Hawaiian Geothermal Fumaroles Contain Diverse and Novel Viruses

Pia Sen[1], Lausanne Lee Oliver[1], Kira S. Makarova[2], Yuri I. Wolf[2], Christina Pavloudi[1,3], Max Shlafstein[1], Jimmy H. Saw[1]

[1]Department of Biological Sciences, George Washington University, Washington DC, USA

[2]Division of Intramural Research, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA

[3]European Marine Biological Resource Centre (EMBRC-ERIC), Paris, France

Co-Corresponding authors: Pia Sen (pia.sen.extremophiles@gmail.com) & Jimmy H. Saw (jsaw@email.gwu.edu)

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Abstract

Microbial communities of geothermal habitats are central to understanding the evolution of life on Earth. Metagenomics has provided insight into the role of viruses in shaping microbial diversity of complex environments. However, identification of novel viruses is constrained by lack of marker genes and low nucleotide similarities between related viral taxa. While microbial and viral diversity have been explored in terrestrial hot springs and hydrothermal vent systems, other volcanic features remain underexplored. Fumaroles (steam vents) are geothermal features that heat groundwater with magma, releasing steam and volcanic gases such as CO_2 and H_2S. Comparatively physicochemically dynamic to hot springs, fumarole temperatures and gas emissions rapidly fluctuate with volcanic activity. Here, we describe viruses identified metagenomically from microbial mats hosted near basaltic fumaroles on the Big Island of Hawai`i. To our knowledge, this is the first systematic survey of fumarole viruses. Our utilization

of a sensitive profile-based approach for identification reveals high viral diversity in fumaroles,

resulting in estimation of two undescribed order-level clades of Caudoviricetes (tailed phages).

Viral metabolic genes provide evidence of viral-mediated adaptation of microbes to fumarole

conditions. We describe patterns of viral diversity that diverge from the Bank model of viral

ecology, hinting at viral dispersal between biofilms and high viral richness and evenness. Lastly,

we provide a description of the first terrestrial geothermal environment dominated by

Microviridae, previously only described in viral communities of deep ocean hydrothermal vents.

This study offers important findings for exploration of viral ecology in extreme environments.

Introduction

Fumaroles (terrestrial steam vents) are the most common, yet least explored geothermal

feature on Earth. Fumaroles are formed when steam and volcanic gases exit cracks in basalt

deposits (Wall et al., 2015). Temperature and volcanic gas emissions fluctuate with volcanic

activity and geological context (Prescott et al., 2022). Located on the volcanically active Big

Island of Hawai`i, the East Rift Zone (ERZ) is a geologically active region in the vicinity of

Hawaii Volcanoes National Park. Along Hawaii Route 130 and between the towns of Pahoa and

Kalapana, there are many physicochemically diverse steam vents, which produce steam that is

mainly composed of water. These fumaroles occur on two lava flows with a wide age range (64-

400 years old) and host phototrophic biofilms dominated by *Cyanobacteria*, *Chloroflexota*,

Deinococcota, and *Alphaproteobacteria* (Prescott et al., 2022). Previous investigation of these

fumaroles reveals microbial succession patterns that are dependent on early colonizing

phototrophs with Actinobacteria and Proteobacteria becoming more abundant in the biofilms as

geothermal activity decreases in the system (King, 2003; Prescott et al., 2022). While microbial

diversity of other geothermal systems is shaped by both interspecific competition and habitat

filtering, microbial diversity of the steam vent biofilms is primarily explained by interspecific

competition which results in an over dispersed microbial community. Functional annotation of

microbial taxa from fumaroles suggests that photosynthesis, CO_2 fixation, and central carbon

metabolism are the primary biogeochemical processes in the biofilms. The presence of taxa

associated with nitrogen and sulfur cycling indicated these processes may be relevant to biofilm

biogeochemical cycling as well. Metals and minerals are made bioavailable in fumaroles by

microbial alteration of basaltic substrate, while CO_2 and H_2S emissions from the vent stimulate

carbon fixation and sulfur oxidation (Wall et al., 2015).

Microbial viruses modulate functional microbial diversity through one of three main

actions: top-down predation of microbial hosts, transduction, or metabolic rewiring during

infection which contributes to metabolic reprogramming of hosts on an ecosystem-level scale

(Tian et al., 2024; Zimmerman et al., 2020). The Bank model of viral ecology describes

ecosystems where a fraction of viruses are active and abundant, while a myriad of other viruses

are inactive and present in low abundances until a shift in environmental conditions (e.g.

seasonal or diel intervals) giving rise to new dominant host-virus pairs as observed in the

succession patterns of environmental viral populations (Breitbart & Rohwer, 2005; Ji et al.,

2024; Liang et al., 2019). The Bank model predicts that a viral community high in vOTU richness

will be low in vOTU evenness. The density dependent 'Kill-the-Winner' (KtW) paradigm

describes virulent viruses infecting the most abundant or fastest growing hosts, while the

'Piggyback-the-Winner' paradigm describes temperate viruses increasing in abundance with

rates of lysogeny (Hu et al., 2025). While relative abundance cannot be taken as confirmation of

viral activity, it is a metric that provides a good indicator of active viral populations (Liang et al.,

2019; Zhou et al., 2025).

Viral biogeography cannot be separated from the biogeography of their microbial hosts,

but is controlled by an interplay of viral characteristics (life cycle, burst and virion size) and

abiotic factors. Geothermal systems are good models for exploring viral biogeography because

their microbial communities are distinct and endemic islands across a broad range of

thermophily (Castelán-Sánchez et al., 2020; Langwig et al., 2025; Power et al., 2018; Salgado

et al., 2025; Teske & Reysenbach, 2015; Zhou et al., 2022). Viral dispersal in geothermal

systems is limited and subject to distance-decay and strong habitat clustering patterns, but only

within hot spring fields (Castelán-Sánchez et al., 2020; Cheng et al., 2022; Hwang et al., 2023;

Salgado et al., 2025; Thomas et al., 2021). Microbial mats (biofilms) in geothermal systems are

low mobility environments with highly specific host-virus pairs (Castelán-Sánchez et al., 2020;

Jarett et al., 2020; Salgado et al., 2025).

79   First, we seek to describe patterns of viral dispersal and diversity across fumarole

microbial populations. While microbial dispersal is known to vary across physicochemical

profiles, geothermal systems of hot springs and hydrothermal vents have consistently been

described as viral and microbial "islands", with limited movement between both geothermal sites

and smaller ranges of specific geothermal features within the broader system (Langwig et al.,

2025; Prescott et al., 2022; Salgado et al., 2025). In such systems with high viral endemism and

low dispersal, highly specific host-virus pairing is observed (Salgado et al., 2025). We gain

insight into dispersal dynamics with viral read mapping and calculations of vOTU relative

abundance. We predict host-virus pairing by matching microbial CRISPR spacers to viral

genomes. If we observe CRISPR-based evidence of abundant vOTUs in an 'arms race' with a

specific microbial population (many spacer matches to a single vOTU) we expect highly specific

host-virus pairing and that there will be corresponding fluctuations in abundances of these viral

and microbial populations across samples (Castledine et al., 2022). This would reflect lytic

fumarole viral populations following the Bank model and adhering to the 'Kill-the-Winner'

hypothesis, which describes a boom-bust dynamic where viruses target the dominant microbial

population, triggering a proportional increase in vOTU abundance as viruses and their hosts

engage in a coevolutionary arms race (Xue & Goldenfeld, 2017). This would reflect lytic

fumarole viral populations following the Bank model and adhering to the 'Kill-the-Winner'

hypothesis, which describes a boom-bust dynamic where viruses target the dominant microbial

population, triggering a proportional increase in vOTU abundance as viruses and their hosts

engage in a coevolutionary arms race (Bucher & Czyż, 2024). Third, we hypothesize that the

viruses within the fumarole biofilms will contain genomic evidence of viral capability to facilitate

microbial adaptation in a dynamic and extreme environment. We predict that viruses will contain

auxiliary metabolic genes that could increase host fitness in volcanic systems.

In this study, we test these hypotheses with metagenomic identification and

characterization of viruses in Pahoa fumarole biofilms from three different geothermal locations

on the Big Island of Hawai`i (steam wall and steam pit within the East Rift Zone (ERZ), and the

Big Ell cave in the Kilauea caldera). We use a sensitive and profile-based approach to identify

and classify vOTUs, many of which are distantly related to known viruses. Further, we

characterize viral genomes to identify shared predicted metabolic traits with viruses previously

described in other early Earth analog geothermal environments. We find that ERZ fumaroles are

a unique system for viral ecology, and demonstrate high viral dispersal and phylogenetic

novelty. The presence of auxiliary metabolic gene (AMG) candidates demonstrates potential for

viral influence on host metabolism. Our study provides insight into the diversification of early life

and patterns of viral and microbial succession (Godin et al., 2023; Hadland et al., 2024).

Results

Sampling and retrieval of viruses from metagenome

Biofilm samples from ERZ fumaroles are described in previous studies (Prescott et al.,

2022; J. H. Saw et al., 2026). Briefly, biofilms that were sampled were several centimeters thick

and grew on the surface of volcanic rock, directly where steam was being released. These

biofilms do not exhibit clear segmentation of layers as observed in some microbial mats, and

display a variety of phenotypes and colors (J. H. Saw et al., 2026). Most Gloeobacter-dominated

biofilms are purple with patches of dark green, while other biofilms are green, yellow, brown,

and grey. While some biofilms have a visible extracellular polysaccharide matrix (EPS) layer

and appear wet, other biofilms are dry or otherwise textured in appearance. While the steam pit

and steam wall are separated by ~100 meters, the Big Ell cave in the Kilauea Caldera is ~35 km

from the steam vents along Hawaii Route 130 (J. H. Saw et al., 2026).

WGS raw sequences from related biofilm features were co-assembled for related

features (20 metagenomes from the 46 collected samples) with the ViralAssembly module of

metaviralSPAdes, producing 400.15 Mbp (12,253 contigs) of assembled sequence data from

the metaviralSPAdes pipeline (Antipov et al., 2020; J. H. Saw et al., 2026). 112.97 Mbp of the

assembled sequence data was generated from Big Ell cave in the Kilauea Caldera (Hawaii

Volcanoes National Park) with 17.87 Mbp (452 contigs) of the sequence data from geothermal

biofilm features within the cave, and 95.11 Mbp (3803 contigs) of sequence data from adjacent

non-geothermal volcanic soil samples. 243.97 Mbp (6596 contigs) of sequence data was

assembled from exposed steam wall biofilms, and 43.21 Mbp (1402 contigs) of sequence data

was assembled from the steam vents along Hawaii Route 130. From these assembled contigs,

the ViralVerify module of metaviralSPAdes identified 754 contigs (6.33 Mbp) as either

confidently (n=76) or uncertainly (n=678) viral (Supplemental Material S1). These contigs were

then used in downstream analysis. VirSorter2 (Guo et al., 2021) identified 119.46 Mbp

(n=16,002) of candidate viral contigs. From there, the VirSorter2 contigs (annotated with DRAM-

V) were filtered for PFAM clan identifiers for the capsid genes (Phage-Coat: CL0373 and

Inovirus-Coat: CL0371) producing 14.29 Mbp of candidate viruses (802 contigs) which were

used for downstream analysis.

