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Identification of novel *Zybavirus* genome sequences and analysis of programmed ribosomal frameshifting motifs in the family *Amalgaviridae*

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The family *Amalgaviridae* comprises monopartite double-stranded RNA viruses that encode two overlapping open reading frames (ORFs), ORF1 and ORF2. A programmed ribosomal frameshifting (PRF) mechanism facilitates the translation of an ORF1+2p fusion protein. Among the three recognized genera (*Amalgavirus*, *Unirnavirus*, and *Zybavirus*), *Zybavirus* remains poorly characterized, with only one approved species, Zygosaccharomyces bailii virus Z (ZbV-Z), and a few unclassified proposed members. In this study, we identified four novel zybavirus-like viral genome sequences, tentatively named Zygosaccharomyces bailii virus Z2 (ZbV-Z2), Cryptops hortensis-associated virus Z1 (ChaV-Z1), Drosophila suzukii-associated virus Z1 (DsaV-Z1), and Sand Creek Marshes virus Z1 (SCMV-Z1), from publicly available transcriptome datasets. Phylogenetic analysis placed ZbV-Z2, ChaV-Z1, and DsaV-Z1 in a well-supported clade with ZbV-Z and Xisha Islands zybavirus, supporting their classification within *Zybavirus*. SCMV-Z1 clustered with seven known viruses in a distinct lineage, which may represent a novel genus within the family *Amalgaviridae*. Comparative analysis of PRF sites in members of *Zybavirus*, *Amalgavirus*, and related clades revealed that UUU_CNN may represent a broader and ancestral consensus +1 PRF motif in this group of viruses. Our study highlights the utility of mining public transcriptome data for novel viral genome discovery and contributes to the refinement of both taxonomic classification and conserved genomic features within this viral family.

KEYWORDS

***Amalgaviridae*, *Zybavirus*, anlovirus, programmed ribosomal frameshifting (PRF), transcriptome mining**

Introduction

The *Amalgaviridae* family comprises monopartite double-stranded RNA viruses classified into three officially recognized genera: *Amalgavirus*, *Unirnavirus*, and *Zybavirus*, as approved by the International Committee on Taxonomy of Viruses (ICTV)[1], and a proposed genus, "Anlovirus" (Krupovic et al., 2015; Depierreux et al., 2016; Pyle et al., 2017). Members of the genus *Amalgavirus* infect plants and include nine ICTV-approved species, along with over 60 additional putative members listed in the National Center for Biotechnology Information (NCBI) Taxonomy database (Lee et al., 2019; Dos Santos et al., 2022; Choi et al., 2023a).

Members of the genus *Unirnavirus* are mycoviruses that infect a range of fungal species (Koloniuk et al., 2015; Zhu et al., 2015; Campo et al., 2016; Kotta-Loizou and Coutts, 2017). Thirteen species have been officially recognized in this genus, including Beauveria bassiana non-segmented virus (BbNV1), Ustilaginoidea virens RNA virus M-A (UvRVM-A), and Colletotrichum gloeosporioides RNA virus 1 (CgRV1) (Kotta-Loizou et al., 2015; He et al., 2022; Suharto et al., 2022).

The genus *Zybavirus* currently includes a single approved species, *Zybavirus bailii*, whose exemplar virus is Zygosaccharomyces bailii virus Z (ZbV-Z). ZbV-Z was identified in the yeast *Zygosaccharomyces bailii*, a species commonly associated with food fermentation and spoilage (Radler et al., 1993; Schmitt and Neuhausen, 1994; Depierreux et al., 2016). Three additional viruses, Exobasidium gracile zybavirus 1 (EgZV1), EgZV2, and Xisha Islands zybavirus (XIZV), have been proposed as unclassified members of this genus (Chen et al., 2022; Teng et al., 2022; Zhang et al., 2022). EgZV1 and EgZV2 were identified in the plant-pathogenic fungus *Exobasidium gracile*, and XIZV was detected in a soil metagenome.

The proposed genus "Anlovirus" is currently represented by Antonospora locustae virus 1 (AnloV1), first detected in *Antonospora locustae*, a microsporidian parasite of insects (Pyle et al., 2017). Another unclassified virus, Hubei partiti-like virus 59 (HplV59), shows high sequence similarity to AnloV1 and may also belong to "Anlovirus" (Shi et al., 2016).

Members of the *Amalgaviridae* family possess genomes with two overlapping open reading frames (ORFs), designated ORF1 and ORF2 (Martin et al., 2011). The product of ORF1 (ORF1p) is not well characterized but is thought to function as a nucleocapsid or replication-associated protein (Isogai et al., 2011; Krupovic et al., 2015). ORF2 encodes an RNA-dependent RNA polymerase (RdRp), which is believed to be expressed as a fusion protein (ORF1+2p) through a programmed ribosomal frameshifting (PRF) mechanism (Firth et al., 2012; Depierreux et al., 2016).

In members of the genera *Amalgavirus*, *Zybavirus*, and the proposed genus "Anlovirus," a conserved motif, UUU_CGN (where the underscore represents the codon boundary of ORF1 and "N" is any nucleotide), has been proposed as a +1 PRF site (Nibert et al., 2016; Lee et al., 2019; Choi et al., 2023a). During ORF1 translation, a phenylalanyl-tRNA ($tRNA^{Phe}$) with the anticodon 3′-AAG-5′ binds to the UUU codon. Occasionally, this tRNA slips forward by one nucleotide to bind a UUC codon in ORF2, skipping a cytosine (C) residue and allowing translation to continue into ORF2, thereby generating the ORF1+2p fusion protein (Nibert et al., 2016; Lee et al., 2019).

In contrast, members of the genus *Unirnavirus* are believed to use a distinct PRF mechanism involving a putative slippery sequence, G_GAU_UUU, located immediately upstream of the ORF1 stop codon (Campo et al., 2016; Depierreux et al., 2016). This sequence is proposed to mediate a −1 PRF, allowing a $tRNA^{Phe}$ bound to the UUU codon in ORF1 to shift backward by one nucleotide to bind another UUU codon in ORF2, thereby producing the ORF1+2p fusion protein.

