sera samples. 27 of 40 human blood samples and twelve of 20 swine samples | Zoonotic |  |
| Ye et al. (105) | * 98.3-100% pairwise nucleotide sequence identities for AGV2 amplicons of 10/54 chicken feather samples and 2/178 human blood samples. | Zoonotic |  |
| Ninomiya et al. (113) | * 73 of 74 chimpanzees in capitivity tested positive for human TTV and TTMV * Coinfection of human TTV, TTMDV and simian TTMDV in one healthy human subject. * Chimpanzee TTV/TTMV phylogenetically clustered with human TTV/TTMV | Zoonotic/Anthroponotic |  |
| Iwaki et al. (114) | * Simian-associated TTVs found in 10.5% of a samples from a Japanese cohort * Phylogenetically distinct from human TTVs. | Zoonotic |  |

# 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