The accuracy of VirSorter2 filtering by PFAM clan was assessed by screening the

remaining 15200 contigs identified as candidate viruses by VirSorter2 for the terminase large

subunit gene (TerL) or another packaging gene (Supplemental Material S2). 320 contigs (~2%

of filtered VirSorter2 output) contained TerL and were manually assessed for viral identity, with

upstream and downstream regions searched for other viral components. Of these contigs, 116

(0.76% of filtered contigs) were determined to be viral (containing both head and packaging

genes). With geNomad (Camargo et al., 2024) we identified 3.41 Mbp (n=107) viral candidates

(Supplemental Material S3). When final viral designations were made and vOTUs were

generated, 24 vOTUs were identified by all of the tools used: VirSorter2, ViralVerify, and

geNomad (Supplemental Figure 01). 15 vOTUs were identified by both VirSorter2 and

ViralVerify, and a single vOTU was identified by both ViralVerify and geNomad. The majority of

vOTUs (n=313) were identified from VirSorter2. 6 vOTUs were only identified by ViralVerify, and

5 vOTUs were identified only by geNomad.

Remote homology-based identification and network analysis reveals diversifying vOTUs

569 viral contiguous sequences (contigs) were identified from 20 fumarole sample

metagenomes (Supplemental Material S4). From these contigs, we performed clustering by

community standards (Roux et al., 2019) and sorted viral sequences into 383 species-rank level

vOTUs (viral populations). 51% of identified fumarole vOTUs are identified as Caudoviricetes

(195 vOTUs), indicating that microbial viruses make up a significant portion of the

metagenomically retrieved dsDNA viruses in fumarole biofilms. 46% of vOTUs (180 vOTUs)

cannot be placed into realms by vConTACT3 v.3.0 despite verification of structural viral genes

on each contig. These unclassified vOTUs may have ecological significance, making up at least

10% of viral relative abundance in the majority (17 out of 20) of samples (Figure 01).

Of the 180 vOTUs which could not be placed into a realm with network analysis, 100 of

the 'no realm' vOTUs were missed by geNomad and filtered out in the VirSorter2 pipeline (Tian

et al., 2024) during the CheckV step (Nayfach et al., 2021) suggesting stringent pipelines may

miss some of the ecologically significant and highly divergent viruses (Camargo et al., 2024;

Liang et al., 2019). The vOTUs were placed into genus-rank viral clusters (VCs) established by

vConTACT3 v.3.0 and were visualized in Cytoscape (v3.10.2) with and without all viruses from

NCBI RefSeq (Goldfarb et al., 2024). Clusters were filtered for quality by topological and

clustering coefficients (>0.85). This network yielded 230 distinct topological groups, including

subclusters, nested subclusters, and singletons within the network (Figure 01). Of these

topological formations, 20 VCs form distinct subclusters (clusters of viral genomes with three or

more genomes without links to the rest of the network). Multiple distinct viral populations

(vOTUs) within a single VC provides evidence of genomic variation and diversification within the

genus (Bolduc et al., 2025). High 'Closeness Centrality' values support the relationships

observed within network clusters and prevent incomplete viral genomes from inflating network

representation of genomic distance between vOTUs (Nayfach et al., 2021; Su et al., 2014).

Resulting network clusters made up entirely of distinct fumarole vOTUs indicates that related

viral populations share many genes with each other while still providing evidence of

diversification between related vOTUs.

While 61% of Caudoviricetes clusters contain one vOTU, six clusters have between 8-24

different vOTUs in a single cluster (Figure 01). One highly abundant cluster, Caudoviricetes

(Cluster B), makes up to 20% of viral relative abundance in three different biofilm samples from

the steam wall. This cluster contains 20 unique vOTUs. The high abundance of related vOTUs

is evidence of potential speciation occurring in abundant viral populations (Liang et al., 2019).

There is also diversity observed within VCs of viruses that cannot be placed into a realm

confidently by vConTACT3, providing tentative evidence of diversification within highly novel

viral genera: out of the 21 VCs (three or more viral genomes within a cluster) which could not be

realm placed, 11 of the 'no realm' filtered VCs contain more than a single vOTU (Supplemental

Material S5). All of these 11 VCs are internally coherent with high connectivity measured by the

NetworkAnalyzer plugin (Su et al., 2014).

Phylogenetic estimation of viral novelty

Fumaroles contain new candidate Caudoviricetes orders

Out of the 196 vOTUs identified as Caudoviricetes by VContact3 assignment, 179

Caudoviricetes vOTUs contained a terminase large subunit (TerL) gene, and thus, were

included in phylogenetic analysis with maximum likelihood (91% of Caudoviricetes vOTUs).

Using 320 first neighbors of fumarole viruses from ICTV MSL40.v1, only 3% of these references

could be placed into an existing order. Less than half (40%) of these references could be placed

into previously identified Caudoviricetes families. The lack of classification capability for the

reference viruses which are most closely related to fumarole vOTUs is evidence of fumarole

viral novelty. Many fumarole viruses form distinct clades, with references often paraphyletic or

sister taxa to fumarole viruses, suggesting that steam vent viruses expand the diversity of

Caudoviricetes. The resulting maximum likelihood tree provides evidence of phylogenetic

diversification and potentially speciation within Caudoviricetes clades. There is evidence of

recent diversification within at least five clades that are almost entirely made up of fumarole

viruses and contain 130 vOTUs (Figure 02). These species-rank vOTUs meet MIUViG

standards necessary for taxonomic proposals (Roux et al., 2019).

We put forward two Caudoviricetes order-level clades containing 30 and 56 unique

fumarole vOTUs respectively. These clades expand the diversity of dsDNA viruses and enable

classification of three previously identified Caudoviricetes families which have not been

assigned to orders previously. The first clade, which we call *Kilaueavirales*, contains the

Casjensviridae and *Herelleviridae* families. The *Casjensviridae* family is made up of

flagellotrophic phages which frequently infect Enterobacteriaceae and can be found in high

abundances in wastewater (Chakraborty et al., 2024) while *Herelleviridae* viruses infect the

Bacillota phylum of bacteria (Barylski et al., 2020). Clade 1 (*Kilaueavirales*) contains a family-

level clade of 30 fumarole virus vOTUs (40 contigs) and 17 previously described RefSeq

Caudoviricetes species which had not previously been assigned to viral families. These

unassigned reference viruses have hosts such as *Xanthomonas*, *Burkholderia*, and

Enterobacter (Black et al., 2025). This family-level clade of 30 fumarole vOTUs and 20 RefSeq

viruses is monotypic with low support (support=60%) to the *Faecalibacterium* phage,

Eponavirus epona. This family-level clade and the *Casjensviridae* family together form a well

supported clade (support=100%) that is a sister group to family *Herelleviridae* (support=98.9%).

Together, this resulting clade is Clade 1 (*Kilaueavirales*) (Supplemental Figure 02).

Clade 2 (*Pahoavirales*) contains 56 fumarole vOTUs, some of which make up clades

which are sister groups to reference viruses (support=90.5%). One sister group within the

Pahoavirales clade contains a fumarole virus with a monotypic relationship (support=89.1%) to

RefSeq *Ralstonia*, *Agrobacterium*, and *Myxococcus* viruses. A sister group to this clade

contains two sister subclades (support=95.8%). In one of these sister subclades, there are 24

fumarole virus vOTUs and viruses from the *Mesyanzhinovviridae* family are shallow-branching

taxa close to tree tips. The second subclade (which is a sister group to the first subclade

containing *Mesyanzhinovviridae)* is mostly made up of fumarole viruses (32 fumarole vOTUs),

but also places two reference viruses (*Dinoroseobacter* and *Microbacterium* RefSeq phages)

within the subclade (support=100%).The shallow branching relationships observed in the

second sister subclade of *Pahoavirales* suggests recent divergence of closely related viruses

without many close relatives (Supplemental Figure 02).

In addition to the 86 Caudoviricetes vOTUs which belong to two previously undescribed

order-level clades, we observe other clades of Caudoviricetes fumarole viruses with some

relation to previously identified viral families (Supplemental Figure 03). One fumarole vOTU is

estimated to be within the same clade of Caudoviricetes incertae sedis viral family

Hodgkinviridae (support=75.1%), which is a family of *Microbacterium* phages (Turner et al.,

2025). We estimate 19 fumarole vOTUs (25 viral contigs) to be a sister group to the

Hodgkinviridae family (support=93.7%). Six fumarole vOTUs are a sister group to the

Straboviridae family (support=87.8%) (Turner et al., 2025). Five fumarole Caudoviricetes vOTUs

are estimated to form a clade with the incertae sedis *Peduoviridae* family (support=85.9%). Two

fumarole vOTUs are part of a clade with the *Zobellviridae* which is an incertae sedis

Caudoviricetes family (support=96.5%). One fumarole vOTU forms a clade with the

Caudoviricetes incertae sedis family *Saffermanviridae* (support=95.6%). The relationships of

fumarole Caudoviricetes to numerous Caudoviricetes incertae sedis families provides evidence

that the inclusion of these viruses in phylogenetic estimation expands the diversity of dsDNA

tailed viruses.

Estimation of diverse Singelaviria and Varidnaviria

Viruses within the Singelaviria realm (class of Laserviricetes) and the Varidnaviria realm

(class of Tectiliviricetes) are assigned to a single class category of

'Laserviricetes|Tectiliviricetes' by vConTACT3. Tectiliviricetes phylogeny was estimated with a

maximum likelihood tree that was constructed using the packaging ATPase gene for six

different fumarole Tectiliviricetes vOTUs (11 viral contigs). Viral species from all five genera

within the *Tectiviridae* family were used as references. The resulting phylogeny shows six

distinct fumarole Tectiliviricetes clades (Supplemental Figure 4). One vOTU is a sister group

with weak support (support = 52.5%) to the Alphatectivirus genus, which contains only the two

species of PRD1 and PRD4. Alphatectivirus is the only genus within the Tectiliviricetes class

that is known to contain plasmid dependent phages (PDPs). This vOTU may be a taxon within

Alphatectivirus, which is potentially worthy of further investigation when exploring the

phylogenetic distribution of related ecological characteristics such as plasmid dependence

(Bignaud et al., 2025). Two fumarole Tectiliviricetes vOTUs which are closely related and have

almost identical packaging ATPase sequences to each other on the amino acid level are a sister

group to the clade containing *Deltatectivirus* and *Epsilontectivirus* (support=93.8%).