RNA viral genome sequences are frequently detected in RNA samples derived from cellular organisms or environmental sources (Bejerman et al., 2020; Chen et al., 2022; Edgar et al., 2022). Analyses of publicly available transcriptomic datasets have substantially expanded our understanding of RNA virus diversity and facilitated the identification of previously unrecognized viral genomes (Shin et al., 2022a; Shin et al., 2022b; Choi et al., 2023b; Choi et al., 2025). In this study, we aimed to identify novel members of the genus *Zybavirus* from Sequence Read Archive (SRA) datasets and to characterize PRF motifs across members of the *Amalgaviridae* family.

Materials and methods

Transcriptome data

The Serratus Explorer[2] was used to identify transcriptome datasets potentially containing zybavirus-like viral genome sequences (Edgar et al., 2022). The ORF1+2p fusion protein of ZbV-Z (GenBank accession ANN12897) was selected as the query. Default settings were used, with an alignment identity range of 45%–100% and a score range of 50 to 100. Datasets were subsequently filtered to include only those with an average read length of at least 100 nucleotides, a paired-end layout, and sequencing performed on the Illumina platform. This filtering process yielded 32 transcriptome datasets, which were then downloaded from the SRA at the NCBI.

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[1] https://ictv.global

[2] https://serratus.io

Identification and annotation of viral genome contigs

Raw transcriptome reads were quality trimmed using Sickle (version 1.33)[3] with parameters "-q 30 -l 55." Trimmed reads were assembled into contigs using SPAdes (version 4.0.0) in "rnaviral" mode[4] (Bushmanova et al., 2019). Assembled contigs were screened by BLASTX to identify those showing similarity to the ORF1+2p fusion proteins of ZbV-Z, EgZV1, EgZV2, and XlZV. When multiple zybavirus-like contigs were present within a single dataset, consensus sequences were generated using CAP3 (version date 02/10/15). Open reading frames (ORFs) were predicted for each contig using ORFfinder[5].

Phylogenetic analysis

Protein sequences were aligned using MAFFT (version 7.526) with the "--auto" parameter[6] (Nakamura et al., 2018). The resulting alignments were filtered using ClipKIT (version 2.4.1)[7] to retain only well-aligned, phylogenetically informative positions (Steenwyk et al., 2020). A maximum likelihood phylogenetic tree was constructed using IQ-TREE (version 3.0.0) with the "-mset WAG,LG,JTT" option[8] (Minh et al., 2020). Bootstrap support values were estimated from 1,000 replicates using the UFBoot2 method (parameter "-B 1000"). The final tree was visualized using MEGA (version 12.0.11)[9] (Kumar et al., 2024). Pairwise sequences identities were calculated using the Sequence Demarcation Tool (SDT) (version 1.3)[10] (Muhire et al., 2025).

Identification of putative fungal ribosomal RNA contigs

To identify ribosomal RNA (rRNA) contigs of potential fungal origin, assembled transcriptome contigs were compared to small subunit rRNA sequences from the EUKARYOME database (version 1.9.4)[11] (Tedersoo et al., 2024) using discontiguous megaBLAST. Contigs with an aligned length of at least 500 bp and nucleotide sequence identity of 90% or higher to fungal small subunit rRNA were retained as putative fungal rRNA contigs.

Prediction of a +1 programmed ribosomal frameshifting site

To identify candidate +1 PRF sites in the newly identified zybavirus-like genome sequences, UUU_CGN-like motifs were systematically searched within the overlapping regions of ORF1 and ORF2. Alignments of PRF sites were visualized using BOXSHADE (version 3.31)[12], and sequence logos were generated using WebLogo (version 3.7.9)[13].

Results

Identification of novel zybavirus-like viral genome sequences

The genus *Zybavirus* currently includes one officially approved species, *Z. bailii* (ZbV-Z), and three unclassified viruses: EgZV1, EgZV2, and XlZV, as listed in the NCBI Taxonomy database (accessed 1 May 2025) (Depierreux et al., 2016; Chen et al., 2022; Teng et al., 2022; Zhang et al., 2022). To expand the known diversity of this genus, we analyzed transcriptome datasets identified using the Serratus Explorer (Edgar et al., 2022), followed by quality trimming, genome assembly, and similarity-based screening. Seven zybavirus-like genome sequences were recovered (Table 1).

Among these, two contigs (SRP327700-2 and SRP313929-1) shared 94.1% and 99.7% nucleotide identity, respectively, with the ZbV-Z reference genome. ZbV-Z was originally identified in the budding yeast *Z. bailii* (Radler et al., 1993; Schmitt and Neuhausen, 1994; Depierreux et al., 2016). The contig SRP327700-2 was derived from transcriptome data of fermented grains used in Baijiu production (Wei et al., 2023). Since the yeast *Z. bailii* participates in the fermentation of Baijiu and Maotai (Xu et al., 2017; Wei et al., 2023), it is likely that *Z. bailii*, or a closely related fungus serving as the viral host, was present in the sample. The second contig, SRP313929-1, originated from a *Drosophila melanogaster* transcriptome (Huang et al., 2021). While fruit flies are unlikely to serve as natural hosts for ZbV-Z, they may carry associated fungi. Supporting this, putative fungal rRNA contigs showing 93.6%–100% identity with the yeast *Z. bailii* rRNA sequences were detected in both datasets (see Supplementary Material S2 for putative fungal rRNA contigs identified across all

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[3] https://github.com/najoshi/sickle

[4] https://github.com/ablab/spades

[5] https://www.ncbi.nlm.nih.gov/orffinder

[6] https://mafft.cbrc.jp/alignment/software

[7] https://github.com/JLSteenwyk/ClipKIT

[8] https://iqtree.github.io/

[9] https://www.megasoftware.net

[10] http://web.cbio.uct.ac.za/~brejnev

[11] https://eukaryome.org

[12] https://launchpad.net/ubuntu/focal/+package/boxshade

[13] http://weblogo.threeplusone.com

TABLE 1 Summary of viral genome contigs identified from transcriptome data.

Virus	Acronym	Contig	Length	Accession	SRA	Sample
Zygosaccharomyces bailii virus Z2	ZbV-Z2	DRP004880-1[a]	3,142	BK069872	DRR089071	*Zygosaccharomyces bailii*
Zygosaccharomyces bailii virus Z2	ZbV-Z2	SRP327700-1[a]	3,153	BK069873	SRR15090909, SRR15090910, SRR15090911, SRR15090912, SRR15090913, SRR15090914, SRR15090915, SRR15090916	fermented grains for Baijiu production
Zygosaccharomyces bailii virus Z	ZbV-Z	SRP327700-2[b]	3,138	BK069874	SRR15090909, SRR15090910, SRR15090911, SRR15090912, SRR15090913, SRR15090914, SRR15090915, SRR15090916	fermented grains for Baijiu production
Zygosaccharomyces bailii virus Z	ZbV-Z	SRP313929-1[b]	3,124	BK069875	SRR14180015, SRR14180055, SRR14180096	*Drosophila melanogaster*
Cryptos hortensis-associated virus Z1	ChaV-Z1	SRP036135-1	3,028	BK069878	SRR1153457	*Cryptops hortensis*
Drosophila suzukii-associated virus Z1	DsaV-Z1	SRP117133-1	2778	BK069877	SRR6019484	wild collected *Drosophila suzukii*
Sand Creek Marshes virus Z1	SCMV-Z1	SRP262753-1	3,158	BK069876	SRR11829260	salt marsh sediment

[a] Two ZbV-Z2 contigs share 99.7% nucleotide identity.

[b] These ZbV-Z contigs share 94.1% and 99.7% nucleotide identity, respectively, with ZbV-Z strain 142 (NCBI accession number NC_075420).

transcriptome datasets analyzed in this study), suggesting that *Z. bailii* or related fungi may serve as hosts of these viruses.

Five additional contigs (DRP004880-1, SRP327700-1, SRP036135-1, SRP117133-1, and SRP262753-1) exhibited sequence similarity to known zybavirus genomes, suggesting that they represent novel zybavirus-like genomes (Figure 1). Two of these, DRP004880-1 and SRP327700-1, originated from transcriptome data of the yeast *Z. bailii* and fermented grains, respectively. These contigs shared 99.7% nucleotide identity and likely represent the same viral genome, herein designated Zygosaccharomyces bailii virus Z2 (ZbV-Z2). The RdRp sequences of ZbV-Z and ZbV-Z2 share 55% amino acid identity, supporting their classification as distinct viral genomes. DRP004880-1 was selected as the representative sequence. ZbV-Z2 encodes a 290-amino acid ORF1p and a 1017-amino acid ORF1+2p fusion protein. A putative +1 programmed ribosomal frameshifting (PRF) site, UUU_CGU, was identified in the overlapping region of ORF1 and ORF2.

The contig SRP036135-1 was recovered from a centipede (*Cryptops hortensis*) transcriptome (Fernandez et al., 2014). Given that all known zybavirus members are mycoviruses, this virus likely infects a fungus associated with the centipede. A fungal rRNA contig sharing 90.1% identity with a Kickxellales fungus was identified in the dataset. This viral genome was named Cryptops hortensis-associated virus Z1 (ChaV-Z1). The ChaV-Z1 RdRp shares 51% amino acid identity with ZbV-Z. The ChaV-Z1 genome sequence contains incomplete ORFs: ORF1 and ORF2 lack portions of the N-terminal and C-terminal amino acid residues, respectively. A putative +1 PRF site, UUU_CCU, was identified, which differs slightly from the canonical UUU_CGN motif.

The contig SRP117133-1 was derived from a *Drosophila suzukii* transcriptome (Medd et al., 2018). Multiple fungal rRNA contigs with 90.4%–100% identity to known fungal sequences were identified, suggesting a fungal origin for the contig. This viral genome was named Drosophila suzukii-associated virus Z1 (DsaV-Z1). Its RdRp shares 46% identity with that of ZbV-Z. The DsaV-Z1 genome sequence has a complete ORF1, while ORF2 lacks part of its C-terminal region. A putative +1 PRF site, UUU_CGA, was identified in the overlapping region of ORF1 and ORF2.

The final contig, SRP262753-1, originated from metatranscriptome data of salt marsh sediment collected from Sand Creek Marshes, Massachusetts, USA. This dataset contained 59 fungal rRNA contigs from diverse species, suggesting a fungal host for the contig. This viral genome, named Sand Creek Marshes virus Z1 (SCMV-Z1), encodes complete ORFs. The SCMV-Z1 RdRp shares 35% amino acid identity with EgZV1. Two potential +1 PRF motifs, UUU_CGU and UUU_CGC, were identified.

All genome sequences identified in this study were deposited in NCBI GenBank under accession numbers BK069872–BK069878 and are available in Supplementary Material S3. Sequencing depth plots of genomic contigs are presented in Supplementary Material S4.