An independent phylogenetic estimation with maximum likelihood was performed for

Laserviricetes using the packaging ATPase gene. This phylogeny contains a single fumarole

vOTU (Supplemental Figure 04) which is a sister taxon with strong support to the *Hukuchivirus*

genus (support=100%). The *Hukuchivirus* genus is made up entirely of viruses infecting the

thermophilic genus, *Thermus*. This vOTU could potentially be considered a member of a new

genus within the family of *Matshushitaviridae*.

Microviridae form a poorly resolved clade with phiX174

A class-level maximum likelihood phylogeny of Malgrandaviricetes was built using the

amino acid sequences of the major capsid protein (MCP) to classify the single fumarole

Microviridae vOTU. The resulting phylogeny produced a poorly resolved clade, resulting in an

apparent polytomy (support=100%) with Enterobacterial coliphage phiX174 (Supplemental

Figure 05). These fumarole viruses and phiX174 are paraphyletic to the other genera of

microviruses in the *Microviridae* family. The high genome conservation of phiX-like viruses (and

more broadly, the *Bullavirinae* subfamily) (Kirchberger & Ochman, 2023) is potentially

explanatory of an apparent polytomy with phiX174 with fumarole *Microviridae*, and suggests that

phylogenetics is insufficient to capture the diversity of fumarole microviruses.

Comparative genomics of abundant Microviridae

A single vOTU of Microviridae was identified, with some genetic variation (subclustering)

observed between phylogenetically identical Microviridae genomes of similar length (Figure 01).

The relative abundance of Microviridae ranges from 22-82% across samples of varied microbial

composition and biofilm phenotype (Figure 01). In almost half of biofilm samples, Microviridae

makes up over half of the viral community, and is most abundant in moderately geothermal

biofilm samples which have overall high viral richness and evenness. The high abundance of

Microviridae in moderately geothermal biofilms is surprising because, to our knowledge, there

has never been a terrestrial environment reported which displays high abundances of

Microviridae. However, abundant Microviridae has been reported from viromic surveys of

viruses in hydrothermal vent sediment, as well as benthic sediment (Cheng et al., 2022;

Yoshida et al., 2018). Despite the more pronounced prevalence of Microviridae in moderately

geothermal biofilms, mesothermal biofilms also display a higher abundance of Microviridae

(>28% viral relative abundance) than both other terrestrial environments and mesothermal

volcanic soil collected adjacent to geothermal features from the Kilauea caldera, suggesting

Microviridae abundance is not simply predictable by measured temperature.

The low abundance of *Enterobacteria* in fumarole biofilms (Metagenome Assembled

Genomes (MAGs) and Amplicon Sequence Variants (ASVs) suggest that these Microviridae

have non-Enterobacterial hosts, and that the apparent polytomy of fumarole viruses with phiX-

174 could be explained by low phylogenetic resolution rather than functional similarity between

environmental *Microviridae* and phiX-174 (J. H. Saw et al., 2026). Our observation of viruses

from the Bullavirinae subfamily in fumaroles is intriguing. While lab models of phiX-like viruses

are heavily studied, the subfamily of Bullavirinae is considered extremely rare in nature and

occupies a specialist niche as a lytic phage with limited capability for uptake of genomic content

(Kirchberger et al., 2022).

Fumarole microviruses were characterized genomically with comparison of whole

genomes (amino acid sequences) with RefSeq *Microviridae*. Network analysis with vConTACT3

demonstrates consistency in length of reference and fumarole *Microviridae* (phiX174 genome

size is 5.386 Kb, fumarole *Microviridae* are 5.463 Kb), supporting that the potential for genome

completion issues does not falsely inflate the degree of genetic difference between fumarole

and reference *Microviridae*. Fumarole *Microviridae* are located within the same nested

subcluster as viruses from the Bullavirinae subfamily, providing evidence of genetic similarity

between fumarole viruses and Bullavirinae viruses beyond simply phylogenetic association. This

overall genome conservation may be explained by limited genomic uptake capabilities of

Microviridae, which occupies a specialist niche as a lytic phage, constraining genetic

rearrangement and mosaicism (Kirchberger & Ochman, 2023). Despite this, fumarole and

RefSeq Microviridae still vary widely with pairwise similarity as low as 40-60% between viruses

(Figure 03). In addition to low overall genome similarity, there is evidence of syntenic changes

between fumarole Microviridae, with differences from RefSeq Bullavirinae species

(Supplemental Figure 06).

The low pairwise similarities and syntenic rearrangement we observe between fumarole

Microviridae is highly unexpected. While innovations do occur in the *Microviridae* genome (gene

overprinting, recombination) the overall high syntenic conservation of the *Microviridae* family is

thought to reflect strong evolutionary constraints for transcriptional timing of *Microviridae* viruses

(Kirchberger & Ochman, 2023).

Biogeography and diversity of vOTUs

Most vOTUs are shared between biofilms

All viral contigs were mapped to samples and normalized by calculating reads-per-

kilobase-per-million (RPKM). Contigs were summed for each vOTU and VC for alpha-diversity

analysis and visualization respectively (Roux et al., 2019; ter Horst et al., 2021). 99.7% of

vOTUs (n=382) are shared between biofilm samples (all but one vOTU). The observation that

most vOTUs are not unique to individual biofilms or sample sites is consistent with predictions

by the Bank model (Breitbart & Rohwer, 2005). However, evidence of viral dispersal is

unexpected within the physicochemical context of spatially separated and geothermally-active

fumaroles that give way to harsh conditions (J. H. Saw et al., 2026). From hyperthermophilic

hydrothermal vents to moderately geothermal hot springs, both microbes and viruses are found

to be overwhelmingly endemic (Jarett et al., 2020; Langwig et al., 2025; Salgado et al., 2025).

Both Hawaiian fumarole-hosted microbial communities (Prescott et al., 2022) and viral

communities show evidence of selective pressure associated with the geothermal activity.

Within Big Ell samples, there are few shared vOTUs between the rock-hosted thermal biofilms

and volcanic soil, collected directly adjacent to the cave wall. No vOTU which is found in over

5% abundance within thermal biofilms has an abundance over 1% in any non-thermal volcanic

soil sample (Figure 01). The vOTU communities of thermal biofilms is suggestive of selection,

rather than random vOTU dispersal (Liang et al., 2019).

Yet, while microbial communities of fumaroles form "islands" with distinct communities

between biofilms of different thermal features (Prescott et al., 2022), shared vOTUs are

observed in unexpectedly high abundances across samples. 9.5% of vOTUs (n=36) are shared

between samples with individual population relative abundance over 1% (Figure 01). 5% of

identified vOTUs (n=19) are shared between geothermal features and sites in abundances over

2%. The abundance of shared vOTUs between fumaroles is inconsistent with descriptions of

distance decay observed in other physicochemically diverse geothermal systems, which are

harsh to varied extents. For hot spring and hydrothermal vent systems, viral community

dissimilarity increases with geographic distance, and fewer shared vOTUs are observed

between distant geothermal fields (regions with localized and relatively high heat flow). Though

the seed-bank model predicts that most of the shared vOTUs will be found in <2% relative

abundance within a given community at any point in time, (Hevroni et al., 2020) we observe

individual fumarole-hosted viral populations that are non-dominant and shared between

samples can make up to 10% of a given biofilm's viral relative abundance, representing a

significant variation from the Bank model.

Perhaps as a consequence of dispersal limitation, specific host virus pairs are often

observed in high abundances within geothermal systems (Guajardo-Leiva et al., 2021a;

Salgado et al., 2025; R. Wu et al., 2023). This has been described as particularly prevalent in

phototrophic thermal biofilms of hot springs, where matrix immobilization of microbes and their

viruses result in dispersal limitations and facilitates tightly coupled host-virus pairs (Jarett et al.,

2020). While it is difficult to determine microbial and viral mobility metagenomically, CRISPR-

based analysis provides evidence of coevolutionary relationships between microbes and their

viruses in line with the Red Queen hypothesis (Common et al., 2019; Koonin & Wolf, 2015; R.

Wu et al., 2023). For the 10% of recovered fumarole vOTUs which were host matched (n=39),

specialized host-virus relationships were determined by the presence of more than three unique

spacer matches between a MAG and a vOTU. Out of the 21% of medium to high quality MAGs

(n=74) which were successfully host matched, 11 CRISPR arrays showed evidence of microbial

coevolution with vOTUs (J. H. Saw et al., 2026). The most spacer matches (n=61) within a

single array comes from a Rhodomicrobiaceae MAG from an unclassified genus. MAGs from

the Chloroflexota families Roseiflexaceae (n=35) and JAJPGT01 (n=34) also provide CRISPR-

based evidence of an antagonistic coevolutionary relationship between specific vOTUs and their

hosts.

Antagonistic coevolution described by 'Kill-the-Winner' is inherently density dependent

(Hu et al., 2025; Salgado et al., 2025). Despite this, not a single of the 14 vOTUs which is

identified as part of a specialized host-virus pair can be found in viral relative abundance over

1%. The associated host phyla abundance also does not correspond to linked vOTU abundance

(Supplemental Material S6) (Prescott et al., 2022; J. H. Saw et al., 2026). This unexpected low

abundance is observed across microbial taxa with specialized pairing to vOTUs. Cyanobacterial

genus *Chlorogloeopsis* is highly abundant in steam wall biofilms, and demonstrates a multi-

spacer relationship with a highly novel paired vOTU which is below relative abundance of 1% in

all samples except for steam wall biofilm PDB5 (9.24% relative abundance) non-thermal

volcanic soil sample HW-02, where it is found in 15.3% relative abundance. These samples are

not in close proximity to each other and have distinct microbial community composition

suggesting that Hawaiian fumarole viruses and their hosts are not necessarily endemic, even

though these shared viruses participate in specialized host-virus pairings (Audrey et al., 2023).

These spacers are not always located in the same array. We observe spacers paired to a vOTU

can be distributed in multiple arrays across the host genome. This phenomena could be

evidence of repeated exposure to a particular vOTU, or be a result of multiple different CRISPR

systems conferring viral resistance (Shmakov et al., 2018). The distribution of vOTU resistance

across two arrays is observed for three Chloroflexota MAGs from the Chloroflexia and UBA6077

classes, which contain various Type I and Type III systems (Supplemental Material S7). These

arrays are not necessarily genomically clustered, with up to ~61 kb observed between these

array pairs.

While the majority of samples do not have high abundances of identified tightly-coupled

virus-host pairs, high relative abundance is observed for two Caudoviricetes vOTUs which are

both prophages belonging to MAGs of *Gloeobacter kilaueensis*. This cyanobacteria is abundant

only in steam pit samples, which is observed for these vOTUs as well. High abundance of

Gloeobacter prophages is only observed as expected in samples dominated by *Gloeobacter*

kilaueensis. Summed vOTUs occur in relative abundances that range from 17-51% in all but

one of the biofilm samples with high abundance of microbial *Gloeobacter kilaueensis*. These

two prophage vOTUs follow the directly proportional abundance trends predicted by the

"Piggyback-the-Winner" hypothesis which describes a microbial community in which co-

abundant vOTUs favor a switch to lysogeny as a viral life cycle strategy, and accordingly,

abundant viral populations are identified as prophages (Hu et al., 2025). The presence of

Gloeobacter prophage vOTUs is in line with this theory. One of these vOTUs is present in

double the abundance of the other vOTU across all samples (Supplemental Material S6). Unlike

many of the other vOTUs with high dispersal inferred (abundance in samples doesn't

correspond to either hosts or microbial taxa) these two vOTUs appear restricted in dispersal to

be consistent with host.