Phylogenetic relationships of novel zybavirus-like viral genomes

To assess the phylogenetic relationships of the newly identified zybavirus-like genome sequences within the family

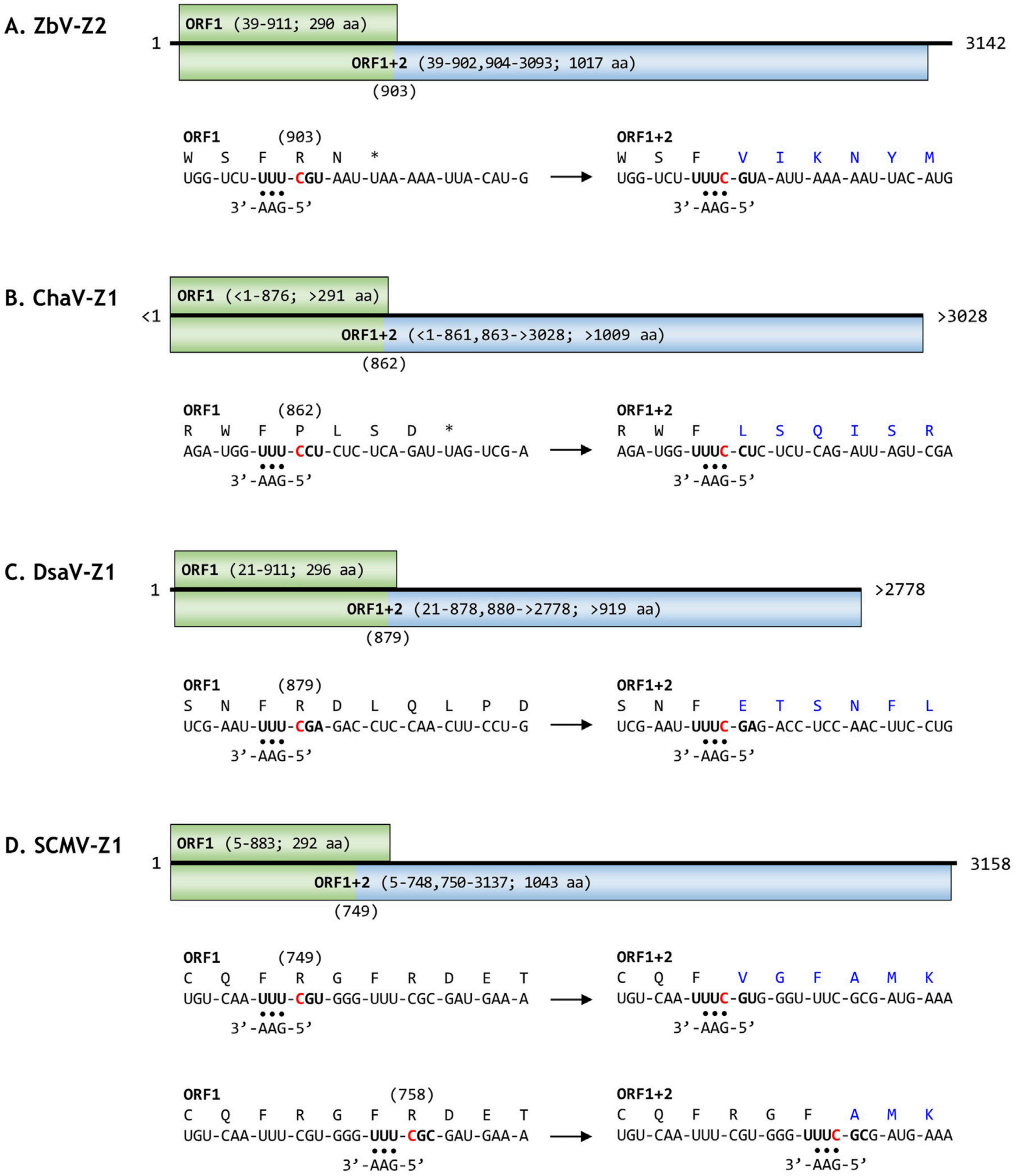


FIGURE 1
Genomic organization of newly identified viral contigs and proposed +1 programmed ribosomal frameshifting (PRF) sites. The genomic organization of the newly identified viral genomes ZbV-Z2 **(A)**, ChaV-Z1 **(B)**, DsaV-Z1 **(C)**, and SCMV-Z1 **(D)** is shown, including open reading frames (ORFs) encoding ORF1p and the ORF1+2p fusion protein. Nucleotide positions and amino acid lengths for each ORF are indicated in parentheses. Putative +1 PRF sites and associated tRNA slippage mechanisms are illustrated below each genome panel. Amino acids translated from the ORF2 reading frame, following frameshifting, are highlighted in blue. Note that ChaV-Z1 ORF1 and ORF2 lack portions of the N-terminal and C-terminal amino acid residues, respectively, and DsaV-Z1 ORF2 lacks a portion of its C-terminal region.

Amalgaviridae, a maximum likelihood phylogenetic tree was constructed using RdRp (ORF2) amino acid sequences (Figure 2). BLASTP searches were performed using the RdRp sequences of ZbV-Z2, ChaV-Z1, DsaV-Z1, and SCMV-Z1 to identify closely related viruses in the NCBI database. A total of 42 representative viruses were selected, including members of the

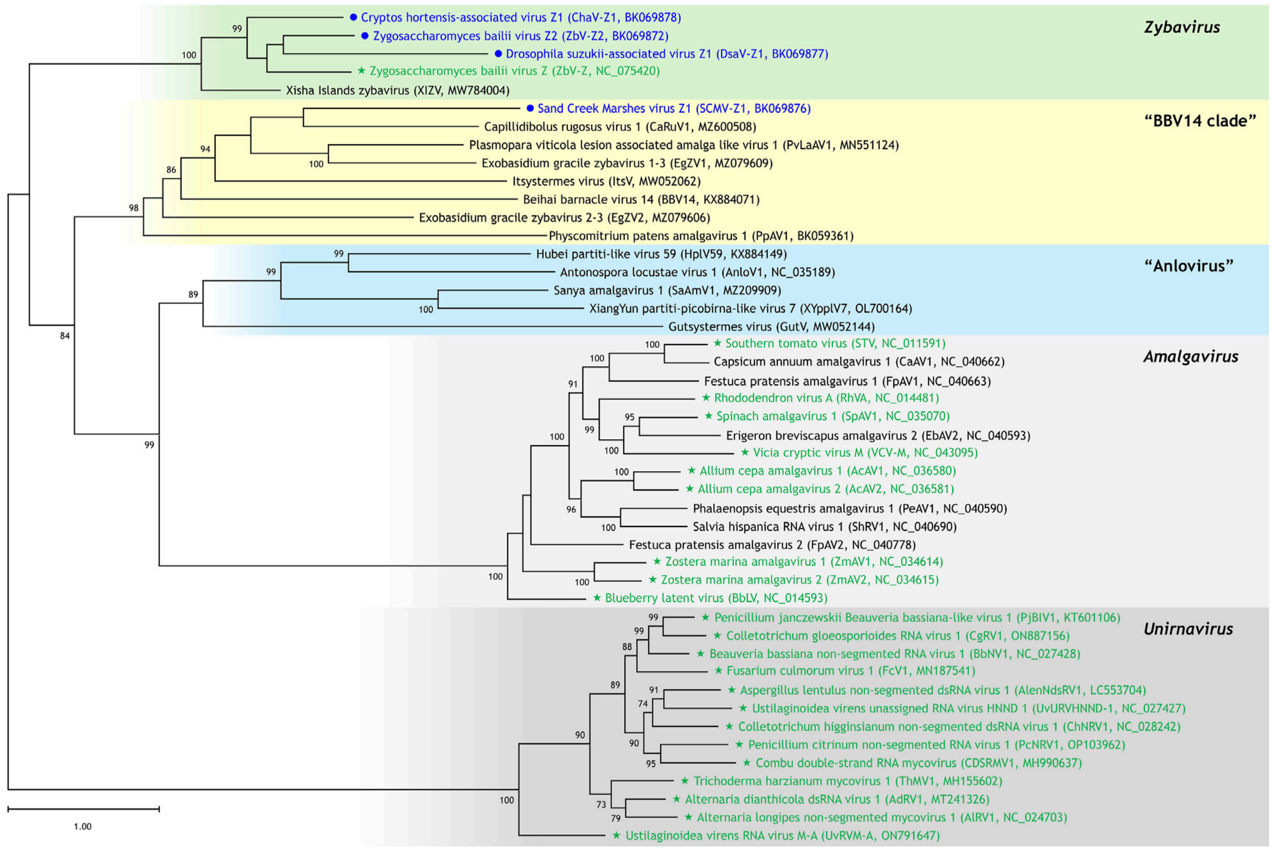


FIGURE 2

Phylogenetic positions of newly identified viral genomes. A maximum likelihood phylogenetic tree was constructed using the "LG + F + I + R5" model based on a multiple sequence alignment of RNA-dependent RNA polymerase (RdRp) sequences encoded by ORF2. The analysis includes viral genomes newly identified in this study (marked with blue circles), officially approved species by the ICTV (marked with green stars), and unassigned viruses (unmarked) within the family *Amalgaviridae*. Bootstrap support values of 70% or greater, calculated from 1,000 resampled datasets, are indicated at the nodes. Abbreviations and GenBank accession numbers are provided in parentheses.

genera *Amalgavirus* and *Unirnavirus*, as well as unclassified viruses within the family *Amalgaviridae*.

Three of the four newly identified viral genomes (ZbV-Z2, ChaV-Z1, and DsaV-Z1) grouped with ZbV-Z and XIZV to form a monophyletic clade with strong bootstrap support (value of 100). This result supports the classification of ZbV-Z2, ChaV-Z1, and DsaV-Z1 as novel members of the genus *Zybavirus*.

The fourth viral genome, SCMV-Z1, clustered with EgZV1 and EgZV2, which are two previously proposed members of *Zybavirus*, in a separate clade distinct from the one containing the type species ZbV-Z. This clade also included five additional unassigned viruses: Beihai barnacle virus 14 (BBV14), Capillidibolus rugosus virus 1 (CaRuV1), Itsystermes virus (ItsV), Physcomitrium patens amalgavirus 1 (PpAV1), and Plasmopara viticola lesion-associated amalga-like virus 1 (PvLaAV1) (Shi et al., 2016; Lay et al., 2020; Vendrell-Mir et al., 2021). This distinct lineage, referred to here as the "BBV14 clade" based on the earliest reported member, may represent a novel genus within the family *Amalgaviridae*. The bootstrap support value for this clade was 98.

The proposed genus "Anlovirus" was represented in the tree by AnloV1 and a closely related virus, HplV59 (Shi et al., 2016; Pyle et al., 2017; Teng et al., 2022; Zhang et al., 2022). Three additional unclassified viruses, Gutsystermes virus (GutV), Sanya amalgavirus 1 (SaAmV1), and XiangYun partiti-picobirna-like virus 7 (XYpplV7), also clustered within this lineage. Although SaAmV1 is currently annotated as a member of the genus *Amalgavirus*, and the other two viruses are listed as unclassified, their phylogenetic placement suggests that they may be more appropriately assigned to "Anlovirus." The bootstrap support value for the "Anlovirus" clade, which includes five viruses, was 89.

The remaining viruses formed two well-supported clades corresponding to the genera *Amalgavirus* and *Unirnavirus*, each with a bootstrap support value of 100. These results are consistent with their current taxonomic classification.

To supplement the phylogenetic analysis, pairwise sequence identities among RdRp amino acid sequences were calculated (Supplementary Material S5). Pairwise identities ranged from 19.9% to 73.0%, with a mean of 29.8%. The sequences were clustered into known genera and proposed clades, consistent with the phylogenetic analysis. The mean pairwise RdRp sequence identities within distinct clades were as follows: *Zybavirus*, 47.5% (range: 42.8%–54.3%); "BBV14 clade," 30.4% (range: 24.1%–41.0%); "Anlovirus," 29.6% (range: 23.9%–43.4%); *Amalgavirus*, 48.6% (range: 41.9%–68.0%); and *Unirnavirus*, 58.5% (range: 47.6%–73.0%). The "BBV14 clade" and "Anlovirus" exhibited greater diversity than the genera *Zybavirus*, *Amalgavirus*, and *Unirnavirus*, suggesting that the former two clades may comprise highly divergent viruses that could be classified into multiple independent genera.