Viral alpha-diversity

409   Chao1 (species richness) and Pielou (species evenness) index values were plotted

410 against each other for each sample (Figure 04). Moderately geothermal biofilms cluster

411 together, regardless of location or microbial distribution within the sample. Relative to other

412 samples, biofilms which are collected from moderately geothermal fumaroles (40-60□) have

413 high richness and evenness. The 17 thermal biofilm samples show a strong positive correlation

414 between vOTU richness and evenness. On the plot of richness to evenness, non-thermal

415 volcanic soil diversity is clearly partitioned from the fumarolic active biofilms, displaying low

416 vOTU richness and high evenness. The low richness is striking when compared to biofilm

417 diversity, because soil has been described as a system that generally has high viral richness

418 and evenness comparative to the viral communities of other systems such as marine and

419 groundwater (Roux & Emerson, 2022; ter Horst et al., 2021).

420   Richness and evenness plots exhibit a clear partitioning of diversity (high

421 richness/evenness in moderately geothermal fumaroles) that suggests diverse viral

422 communities may be characteristic of active fumaroles. The biofilms which display this high

423 species richness and evenness are dominated by different microbial taxa, suggesting that

424 microbial taxonomy alone is insufficient to explain viral diversity (Figure 04). Microbial diversity

425 indexes of biofilms were assessed using ASVs (Prescott et al., 2022; J. H. Saw et al., 2026).

426 Unlike with viruses, there is no visually evident clustering of temperature-associated diversity

427 (Figure 04). The decoupling of microbial and viral diversity is observed across samples of varied

428 temperature ranges and microbial compositions. The samples with high viral richness and

429 evenness are composed of diverse phyla (Figure 04). Interestingly, these dominant phyla all are

430 known to occupy varied niches and display varied metabolic traits, suggesting microbial

431 composition alone is insufficient to be the primary explanatory driver of viral diversity within

432 these biofilms (Prescott et al., 2022).

When the relationship between richness and temperature was assessed, a quadratic

model was found to be the best fit to explain this relationship. While the 4-parameter cubic

model had the lowest residual sum of squares (RSS) value (RSS=36846.4), the quadratic

model had the lowest BICc value (BICc=161.8) indicating that the quadratic model is the best fit

for describing the relationship between temperature and viral richness for non-thermal volcanic

soil and fumarolic biofilms, ranging in temperature from mesophilic to highly geothermal (>70°C)

(Prescott et al., 2022; J. H. Saw et al., 2026). These tests support the significance of a non-

linear (quadratic) relationship between temperature and viral richness in the Hawaiian fumarole

system, with the highest vOTU richness observed at ~50°C. The extent to which microbial life

adheres to Humboldt's theory of a temperature-diversity gradient has only been explored in a

handful of geothermal environments, with hot springs making up the majority of geothermal

systems studied for this (Sharp et al., 2014). In some moderately geothermal circumneutral hot

springs, the highest species richness of bacteria has been observed at 50°C, with increasing

temperatures associated with decreased species richness (Castelán-Sánchez et al., 2020).

While the bacterial diversity of fumarolic biofilms observed does not clearly follow this trend,

viral diversity demonstrates a surprisingly consistent temperature-diversity gradient which is

independent of microbial diversity (Prescott et al., 2022; Sharp et al., 2014). This indicates that

moderately geothermal features of fumaroles are potential biological hot spots for viruses, with

high biodiversity of microbial/viral life which is observed across different geothermal systems at

similar temperatures.

The R-squared value (R-squared=0.7292) indicates temperature explains ~73% of

variance in viral richness, and is determined to be significant with the p-value of the F-statistic

(p=1.619e-05) (Figure 04). Normality of residuals was supported by QQ and density plots, and

the Shapiro-Wilk test confirms that the quadratic model meets normality assumptions

(W=0.94514, p-value=0.2992) (Supplemental Figure 07). Though vOTU evenness varies greatly

between samples (from Pielou=0.21-0.6), temperature cannot explain the great variation in viral

population evenness (Supplemental Figure 07). While there is a significant relationship between

vOTU richness and temperature (consistent with the cluster of high viral richness and evenness

for moderately geothermal biofilm samples) the evenness of these samples cannot be described

as temperature dependent, or consistent with any specific phylogenetic distribution, indicating

complex biogeographic factors may explain viral diversity of biofilms.

CRISPR defends fumarole bacteria against a breadth of unknown elements

CRISPR spacer targets (n=302) were identified for arrays from 13 different microbial

phyla (Supplemental Material S7). Matches are found for 20% of all fumarole-assembled MAGs

(n=74) (J. H. Saw et al., 2026). The majority of spacer matches (n=120) are for arrays that come

from Chloroflexota MAGs (classes:UBA6077, Ktedonobacteria, and Anaerolineae). Despite this,

only 70% of the Chloroflexota targets identified are vOTUs (n=9). Additionally, Chloroflexota

spacer matches were identified for 1 plasmid (0.65% of all spacer matches) and 33 unknown

elements. Beyond the Chloroflexota phylum, spacer targets are not limited to viruses either.

Even though the majority of matches identified were to vOTUs targeted by spacers (74.2% of

spacer matches) viruses were only 44% of the matched target elements.

These unknown elements represent possible other miscellaneous mobile genetic

elements (conjugative/integrative elements, transposons, insertion sequences) (Tokuda &

Shintani, 2024) or chromosomally integrated elements (Shmakov et al., 2017). Even though the

majority of spacer targets are non-viral, these unknown element matches make up only 20.86%

of total spacer matches. This suggests that the microbial populations of fumaroles utilize

CRISPR to defend themselves against a wide breadth of unknown elements, and that CRISPR

may play a minimal role in any sustained antagonistic coevolution occurring between non-viral

elements and microbes in the fumarole biofilms. (Martynov et al., 2017). One exception to this,

however, is multiple spacer matches (n=8) between an unknown element and a Chloroflexota

MAG (class UBA6077). Seven of these matches to the unknown element are part of a Type IA

CRISPR system, while one spacer match is contained within a different array that is part of a

Type IE CRISPR system.

Identification of putative AMGs and accessory viral genes

AMG mediated adaptation to heavy metal toxicity and resource limitation

Secondary curation of baseline DRAM-v AMG summary hits (212 genes) was performed

to eliminate viral life cycle and host-associated genes from AMG candidates. This yielded 72

putative AMGs and 70 accessory (moron) genes associated with defense against mobile

genetic elements (Brüssow et al., 2004). The majority of AMG candidates and accessory

defense genes we identify are from viruses in fumarole biofilms - only one accessory defense

gene and one AMG candidate is observed in viruses assembled from non-thermal soil. Overall,

7% of vOTUs (n=27) identified from fumarole metagenomes contain AMG candidates. These

low AMG frequencies have been observed in other geothermal systems such as hydrothermal

vents (Langwig et al., 2025). The majority (74%) of AMG-carrying vOTUs are classified as

Caudoviricetes, and the remaining vOTUs with AMG candidates cannot be assigned to realms

(Figure 05). Five vOTUs contain AMG-candidates related to energy metabolism (boosting ATP-

generation). Three of these vOTUs encode NifU-like IscA (scaffold and iron carrier) which is a

gene involved in Fe-S cluster biosynthesis. One vOTU contains AMG candidate gene lactate

dehydrogenase (reversible enzyme that converts pyruvate to lactate to generate NAD+ for

glycolysis, or converts pyruvate to lactate, feeding the TCA cycle). Another vOTU contains

putative AMG succinate dehydrogenase cytochrome b558, which, to our knowledge, has only

been described as an AMG in giant virus family Mimiviridae (Blanc-Mathieu et al., 2021; Lamb

et al., 2024).

The majority of putative AMGs identified in fumarole viruses encode transporter-related

gene products (Supplemental Material S8). Transporter types include: divalent metal (DMT)

transporters, RND efflux pumps, ABC-transporters, and Major facilitator superfamily (MFS)

transporters. Ten fumarole vOTUs contain AMG candidates that can be linked to cellular

detoxification from heavy metals, such as metallothioneins, Zn2+/Cd2+ exporting ATPases, and

heavy metal efflux proteins including transporter TolC. The TolC transporter functions with efflux

of transition metals, including first row transition metals which are present in basalt (Farias et al.,

2015). The transporters observed in fumarole viruses have also been described in AMG

candidates from viruses in hydrothermal vents and heavy metal enriched soils with similar

elemental composition to basaltic surfaces described in the ERZ volcanic system (Prescott et

al., 2022; Sun et al., 2023; Thomas et al., 2021). Other fumarole virus transporters are

protective proteins (such as Fe-Mn superoxide dismutase) that neutralize reactive oxygen

species (ROS) which can also be generated by metal induced cellular stress. Fumarolic non-

transporter AMG candidates (such as rubrerythrin) also neutralize ROS, which usually confers

host survival in microaerophilic or anoxic environments (Cardenas et al., 2016).

Other AMG candidates relate to nutrient deficiency. One vOTU contains sporulation

maturation protein CgeB which is sometimes observed in Bacillus subtilis prophages where it is

part of the Cge operon which modifies the surface properties of the spore (Shuster et al., 2019).

One putative vOTU contains a carbohydrate ABC transporter permease (Trehalose transport

system permease protein SugB) which is involved in trehalose recycling within the cell (Pohane

et al., 2021). One fumarole vOTU (prophage of Gloeobacter kilaueensis) encodes a gene highly

similar to polyhydroxybutyrate depolymerase (phaZ). Polyhydroxybutyrate depolymerase is an

intracellular enzyme which breaks down polyhydroxyalkanoates (PHAs) or Polyhydroxybutyrate

(PHB) biopolymers which can be used as carbon sources. Eight unique vOTUs contain AMG

candidates related to carbon utilization. Two Caudoviricetes vOTUs contain five different carbon

utilization genes that could possibly be involved in PHA biosynthetic pathways, converting short-

chain carboxylic acids (such as acetate, propionate,lactate, and butyrate) into PHA (Yao et al.,

2025). Alternatively, these five genes could be involved in beta oxidation of fatty acids, which

have been described in the giant virus family Mimiviridae (Brahim Belhaouari et al., 2022). The

presence of these five AMG candidates in Caudoviricetes as observed in two fumarole

Caudoviricetes vOTUs is unusual because Caudoviricetes hosts are prokaryotic while

Mimiviridae hosts are eukaryotic (Duda & Teschke, 2019). Another putative AMG contained

within one of these Caudoviricetes vOTUs is gene 2-methylcitrate dehydratase (prpD) which

catabolizes the third step of the methylcitrate cycle for propionate catabolism, and has been

described as an AMG in hydrothermal vents (Zheng et al., 2021). Host linking with experimental

verification of transcriptional activity and gene product expression is necessary to ascertain if

these putative AMGs are involved in beta oxidation of fatty acids and lipid catabolism pathways,

or PHA biosynthesis pathways.