Analysis of programmed ribosomal frameshifting sites

The UUU_CGN sequence has been proposed as a slippery consensus motif that induces +1 PRF during translation of the ORF1+2p fusion protein in members of the genus *Amalgavirus* (Nibert et al., 2016). This motif was also identified within the overlapping region of ORF1 and ORF2 in three of the newly identified viral genomes in this study: ZbV-Z2, DsaV-Z1, and SCMV-Z1. In contrast, the viral genome of ChaV-Z1 contained a UUU_CCU sequence, which differs slightly from the canonical UUU_CGN motif.

To evaluate the conservation of +1 PRF motifs, we analyzed 18 viral genomes classified in this study as members of the genus *Zybavirus* (five viruses), the "BBV14 clade" (eight viruses), and the proposed genus "Anlovirus" (five viruses). Among the five viruses assigned to *Zybavirus*, three (DsaV-Z1, ZbV-Z, and ZbV-Z2) possessed the UUU_CGN motif. The remaining two, ChaV-Z1 and XIZV, contained UUU_CCU and UUU_CUU motifs, respectively (Figure 3A). Among the eight viruses grouped in the "BBV14 clade," six (CaRuV1, EgZV1, EgZV2, PpAV1, PvLaAV1, and SCMV-Z1) contained the canonical UUU_CGN motif. The other two, BBV14 and ItsV, contained UUU_CUU and UUU_CUA sequences, respectively (Figure 3B). Among the five viruses classified as members of the proposed genus "Anlovirus," only GutV contained a UUU_CGN motif. The remaining viruses included AnloV1 and HplV59 with UUU_CUU, SaAmV1 with UUU_CAA, and XYpplV7 with UUU_CCG (Figure 3C).

Overall, ten of the 18 analyzed genomes contained the UUU_CGN motif. The remaining eight genomes retained a UUU codon followed by a C nucleotide. These findings suggest that UUU_CNN may represent a broader consensus +1 PRF motif for this group of viruses.

For comparison, we also examined the +1 PRF motifs in members of the genus *Amalgavirus*. Most species retained the UUU_CGN motif, as previously reported (Figure 3D), although some deviations have been noted in earlier studies (Nibert et al., 2016).

In contrast, members of the genus *Unirnavirus* are thought to employ a −1 PRF mechanism. A conserved slippery sequence, G_GAU_UUU, has been proposed to mediate this translation event (Campo et al., 2016; Depierreux et al., 2016). Among the 13 ICTV-approved species of *Unirnavirus*, ten contained the G_GAU_UUU motif, while two contained G_GAU_UUA and one had G_GAU_UUC (Figure 3E). Although G_GAU_UUU is the most frequently observed motif, the broader consensus sequence G_GAU_UUN may better encompass the diversity within this genus.