Accessory vOTU genes may confer resistance to non-self viruses

In addition to AMG candidates, 32 vOTUs (8% of fumarole derived vOTUs) contain

virally encoded defense genes, which can potentially provide antiviral defense for the microbe

(increasing host fitness), or facilitate superinfection exclusion against other viruses. 78% of the

defense-containing vOTUs (n=25) were classified as Caudoviricetes, and the remaining

defense-containing vOTUs (n=7) could not be assigned to a realm classification. Queosine

biosynthesis genes (7-Deazaguanine modification) and (cytosine-5)-methyltransferase (dcm)

were initially flagged by DRAM-v as metabolic AMG candidates. However, they were manually

reclassified as defense-related because that is more likely reflective of functional role (Hutinet et

al., 2019; Wang et al., 2024).

Fumarole viruses contain a range of defense and anti-defense related genes: anti-

CRISPR, anti-Thoeris, and 7-Deazaguanine modification genes were annotated by DRAM-v.

Virally encoded toxin/antitoxin (TA) genes from Type I-IV systems were observed in ~5% of total

fumarole vOTUs (n=18) (Supplemental Material S8). For most of the vOTUs (n=10) containing

TA systems, both toxin and antitoxin genes of the same system type are positioned with no

more than five genes between components, suggesting that at least some fumarole viruses

encode functional TA systems. Two vOTUs contain CRISPR-associated proteins. One vOTU

(vOTU 189) does not have an identified host and encodes a minimal Type I-C CRISPR-Cas

Cascade system, which contains only three unique Cas proteins: Cas5d, Cas8c, and Cas7. This

minimal Cascade encodes an RNA-guided multi-subunit CRISPR-Cas surveillance complex

which targets foreign DNA for destruction (O'Brien et al., 2022).

A different vOTU (vOTU 212) contains a minimal Type IV system (genes Cas6, Csf3,

and Csf2) and an unassociated CRISPR array. Type IV systems are primarily encoded by

plasmids and more occasionally observed on prophages (Moya-Beltrán et al., 2021). Type IV

CRISPR systems are enigmatic in mechanism, and usually lack a CRISPR array. They are

hypothesized to modify existing CRISPR-Cas activity of hosts which already contain CRISPR

systems (Koonin & Makarova, 2017). Accordingly, vOTU 212 is targeted by spacers within a

Chloroflexota (class UBA6077) MAG which contains both Type IA and Type IE systems (J. H.

Saw et al., 2026). In addition to the Type IV effector proteins, vOTU 212 encodes a CRISPR

array. The maintenance of both an array and Type IV system (which characteristically, lack

associated CRISPR arrays) (Pinilla-Redondo et al., 2020) is suggestive of inter-viral competition

which has resulted in the use of CRISPR as a method for viruses to prevent superinfection

exclusion (Muscatt et al., 2022).

573 Discussion

Fumaroles contain novel and diversifying viruses

Our approach to viral identification that utilizes sensitive, profile-based methods

(Steinegger et al., 2019) for annotation of structural viral gene components reveals novel

viruses from fumarole biofilms and volcanic soil. Our approach identifies viruses using amino

acid sequences encoding viral structure components (capsid-associated and packaging genes)

which are highly divergent (Söding et al., 2005). Our recovery of phylogenetically novel viruses

from biofilm metagenomes and our subsequent careful manual verification supports our

workflow which utilizes remote homology protein prediction for identifying structural virion

components. This can, in turn, be used for identification of viruses in a high-throughput

processing pipeline with existing tools (Felipe Benites et al., 2024) without extensive false

positives (Supplemental Material S4).

Our approach yields recovery of highly novel viruses (Bolduc et al., 2025) which we find

to be sometimes highly abundant relative to other viral populations (Figure 01). This is predictive

of either functional significance for these novel and abundant viruses, or represents significant

population dormancy (Liang et al., 2019). Because viruses do not all share a single gene that is

common to all species (Harris & Hill, 2021), there is not a way to confidently place all viruses of

microbes on a single phylogenetic tree of life. Thus, it is not possible to phylogenetically

estimate viruses which cannot be classified into an existing realm (Koonin & Wolf, 2012). Our

assessment of viral diversification for these 'no-realm' viruses relies on gene-sharing network

analysis, which provides a method for comparison of genomic distance between viruses which

cannot be otherwise taxonomically placed. While uncertainty in phage genome completion

(vOTUs of variable length within a cluster) and chimeric or mosaic assemblies can constrain

comparisons of viral genome similarity, our utilization of NetworkAnalyzer metric of 'Closeness

Centrality' (Su et al., 2014) minimizes the effects of incomplete viruses producing erroneous

inferences of genomic distance (Bolduc et al., 2017). Our observation of subclusters for these

highly novel viruses, which are not placed into a realm by vConTACT3 and do not exhibit gene

sharing with previously described viruses, is evidence that there may be genomic diversification

between these novel viruses. Yet, conclusions about associated viral speciation for these highly

novel populations are limited by our inability for phylogenetic estimation without realm

classification.

The majority of recovered viruses are from the Caudoviricetes class. This could possibly

be a result of detection facilitated by high representation of Caudoviricetes genomes within

databases of known viruses (Weinheimer et al., 2025). As a result, our conclusions about

evolutionary patterns in other viral classes are limited by the low genomic diversity of recovered

fumarole vOTUs from the Laserviricetes, Tectiliviricetes, and Malgrandaviricetes classes. Even

though Caudoviricetes are the most characterized phage class, our identification of fumarole

viruses significantly expands the diversity of the class. Out of the 179 Caudoviricetes vOTUs

included in phylogenetic estimation, 86 fumarole vOTUs belong to two previously undescribed

order-level clades within the Caudoviricetes class (Supplemental Figure 02). The 93 other

fumarole Caudoviricetes vOTUs have varied levels of relation to previously described viral

families. Phylogenetic estimation exhibits that many of the fumarole-derived Caudoviricetes

vOTUs are related to families which are primarily incertae sedis within the Caudoviricetes class

such as *Hodgkinviridae*, *Peduoviridae*, *Zobellviridae*, and *Saffermanviridae* (Supplemental

Figure 03). This suggests that the inclusion of novel viruses may help resolve the phylogenetic

positions of problematic taxa within the Caudoviricetes class. Our observation of distinct

Caudoviricetes fumarole vOTUs which have close phylogenetic relationships (Figure 02)

suggests not only genomic diversification, but also speciation, could be occurring between

fumarole Caudoviricetes populations.

Abundant Microviridae may be a signature of geothermal environments

Microviridae are known to be diverse and globally distributed across a variety of

environments (sediment, marine, animal, insect, and humans) (Kirchberger et al., 2022;

Kirchberger & Ochman, 2023; Roux et al., 2012). Despite this, there has never been a

description of a terrestrial environment in which Microviridae is the most abundant viral

population, or otherwise represents a substantial portion of viral abundance in terrestrial or

marine (oceanic zone) environments. Benchmarking with mock communities representative of

open ocean marine and freshwater environments are consistent with observations that ssDNA

viruses make up a very small portion (<5% total DNA viruses) of total viral content, and are

much less abundant than dsDNA viruses (Kirchberger et al., 2022; Kirchberger & Ochman,

2020). The other environment in which Microviridae has been described as highly abundant is in

deep ocean hydrothermal vents sediments (Castelán-Sánchez et al., 2019; Cheng et al., 2022;

He et al., 2017; Y. Wu et al., 2024). It is possible that viruses belonging to Microviridae can be

adapted to thermal conditions as an ssDNA virus. Phage phiX-174 has been demonstrated to

be most stable at 37 - 44°C and is predicted to be favored over dsDNA and RNA phages under

thermal stress because of the increased access that ssDNA viruses have to mutational diversity

(Greenrod et al., 2025). Alternatively, it is possible that Microviridae has the capability to

circularize which would enable abundance at higher temperatures (Laanto et al., 2017).

However, the ERZ fumaroles with highest Microviridae abundances (50-70°C) are over three

times cooler than sediment of Southwest Indian Ocean hydrothermal vents (temperatures as

high as 400°C), where Microviridae has been identified as the most abundant virus (Cheng et

al., 2022).

Out of ERZ fumarole viral communities, moderately geothermal and high geothermal

biofilms are described as having the highest Microviridae abundances out of all samples.

However, mesothermal fumarole biofilms are observed to have high Microviridae abundance

(22-28%) which is higher than described in other terrestrial and marine environments (Figure

01). This may be explained by the high abundance of Microviridae in mesothermal fumaroles

experiencing rapid fluctuations in fumarole temperature. Mesothermal fumaroles which harbor

high Microviridae abundance may reflect a lag in decreasing abundance of ssDNA microvirus

abundance as the biofilm communities adapt to lower temperatures. Volcanic activity of the ERZ

varies widely across small spatial scales and fluctuates rapidly within short time spans (minute

to minute), making it possible that the steam pit mesothermal fumaroles are dying geothermal

features, while the ~100 m adjacent steam wall is more geothermally active. Many fumaroles

are ephemeral and experience more variation in activity as they near the end of their

geothermal life span (Madonia, 2020).

Viromics is known to overestimate the abundance of ssDNA viruses, and the perception

of high abundance is often a sequencing artifact. Many viromics extraction approaches may

utilize Adaptase prior to linker amplification and produce a quantitative bias, leading to

erroneous predictions of microvirus abundance (Roux et al., 2016). Alternatively, many viromics

DNA preparation methods for low biomass viromics may destroy Microviridae DNA (Gregory et

al., 2019; Y. Wu et al., 2024). Our approach utilizes bulk metagenomics prior to sequencing,

which may explain the abundance of microviruses we observe. We hypothesize that microvirus

diversity is underpredicted in geothermal environments, and temperature may be a selective

pressure which favors ssDNA viruses (Roux et al., 2016). Studies of other terrestrial geothermal

systems have not yielded similar observations of dominant Microviridae. However, some of

these other systems (such as terrestrial hot springs) are often explored in the context of low

biomass sediment that requires amplification or alternative methods of extraction which may

destroy microviruses (Gregory et al., 2019). Alternatively, these geothermal systems are

investigated with methods of viromics that destroy ssDNA viral representation in the sample

(Roux et al., 2012). Fumarole biofilms may not be the only terrestrial system whose viral

community contains dominant *Microviridae*, but the high fumarole biofilm biomass which is

utilized for DNA extraction may reveal this characteristic of thermal viral communities which is

otherwise obscured by methodological decisions which are made when investigating the

microbial and viral diversity of other terrestrial thermal systems.

Fumarole *Microviridae* are highly novel with pairwise genome similarities as low as 40-

60% (Figure 03) as well as syntenic rearrangement between viruses within the fumarole biofilms

and compared to reference *Microviridae* (Supplemental Figure 06). However, there is a need for

comparative genomic approaches when analyzing highly conserved ssDNA viruses to make

conclusions about the degree of novelty introduced with fumarole *Microviridae*. Network

analysis with vConTACT3 demonstrates multiple *Microviridae* form a single subcluster which is

unexpected because clusters usually represent genus level rank (Bolduc et al., 2017, 2025).

This suggests that environmental *Microviridae* are highly conserved and/or significantly under-

described.

Viruses are widely shared between geothermal features of ERZ fumaroles

There is evidence that viral dispersal has consequences for fumarole microbial

evolution. Antagonistic coevolutionary relationships between hosts and viruses are often

described through a 'Kill-the-Winner' dynamic, where both host and virus engaging in an active

arms race are abundant relative to other viral and microbial populations. Relatedly, viral

dispersal is thought to be host-dependent (Langwig et al., 2025) and associated with microbial

abundance (Salgado et al., 2025). Despite these expectations, we observe many of the shared

vOTUs are only found in low abundances within samples. For microbes that appear to utilize

CRISPR as a defense mechanism and have identified arrays, vOTUs were determined to be

part of a 'specialized' host-virus pair if there were more than three spacers linking a vOTU to a

single microbial host (indicative of antagonistic coevolution). The 14 vOTUs which have

specialized pairings with microbial hosts are not found in viral relative abundance over 1%

within any sample. This suggests that low abundance viruses and hosts are still antagonistically

coevolving and dispersal. Host-dependence and abundance are insufficient factors to predict

limited dispersal of viruses in fumaroles. However, it should be noted that many microbial taxa

do not rely on CRISPR for viral defense, and viral novelty constrains capability for host

identification of 90% of identified fumarole vOTUs (Tan et al., 2024). Yet, even when simply

searching for patterns of concurrent variation between abundances of unmatched vOTUs and

hosts, no high abundance concurrent variation that would suggest an expected 'Kill-the-Winner'

trend was observed (Supplemental Material S6).

Most vOTUs being shared between fumarole biofilms is highly unexpected. For hot

springs and hydrothermal vents, individual vents and pools are biological "islands" with distinct

communities between features (Castelán-Sánchez et al., 2019). Microbial communities of

Hawaiian fumarole vents exhibit distinct communities as well (Prescott et al., 2022). In hot

spring and hydrothermal vent systems, viruses display a distance decay effect with fewer

shared viral populations observed with increasing distance between features (Jarett et al.,

2020). The majority of viruses being shared between samples is even more unexpected when

the context of phototrophic biofilm matrixes is considered (Castelán-Sánchez et al., 2020; Jarett

et al., 2020). Compared to other mediums of geothermal systems (sediment or water),

geothermal biofilms with strict spatial stratification are expected to have even less dispersal,

with deterministic selection occurring on viruses within the microscale of centimeters within the

biofilm (Guajardo-Leiva et al., 2021a). This is particularly remarkable considering the context of

this comparison for fumarole biofilms to hot spring phototrophic biofilms with similar

biogeochemical cycling processes and organismal characteristics (Guajardo-Leiva et al., 2021b;

Salgado et al., 2025).

The high viral dispersal that is suggested by many shared vOTUs could be explained by

unique dispersal mechanisms of fumaroles that diverge from the methods of dispersal seen in

hot spring and hydrothermal vent systems. Steam (groundwater heated by magma) may be a

mechanism for viral dispersal (Wall et al., 2015). Steam may enable biofilms to act as

"extremophile collectors" which retain viruses within biofilms (Prescott et al., 2022; Wall et al.,

2015). This suggests Hawaiian fumaroles in the ERZ have unique ecological and complex

biogeographic characteristics with high viral dispersal informing these communities. By

elucidating the metabolic and phylogenetic diversity of fumarole viruses, we have identified ERZ

fumaroles on the Big Island of Hawai`i as a promising and unique early Earth analog

environment to investigate viral ecology.

Gloeobacter prophage may increase host fitness

The steam pit sample site is dominated by *Gloeobacter*, despite the close proximity and

presence of other oxygenic phototrophic Cyanobacteria (J. H. Saw et al., 2026) which are

faster-growing and photosynthetically efficient compared to *Gloeobacter* species which contain

reduced photosystems (Grettenberger, 2021). Two vOTUs (Supplemental Figure 08) identified

as *Gloeobacter kilaueensis* prophages by global alignment to *Gloeobacter* MAGs (J. H. Saw et

al., 2026; J. H. W. Saw et al., 2013) are highly abundant and co-vary with *G.kilaueensis* in

abundance for all but one of the biofilm samples dominated by *G.kilaueensis* (Figure 01). The

high abundance of both host and prophage suggests that these two vOTUs follow a "Piggyback-

the-Winner" pattern of viral ecology (Hu et al., 2025). Across all vOTUs, these are the only host

matched viruses which directly increase in abundance with corresponding host taxa

(Supplemental Material S6).

The only sample site which contains *Gloeobacter* dominated biofilms is the steam pit.

The steam wall sample site, located ~100 m from the pit, is dominated by other oxygenic

phototrophic cyanobacteria and is more exposed to light than the shaded steam pit.

Gloeobacter lacks a thylakoid membrane and is susceptible to light induced stress, resulting in

metabolic disruption and cellular damage from oxidative stress (J. H. W. Saw et al., 2013). Our

recovery of these vOTUs is the first description of viruses known to infect the *Gloeobacter*

genus, which is of astrobiological significance due to its evolutionary role as an ancient

cyanobacterium (Prescott et al., 2022; J. H. W. Saw et al., 2013). *Gloeobacter kilaueensis* is a

highly conserved species with MAGs sharing 99% ANI (J. H. Saw et al., 2026; J. H. W. Saw et

al., 2013). The presence of multiple vOTUs within steam pit biofilms suggests these prophages

introduce genetic variation to the *Gloeobacter* host and facilitate genome evolution of the

Gloeobacter kilaueensis species. CRISPR arrays in *Gloeobacter* MAGs contain a spacer match

to a single vOTU of the genus-rank cluster. The match to a single vOTU within the genus could

indicate reduced reliance on CRISPR as defense against the prophage or that the host no

longer benefits from defense against vOTUs as they diversify in close proximity without

geographic partitioning.

In addition to susceptibility to oxidative stress, *Gloeobacter*'s energy inefficiency makes

its high relative abundance in biofilms highly unexpected. All membrane-based energy

metabolism occurs within a single layer of the cytoplasmic membrane, restricting surface area

and metabolic rates. *Gloeobacter*'s reduced photosystems, light sensitivity, and inefficient

RuBisCO (slow carboxylation rate) also limit growth of the genus compared to other oxygenic

Cyanobacteria (Raven & Sánchez-Baracaldo, 2021). Energetically, it is possible that the

putative carbon utilization AMG polyhydroxybutyrate depolymerase (*phaZ*) confers host fitness

for *Gloeobacter*, contributing to its dominance in steam pit biofilms and optimizing host carbon

utilization. Polyhydroxybutyrate depolymerase is an intracellular enzyme which breaks down

polyhydroxyalkanoates (PHAs) or polyhydroxybutyrate (PHB) biopolymers. The ability to utilize

PHB biopolymers may provide a mobilizable reserve of carbon and energy to *Gloeobacter*,

enabling efficient resource management (granules storing nutrients without altering internal

osmotic pressure or loss of compounds through leakage) and increasing prophage-mediated

Gloeobacter survival over other competitors. The vOTU containing the *phaZ* AMG candidate is

doubled in abundance compared to the other *Gloeobacter* prophage vOTU, suggesting that

there is a comparative fitness benefit to the prophage maintaining the putative AMG.

There is evidence of the Gloeobacter prophage evading host defenses. The presence of

what appears to be a self-targeting spacer shows that CRISPR systems of the host may prevent

prophage activation or lytic activity (Supplemental Material S7). Alternatively, the prophage may

carry an anti-CRISPR protein that inhibits host CRISPR-Cas systems (*Gloeobacter* MAGs

contain both Type I and Type III CRISPR-Cas systems). The biofilm sample observed which

had both high abundance of *Gloeobacter* (J. H. Saw et al., 2026) and a low abundance of the

Gloeobacter-linked vOTUs was collected from a sunlight-exposed area, with a lower light

intensity than the steam wall sample site. High-light stress in exposed biofilms may compromise

Gloeobacter cells with metabolic disruption or cellular damage, destabilizing prophage

maintenance and reducing their relative abundance in the sample (Figure 01). Oxidative stress

could also trigger prophage excision and loss (Middelboe et al., 2025). The substantial decrease

of prophage content in biofilms despite the host remaining abundant suggests that light-induced

stress in Gloeobacter may limit lysogeny (consistent with "Piggyback-the-Winner" models)

influencing viral turnover and potentially altering persistence of lysogenic viral populations.

Active fumaroles host diverse viral communities.

Soil has been described as a medium that contains one of the most diverse and complex

viral communities with high richness and evenness suggesting significant levels of viral activity

(Roux & Emerson, 2022), In particular, young volcanic soil is known to harbor incredibly diverse

microbial and viral communities as a result of the soil's nutrient rich physicochemical properties

(Aguila-Torres et al., 2025; Huang et al., 2024; Mihai et al., 2023). Despite these previous

descriptions of high volcanic soil viral richness, we discover that the biofilms of active (thermal

and steam emitting) fumarole features contain communities with vOTU richness that is

significantly higher than vOTU richness of non-thermal volcanic soil from the Kilauea caldera

Big Ell cave (Figure 04). Further, some of the biofilms also contain high vOTU evenness that is

comparable to the evenness of volcanic soil. Because biofilms with high viral richness and

evenness do not contain a specific microbial phylogenetic profile, we suggest that the

environmental context of Hawaiian fumaroles as a geothermal system contributes significantly

to the overall viral diversity. There is not a clear distance decay effect or geographic partitioning

of diversity indexes, indicating that localized biogeographic context is significant in explaining

viral diversity. The structural properties of biofilms and microbial metabolism within fumarole

features (increased nutrient availability through basaltic bioleaching, metabolism of emitted

gases, and temperature) may all contribute to fumarole viral diversity.