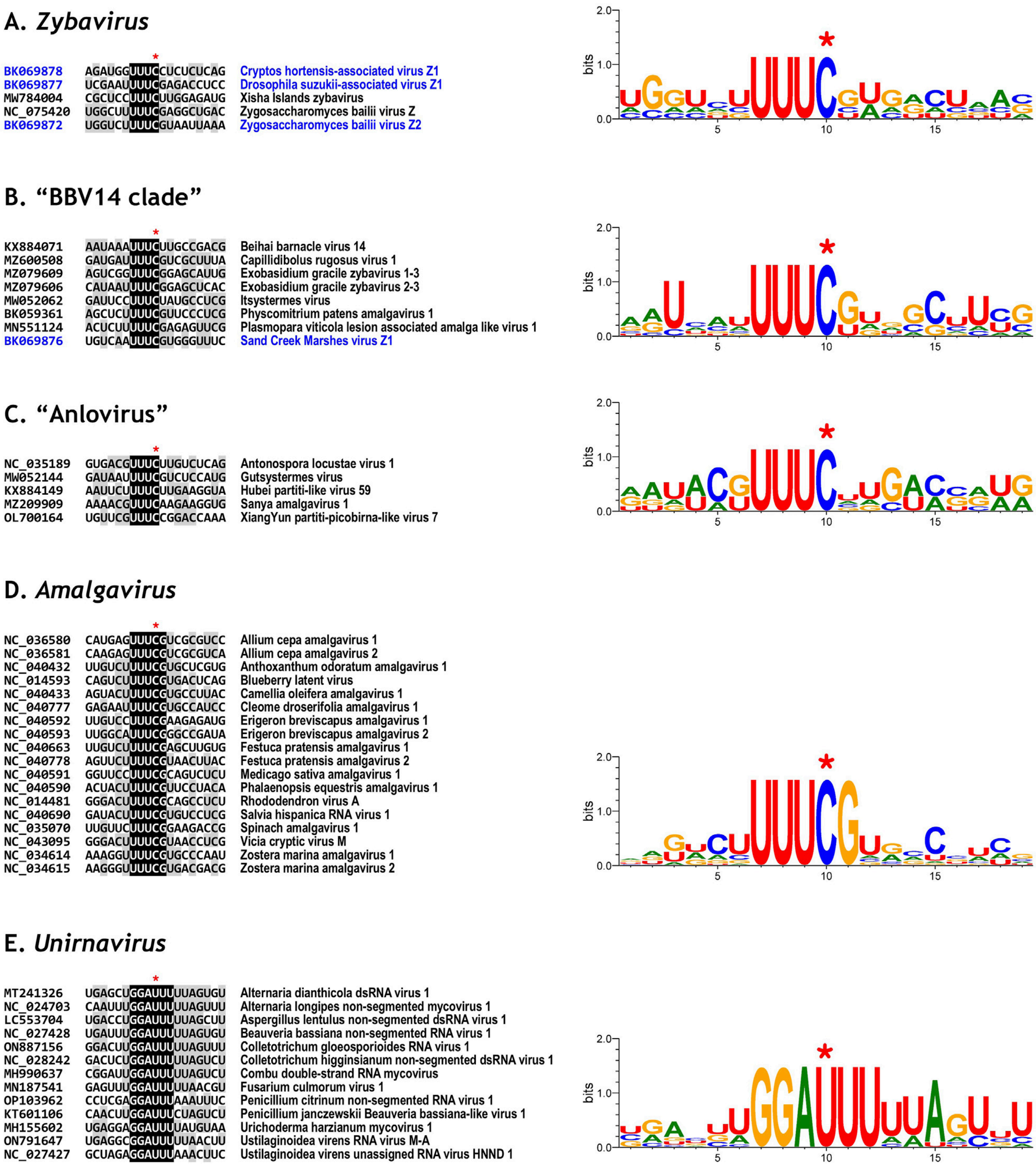
Discussion

In this study, we expanded the known diversity of the genus *Zybavirus* and the family *Amalgaviridae* by identifying four novel zybavirus-like viral genome sequences (ChaV-Z1, DsaV-Z1, SCMV-Z1, and ZbV-Z2) from publicly available transcriptome datasets. Prior to this work, ZbV-Z was the only officially approved species, and three unclassified viruses (EgZV1, EgZV2, and XIZV) had been proposed as members of this genus (Depierreux et al., 2016; Chen et al., 2022; Teng et al., 2022; Zhang et al., 2022). Our findings provide evidence for three additional members of the genus *Zybavirus* and indicate the presence of a distinct taxonomic group within the family *Amalgaviridae* that may warrant reclassification.

The RdRp proteins encoded by the genome sequences of ChaV-Z1, DsaV-Z1, and ZbV-Z2 clustered phylogenetically with those of ZbV-Z and XIZV. These five viral genomes formed a strongly supported monophyletic group with a bootstrap value of 100, consistent with their classification within the genus *Zybavirus*.

In contrast, SCMV-Z1 clustered with EgZV1, EgZV2, and five other unassigned viruses (BBV14, CaRuV1, ItsV, PpAV1, and PvLaAV1). This clade, referred to here as the "BBV14 clade," was also strongly supported (bootstrap value of 98) and was clearly distinct from the lineage containing the type species of *Zybavirus*. Although EgZV1 and EgZV2 were previously proposed as *Zybavirus* members (Teng et al., 2022; Zhang et al., 2022), their consistent grouping within the "BBV14 clade" and their sequence divergence from ZbV-Z suggest that this clade may represent a novel genus within the family.

Although ChaV-Z1, DsaV-Z1, and SCMV-Z1 are closely related to known mycoviruses, they were identified in transcriptome datasets derived from a centipede, a fruit fly, and salt marsh sediment, respectively (Fernandez et al., 2014; Medd et al., 2018). The presence of fungal rRNA sequences in these datasets suggests that fungi are the likely hosts of these viral genomes, although experimental confirmation is currently not feasible.



A. *Zybavirus*

BK069878	AGAUGGUUUCCUCUCUCAG	Cryptos hortensis-associated virus Z1
BK069877	UCGAAUUUUCGAGACCUCC	Drosophila suzukii-associated virus Z1
MW784004	CGCUCCUUUCUUGGAGAUG	Xisha Islands zybavirus
NC_075420	UGGCUUUUUCGAGGCUGAC	Zygosaccharomyces bailii virus Z
BK069872	UGGUCUUUUCGUAAUUAAA	Zygosaccharomyces bailii virus Z2

B. "BBV14 clade"

KX884071	AAUAAAUUUCUUGCCGACG	Beihai barnacle virus 14
MZ600508	GAUGAUUUUCGUCGCUUUA	Capillidibolus rugosus virus 1
MZ079609	AGUCGGUUUCGGAGCAUUG	Exobasidium gracile zybavirus 1-3
MZ079606	CAUAAUUUUCGGAGCUCAC	Exobasidium gracile zybavirus 2-3
MW052062	GAUUCCUUUCUAUGCCUCG	Itsystermes virus
BK059361	AGCUCUUUUCGUUCCCUCG	Physcomitrium patens amalgavirus 1
MN551124	ACUCUUUUUCGAGAGUUCG	Plasmopara viticola lesion associated amalga like virus 1
BK069876	UGUCAAUUUCGUGGGUUUC	Sand Creek Marshes virus Z1

C. "Anlovirus"

NC_035189	GUGACGUUUCUUGUCUCAG	Antonospora locustae virus 1
MW052144	GAUAAUUUUCGUCUCCAUG	Gutsystermes virus
KX884149	AAUUCUUUUCUUGAAGGUA	Hubei partiti-like virus 59
MZ209909	AAAACGUUUCAAGAAGGUG	Sanya amalgavirus 1
OL700164	UGUUCGUUUCCGGACCAAA	XiangYun partiti-picobirna-like virus 7

D. *Amalgavirus*

NC_036580	CAUGAGUUUCGUCGCGUCC	Allium cepa amalgavirus 1
NC_036581	CAAGAGUUUCGUCGCGUCA	Allium cepa amalgavirus 2
NC_040432	UUGUCUUUUCGUGCUCGUG	Anthoxanthum odoratum amalgavirus 1
NC_014593	CAGUCUUUUCGUGACUCAG	Blueberry latent virus
NC_040433	AGUACUUUUCGUGCCUUAC	Camellia oleifera amalgavirus 1
NC_040777	GAGAAUUUUCGUGCCAUCC	Cleome droserifolia amalgavirus 1
NC_040592	UUGUCCUUUCGAAGAGAUG	Erigeron breviscapus amalgavirus 1
NC_040593	UUGGCAUUUCGGGCCGAUA	Erigeron breviscapus amalgavirus 2
NC_040663	UUGUCUUUUCGAGCUUGUG	Festuca pratensis amalgavirus 1
NC_040778	AGUUCUUUUCGUAACUUAC	Festuca pratensis amalgavirus 2
NC_040591	GGUUCCUUUCGCAGUCUCU	Medicago sativa amalgavirus 1
NC_040590	ACUACUUUUCGUUCCUACA	Phalaenopsis equestris amalgavirus 1
NC_014481	GGGACUUUUCGCAGCCUCU	Rhododendron virus A
NC_040690	GAUACUUUUCGUGUCCUCG	Salvia hispanica RNA virus 1
NC_035070	UUGUUCUUUCGGAAGACCG	Spinach amalgavirus 1
NC_043095	GGGACUUUUCGUAACCUCG	Vicia cryptic virus M
NC_034614	AAAGGUUUUCGUGCCCAAU	Zostera marina amalgavirus 1
NC_034615	AAGGGUUUUCGUGACGACG	Zostera marina amalgavirus 2

E. *Unirnavirus*

MT241326	UGAGCUGGAUUUUAGUGU	Alternaria dianthicola dsRNA virus 1
NC_024703	CAAUUUGGAUUUUAGUUU	Alternaria longipes non-segmented mycovirus 1
LC553704	UGACCUGGAUUUUAGCUU	Aspergillus lentulus non-segmented dsRNA virus 1
NC_027428	UGAUUUGGAUUUUAGUGU	Beauveria bassiana non-segmented RNA virus 1
ON887156	GGACUUGGAUUUUAGUUU	Colletotrichum gloeosporioides RNA virus 1
NC_028242	GACUCUGGAUUUUAGUCU	Colletotrichum higginsianum non-segmented dsRNA virus 1
MH990637	CGGAUUGGAUUUUAGUCU	Combu double-strand RNA mycovirus
MN187541	GAGUUUGGAUUUUAACGU	Fusarium culmorum virus 1
OP103962	CCUCGAGGAUUUAAAUUUC	Penicillium citrinum non-segmented RNA virus 1
KT601106	CAACUUGGAUUUCUAGUCU	Penicillium janczewskii Beauveria bassiana-like virus 1
MH155602	UGAGGAGGAUUUUAUGUAA	Urichoderma harzianum mycovirus 1
ON791647	UGAGGCGGAUUUUUAACUU	Ustilaginoidea virens RNA virus M-A
NC_027427	GCUAGAGGAUUUAAACUUC	Ustilaginoidea virens unassigned RNA virus HNND 1

FIGURE 3
Analysis and comparison of programmed ribosomal frameshifting (PRF) sites among members of the family *Amalgaviridae*. Alignments of PRF site sequences are shown for the genus *Zybavirus* **(A)**, the "BBV14 clade" **(B)**, the proposed genus "Anlovirus" **(C)**, the genus *Amalgavirus* **(D)**, and the genus *Unirnavirus* **(E)**. Residues identical across all sequences are highlighted with a black background, and those conserved in at least half of the sequences are shown with a gray background. Sequence logos illustrating conservation patterns, derived from the aligned sequences, are shown on the right. A red asterisk marks the C residue skipped during +1 PRF translation **(A–D)** or the U residue translated twice during −1 PRF translation **(E)**.

Phylogenetic analysis also supports the distinctiveness of the proposed genus "Anlovirus," which includes AnloV1, HplV59, and three additional unclassified viruses: GutV, SaAmV1, and XYpplV7. Although SaAmV1 is presently annotated as a member of *Amalgavirus*, its RdRp sequence groups more closely with that of AnloV1. The consistent clustering of these five viruses into a separate lineage, supported by a bootstrap value of 89, reinforces the recognition of "Anlovirus" as a distinct genus within the family *Amalgaviridae*, as previously proposed (Pyle et al., 2017).

The analysis of +1 PRF motifs further supports these groupings. Among the 18 viral genomes classified as members of *Zybavirus*, the "BBV14 clade," or "Anlovirus," ten possessed the canonical UUU_CGN motif, while the remaining eight contained related variants conforming to a UUU_CNN pattern. These results suggest that UUU_CNN may serve as a broader consensus sequence for +1 PRF sites in these viral genomes. Given that most members of the genus *Amalgavirus* retain the UUU_CGN motif, we hypothesize that ancestral members may also have used the more general UUU_CNN motif, with the UUU_CGN sequence becoming preferentially selected over evolutionary time.

In contrast, members of the genus *Unirnavirus* predominantly carry the G_GAU_UUU motif and employ a −1 PRF mechanism rather than a +1 PRF mechanism (Campo et al., 2016; Depierreux et al., 2016). This stark divergence from other members of the family *Amalgaviridae* remains puzzling. Due to these differences, the establishment of a separate family, "Unirnaviridae," was previously proposed (Kotta-Loizou and Coutts, 2017; Mahillon et al., 2020). Nevertheless, the genus *Unirnavirus* was recently established within the family *Amalgaviridae* by the ICTV. Although the PRF mechanisms differ, the UUU codon in ORF1 appears to play a central role in both. In the +1 PRF mechanism, a tRNA^{Phe} binds to the UUU codon and slips forward at a UUU_CGN or UUU_CNN motif. In the −1 PRF mechanism, the same tRNA^{Phe} is proposed to slip backward at the G_GAU_UUU motif. This observation raises the possibility that transitions between +1 and −1 PRF mechanisms may have occurred during the evolution of the family *Amalgaviridae*.

In conclusion, this study provides new insights into the diversity and evolutionary relationships within the family *Amalgaviridae*. The identification of three additional *Zybavirus* members and the delineation of the "BBV14 clade" highlight the utility of mining public transcriptome datasets for virus discovery. These findings contribute to a more comprehensive understanding of *Zybavirus* diversity and support future efforts to revise the taxonomy of the family *Amalgaviridae*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

HP and YH performed bioinformatics analysis; YH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/av.2025.14882/full#supplementary-material

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