The statistically significant relationship between temperature and viral richness has not

been described previously in a geothermal system other than hot springs (Castelán-Sánchez et

al., 2020; Sharp et al., 2014). The steady increase of viral diversity in fumaroles which peaks at

50°C and decreases in high geothermal biofilm samples emitting steam (>70°C) provides

compelling evidence that viral diversity in some geothermal systems can adhere to a

temperature-diversity gradient, and that biodiversity hotspots in geothermal environments are

are not limited to "Humboldt's spa" of hot springs (Sharp et al., 2014). It is known that

temperature influences structural biofilm properties, regulating processes such as viral particle

attachment and mineral precipitation (Simmons et al., 2020). Indeed, temperature dependent

biomineralization has been described within moderately geothermal ranges (Kostešić et al.,

2023). These structural alterations could potentially turn biofilms into physical refugia for virion

particles, increasing the density of virion particles with immobilization and in turn increasing

metagenomically identified viral diversity for species richness while potentially increasing

species evenness under specific physicochemical conditions which influence biofilm properties

(Decho & Gutierrez, 2017). The physical proximity of viruses within the biofilms exhibiting

thermal-induced structural properties could allow these viruses to act as "gene highways"

(Thomas et al., 2021) and facilitate microbial adaptation to the dynamic and harsh conditions of

a volcanic system (Supplemental Material S8). Even though moderately geothermal samples

have high viral richness and evenness and there is a unimodal relationship between

temperature and species richness, microbial diversity indexes of samples do not follow the

same clear trend (Figure 04).. The decoupling of fumarole virus and microbial diversity could

reflect temporal lag and community turnover of viruses, or dormant but inactive viral populations

retained at higher abundances than expected within biofilms.

If the viral richness and evenness within biofilms is representative of active viral

populations, this may facilitate increased opportunity for viral competition while increasing the

fitness conferred by superinfection exclusion (Muscatt et al., 2022; Pinilla-Redondo et al., 2020).

This would contribute to explaining diverse and frequent genomic observations of TA systems

(encoded in ~5% of fumarole vOTUs) and other defense related proteins in fumarole viruses

(Supplemental Material S8).

Accessory viral genes may aid adaptation to basaltic surface and resource limitation

Biofilms contain the majority of putative AMGs. Of identified fumarole vOTUs, only 7% of

fumarole vOTUs contain AMG candidates which is consistent with observations of AMGs as

rare in other geothermal environments such as hydrothermal vents (Langwig et al., 2025).

However, more samples with broader geographic distribution are necessary to make

conclusions about AMG prevalence in systems with high microbial endemism anticipated

(Castelán-Sánchez et al., 2019; Langwig et al., 2025; Salgado et al., 2025; Thomas et al.,

2021). The presence of diverse AMG candidates seen within vOTUs suggests that these

viruses may function as a genomic reservoir for microbial hosts and facilitate their persistence in

adverse and nutrient limited conditions (Supplemental Material S8). Basalt hosted microbial

communities in hydrothermal vents are known to engage in bioleaching with enzymatic

dissolution of substrate in hydrothermal vents, releasing metals in the process (Bergsten et al.,

2021; Thomas et al., 2021). The presence of ten different vOTUs with putative AMG involved in

heavy metal detoxification would suggest that viruses play a significant role in facilitating

microbial adaptations to high metal concentrations released from the dissolution of basalt

(Figure 05). This explains the presence of detoxifying metal efflux and ROS neutralization

putative AMGs, which are often seen in hydrothermal vents and heavy metal contaminated soil

where similar processes may occur(Cheng et al., 2022; Farias et al., 2015). While most of these

transporters are predicted to facilitate heavy metal resistance, some fumarole AMGs candidates

likely confer fitness for the host in other ways. MFS transporters may alternatively play a role in

conferring antibiotic resistance for the host, while biopolymer transport genes are also observed.

Other AMG candidates increase host fitness in resource limited conditions, primarily with

genes that participate in carbon utilization pathways. PHA/PHB metabolism in particular may

play an important role in microbial response to adverse conditions. Polyhydroxybutyrate

depolymerase is an AMG candidate in a *Gloeobacter* prophage vOTU which is highly abundant

in steam pit samples (Supplemental Material S8). Two other vOTUs contain carbon utilization

putative AMGs which could be involved in either beta oxidation of fatty acids or PHA

biosynthetic pathways (conversion of short-chain carboxylic fatty acids). In resource limited

conditions with nutrient depletion, PHA and PHB utilization could provide microbes the ability to

sequester and mobilize carbon and energy sources, aiding survival in dynamic and volcanism

dependent physicochemical conditions of fumaroles. Chitin degrading genes (chitinase and

chitosanase) are contained by three different fumarole vOTUs and are AMG candidates.

Chitinase has been described as an AMG in prophage encoded chitinase within hydrothermal

vents, where the AMG is involved in substrate dependent lysis-lysogeny switches of viruses. It

is known that carbohydrate degradation capacity is a driver of microbial community structure in

hadal zones, offering a possible explanation for how these putative AMGs could confer host

fitness in nutrient limited and dynamic environments such as fumarole biofilms (Middelboe et al.,

2025). Putative AMGs related to nutrient limitation suggest viral AMGs confer host fitness with

AMGs related to resource acquisition and scavenging (Monier et al., 2012; Tian et al., 2024;

Zheng et al., 2021).

Sporulation related AMG candidates may facilitate survival in adverse conditions via

dormancy, which has primarily been observed as an AMG in low oxygen zones, heavy metal

contaminated soil, and sub-freezing environments (Brown et al., 2022; Jurgensen et al., 2022;

Sun et al., 2023). These putative AMGs may help their host by facilitating microbial sporulation

when conditions are resource limited until nutrients are available for host reactivation. The

selection for microbial survivability under resource limitation may incentivize acquisition of

viruses with these AMG candidates. However, identification of viral metabolic genes is

constrained by sequence length (contigs under 10 kb). Biochemical characterization or

transcriptomic changes provide evidence for confident AMG identifications, and should be

performed for any structurally novel predicted AMGs. This is particularly true for CAZyme genes

which are poorly conserved on a structural level (Martin et al., 2025).

Methods

Sample collection and sequencing

Detailed methods on sample collection and sequencing have been described in two

previous studies (Prescott et al., 2022; J. H. Saw et al., 2026). Briefly, a total of 46 biofilm and

soil samples were collected in an expedition in 2019 from steam vent features found within

Kilauea Caldera inside Hawaii Volcanoes National Park and within East Rift Zone along Hawaii

Route 130. DNA from samples were extracted using ZymoBIOMICs mini prep kit, quantified with

Qubit HS dsDNA assay, then sent for sequencing at two sequencing centers: ASGPB at

University of Hawaii at Manoa for 16S amplicon sequencing and CQLS at Oregon State

University for whole-genome shotgun (WGS) sequencing using Illumina MiSeq and HiSeq,

respectively. Some of the amplicon data has been analyzed in a previous study that excluded

the cave soil samples from Big Ell (Prescott et al., 2022). BBTools script bbduk.sh (Bushnell,

2014) was used for quality control prior to assembly. Adapters were trimmed from the right ends

of the reads, with reads shorter than 50 bp discarded (--minlen=50). Adapters were trimmed

based on paired overlap (tbo=t) with low quality bases trimmed on both ends of reads (--

qtrim=rl) with low quality bases discarded (--trimq=20).

Identifying viral sequences

The ViralAssembly module of metaviralSPAdes v3.15.5 (Antipov et al., 2020) was used

for viral assembly and mismatch correction (k-mer lengths of 21, 33, 55, and 77). Assembled

contigs were input into ViralVerify v1.1.0 (Antipov et al., 2020) (Naive Bayesian classifier on

hmmsearch gene predictions), geNomad v1.11.2 (Camargo et al., 2024) (hybrid approach of

alignment-free models and gene models with neural networks for classification), and VirSorter2

v2.2.4 (Guo et al., 2021) (multi-classifier with HMM search for viral hallmark genes) for initial

identification. These tools employ a diversity of methods for viral identification (Antipov et al.,

2020; Bolduc et al., 2025; Camargo et al., 2024; Guo et al., 2021). Viruses were then identified

by identifying both a head and packaging gene in each candidate viral contig. Default

parameters were used for ViralVerify with the '-hmm' flag. geNomad was run with the '--end-to-

end' flag. As per community standards for VirSorter2, the default score of 0.5 (low-cutoff) was

used for maximum sensitivity and filtering. CheckV v1.0.3 (Nayfach et al., 2021) was then used

on extracted contigs (end_to_end option) to remove false positives and trim host regions of

proviruses prior to running VirSorter2 again on trimmed sequences (Guo et al., 2021; Tian et al.,

2024). Lastly for the VirSorter2 pipeline, a secondary screening approach was employed on

domain architecture DRAM-v annotations searched for capsid associated proteins (Phage-coat

PFAM clans CL0373 or CL0371). PFAM clans account for evolutionary relationships which

cannot be encompassed by a single HMM profile of DRAM-v (Finn et al., 2016; Kashif-Khan et

al., 2024).

Open reading frames (ORFs) were called in metagenomic ('-meta') mode with Prodigal

v2.6.3 (Hyatt et al., 2010). Gene calls were annotated with HHblits (Steinegger et al., 2019)

(uniprot_sprot_vir70 database) with flag '-n 3' iterations for remote homology prediction.

Annotations were filtered for E value (<1E-1) and probability (>50%). The first 10 hits for each

Coding DNA Sequence (CDS) were screened for head and packaging associated genes using

an automated python script which iterated over annotations by contig. A list of 33 gene ontology

(GO) terms identified as head or packaging genes was curated from the QuickGO display

(Binns et al., 2009). UniProtKB annotations which correspond to GO terms for virion assembly

and maturation were extracted and curated to exclude baseplate and tail gene products (The

UniProt Consortium, 2022). The resulting list of 332 UniProtKB codes were used for the Python

search script which flagged contigs containing both head and packaging associated gene

products which were then manually verified. All manual checks for gene functional prediction

were performed with a combination of BLAST(Camacho et al., 2009), UniProt BLAST, and

HHPred (Swissprot DB) (Söding et al., 2005).

Viral clustering

Viral sequences were also grouped into roughly genus level viral clusters (VCs) using

vConTACT3 v.3.0 with database (v220) domain restricted to "prokaryotes" (Bolduc et al., 2025).

Graph output (.cyjs) was imported into Cytoscape (v3.10.2) and the NetworkAnalyzer plugin

was used to evaluate the quality of clusters (Su et al., 2014). 'Clustering Coefficient' and

'Topological Coefficient' metrics were filtered to be equal to or greater than 0.85 (Tian et al.,

2024). Subclustering (virus cluster of three or more nodes without links to the rest of the

network) is observed for 20 VCs. Packaging and head related genes of viruses that were unable

to be taxonomically assigned into a realm by vConTACT3 v.3.0 were manually verified using

BLAST, UniProt BLAST, and remote homology prediction tool HHPred. Genes for major capsid

protein and TerL subunit were annotated (HHblits) and clustered (BLAST 2.14.1+) to assess

cluster success with viral sequences of variable length (variation > 1 kb).

Generating vOTUs

Viruses were clustered into species-rank vOTUs reflecting viral populations using

Minimum Information about an Uncultivated Virus Genome (MIUViG) standards with

recommended thresholds (95% ANI and 85% AF) for clustering parameters (Roux et al., 2019).

Contigs were first compared with nucleotide BLAST (v2.11.0+) and pairwise comparisons were

generated by blasting all sequences together ("-outfmt '6 std qlen slen' -max_target_seqs

10000"). Finally, clustering of viral sequences was carried out using scripts anicalc.py and

aniclust.py ("--min_ani 95 --min_tcov 85 --min_qcov 0") which are distributed as part of the

CheckV package (Nayfach et al., 2021).

Calculating vOTU diversity and abundance

Individual viral contigs were mapped using Bowtie2 (Langmead & Salzberg, 2012) with

the bowtie2-build function to create .sam files. The .sam files were converted to .bam files then

sorted, indexed, and counted (samtools idxstats) with SAMtools (Li et al., 2009). Relative

abundance was calculated with reads per kilobase per million mapped reads (RPKM) for each

viral contig and then summed for all contigs for each vOTU (Yan et al., 2025). vOTU richness

and evenness were calculated with the vegan package v2.7-2 (Oksanen, 2025) in R. Richness

was assessed for each sample by finding the Chao1 index using the sample vOTU relative

abundance table, which was calculated with the specnumber() function. Shannon diversity was

calculated for each sample with the diversity() function from the sample relative vOTU

abundance table. Pielou's evenness was calculated by dividing the Shannon index by the ln() of

Chao1. The relationship between the temperature of samples and vOTU diversity indexes was

assessed for significance with a non-parametric least squares regression, and then assessed

for overfitting with a Bayesian information criterion test with sample correction (BICc) due to

sample size (n=20). Linear, quadratic, and cubic models were attempted, and the lowest BIC

value indicated the best fit model. Normal distribution and constant variance of residuals was

supported with density and QQ-plots for both relationships (Supplemental Figure 07). Normality

for models of temperature and related diversity indexes was confirmed with the Shapiro-Wilk

test.

Phylogenetic classification of viruses

Phylogenetic estimation was guided by vConTACT3 v.3.0 (Bolduc et al., 2017, 2025).

Phylogenies were estimated using maximum likelihood independently for classes

Caudoviricetes, Malgrandaviricetes, Laserviricetes, and Tectiliviricetes. Caudoviricetes

references were chosen by selecting first neighbors ('first neighbors of select nodes' Cytoscape

v3.10.2) of vConTACT3 v.3.0 assigned fumarole Caudoviricetes (Shannon et al., 2003). For

Malgrandaviricetes, Laserviricetes, and Tectiliviricetes phylogenies, all known references were

selected from ICTV MSL40.v1 and ORFs were called with Prodigal v2.6.3 (Hyatt et al., 2010).

All references were annotated as described above with HHblits to identify marker genes. The

marker genes were TerL, major capsid, and packaging ATPase marker genes for the

Caudoviricetes, Malgrandaviricetes, Laserviricetes,Tecitiviricetes trees respectively with 1000

iterations of tree topology, branch lengths, and substitution model parameters (Grybchuk et al.,

2024; Katoh et al., 2002). Trimming of the multiple sequence alignment was performed with

trimAl v1.5.rev0 with a gap threshold of 30% (Capella-Gutiérrez et al., 2009). IQTree v2.4.0 was

used for phylogenetic reconstruction, with the evolutionary model Q.pfam+F+I+G4 selected as

the best model for *Caudoviricetes* (Nguyen et al., 2014). The model Q.pfam+G4 was used for

Malgrandaviricetes and *Laserviricetes* trees, and the Q.pfam+I+G4 model was used for

Tectiliviricetes. 1000 bootstrap estimates were used for branch support estimation. All

phylogenies were midpoint rooted and visualized with iTOL v7 (Letunic & Bork, 2006).

Host matching with CRISPR spacers

Putative host prediction was performed with CRISPR spacer matching. CRISPR-Cas

systems were identified from the binned nucleic acid sequences with <5% contamination using

the standalone tool of CRISPRCasFinder as a Singularity image (Couvin et al., 2018). High

confidence arrays were identified by using '-levelMin 3' parameter. Arrays adjacent to Cas

systems within 10,000 base pairs (bp) were identified with the '-ccc' parameter. Spacers were

extracted from high confidence arrays using minCED and clustered with a cutoff of 100%

identity using cd-hit-est (directionality assessed with parameter '-r 1') to dereplicate spacer

sequences (Bland et al., 2007; Li & Godzik, 2006). Targets with host matches were sorted into

three categories of mobile elements during the viral identification workflow: plasmid, viral, or

unknown mobile genetic element (MGEs). MGEs that did not have chromosomal, plasmid, or

viral indicators (structural genes) were characterized as other MGEs. Chromosomal fragments

were confidently excluded by scanning matches and eliminating targets with either entire arrays

or multiple shared spacers and neighboring sequences which were marked as 'suspicious'

matches (Supplemental Material S7). Viruses identified as viral and plasmid hits or uncertain

viral and plasmid sequences (ViralVerify, VirSorter2, and geNomad) were aligned with BLAST

(2.14.1+). Matches were filtered for ratio *nident* (identical bp matches) to *qlen* (spacer length) >

0.89 to be considered operationally valid.

Matches that could be plasmid-encoded arrays or chromosomal fragments were

eliminated as follows (Pinilla-Redondo et al., 2021): CRISPRDetect was used to identify arrays

in chromosomal arrays and target elements which were then aligned with BLAST (2.14.1+).

Identical matches between chromosomal and plasmid arrays were discarded immediately. If

there were multiple shared spacers and neighboring sequences (spacers and repeat element)

the hit was designated as suspicious (Supplemental Material S9). Target sequences were

searched for ORFs using Prodigal (v2.6.3) and .faa (amino acid sequence) and .sco (ORF

coordinates) output files were used in conjunction with BLAST (2.14.1+) match coordinates to

identify which ORF was targeted by spacers. Targeted ORFs (coding sequences (CDS) or non-

coding ORFs) were annotated using BLAST, UniProtBLAST, and HHPred.

Viral genome annotation (metabolic and accessory genes)

Three categories of genes were systematically recovered from vOTUs: auxiliary

metabolic genes (AMGs) that may have a role in host metabolism during the infection cycle,

viral resistance genes, and viral defense system related genes. Viral resistance and defense

genes are non-essential accessory genes (morons) which provide an adaptive advantage to

either the phage or host (Kang et al., 2024). Annotation for functional gene product prediction

was performed with DRAM-v v0.1.2 (Shaffer et al., 2020) on all viral sequences. Final vOTUs

were processed with VirSorter2 v1.0.1 using the -prep-for-dramv flag then annotated with

DRAM-v to identify viral metabolic genes. Gene curation was performed by filtering and

manually scanning both the DRAM-v AMG summary (275 gene predictions) and total annotation

output (20,666 genes) for AMGs and accessory genes. Gene product annotations were

discarded for genes that did not have an auxiliary score <4, or had DRAM-v auxiliary flags of

(V) viral structural component, (A) attachment, (P), viral specific MEROPS peptidase, or (F)

'near end of contig' (ter Horst et al., 2021; Tian et al., 2024). To eliminate the candidate genes

within host-like gene operons (possible miscalled proviral ends) all candidate genes were

scanned for flanking viral genes using DRAM-v flag (V) for structural virion components,

annotations such as portal, tail, baseplate, capsid, and structural or replicative genes with

VOGDB flags (Xs;Xr). This left 212 putative AMG candidates (Supplemental Material S8).

To be conservative in AMG prediction, secondary manual curation was performed on

putative AMG candidates to eliminate genes that could be involved in viral life cycle processes

(ter Horst et al., 2021; Trubl et al., 2018). This led to removal of putative AMGs involved in

nucleotide or amino acid metabolism (DRAM-v and KEGG-MAPPER pathways and modules).

Putative AMGs were also discarded if their annotation was overly general (genes annotated as

glycosyltransferases, peptidases, glycosylhydrolases, adenylyltransferases, methyltransferases,

and ribosomal proteins). Attempts were made to obtain a more specific gene prediction with

BLASTp before discarding (Martin et al., 2025; ter Horst et al., 2021). To predict accessory

genes, a secondary manual curation was performed by searching non-metabolic annotations

that did not have the (M) auxiliary flag in the DRAM-v master annotation spreadsheet output.

Participation in life cycle pathways was assessed using methods described above, and resulting

accessory gene candidates were manually inspected.

Prophage annotation and boundaries were performed by aligning viral contigs to MAGs with

BLAST+ alignment (2.14.1+). Prophage boundaries were identified in viral contigs PHASTEST

and manually checked for flanking viral genes on viral contigs using annotation methods

described in the workflow for viral identification (Wishart et al., 2023). ORFs were called with

Prodigal (v2.6.3) and annotated with Pharokka (v1.7.5) identifying phage orthologous groups

(PHROGs) with local alignment (Bouras et al., 2022). Gene diagrams (genomic context

determined with MMSeq2 and pyHMMer) were plotted using LoVis4u (Egorov & Atkinson, 2025;

Steinegger & Söding, 2017). Proteomic similarity matrix for *Microviridae* viruses was built with

MMSeq2 alignments through LoVis4u.

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Data availability

1065   All raw data used in this study have been deposited in the European Nucleotide Archive (ENA)

1066   under project accession number PRJEB52128. Phylogenetic IQ-Trees, viral candidate contigs,

1067   and final viral contigs are available on Zenodo at DOI: https://doi.org/10.5281/zenodo.19378070

Code availability

1068   Scripts to run analyses and viral identification pipeline custom scripts are available at
1069   https://github.com/pia-sen/Hawaii_fumarole_viruses

Author contributions

1070   J.H.S. designed and led the sampling and sequencing. P.S. and J.H.S. determined the scope of

1071   analysis for this project. P.S, L.O, C.P. and M.S. performed data analyses, K.S.M. and Y.I.W.

1072   supervised and guided development of the viral identification pipeline, P.S. L.O. and J.H.S wrote

1073   the manuscript, and all authors reviewed and approved the final version of this paper.

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Figure 01. **A)** Gene-sharing network of confidently identified fumarole viral contigs visualized in Cytoscape. Nodes represent full viral sequences. Grey nodes are RefSeq viruses from the vConTACT3 v220 database, while colored nodes are fumarole viruses, grouped by vConTACT3 taxonomic predictions. Categories indicate the lowest confident taxonomic level assigned to each cluster; highly abundant clusters are colored independently of taxonomy. Edges represent the strength of gene-sharing between viruses, determined with protein clusters. All shown clusters have high clustering coefficients (>0.85), as calculated using the Cytoscape NetworkAnalyzer plugin. **B)** Relative abundance of identified viruses in all 20 samples, colored by cluster classification. Relative abundance (RPKM) was calculated for each individual viral contig and grouped by the taxonomic designation of clusters for visualization. Clusters found in notable abundance were colored and denoted independently, regardless of predicted taxonomy. Relative abundance of vOTUs used for statistical analysis is available as Supplemental Material S6.

Figure 02. Maximum likelihood phylogenetic tree of Caudoviricetes terminase large subunit (TerL) amino acid gene sequences. Red node labels correspond to TerL sequences annotated in fumarole viral contigs. Black node labels correspond to RefSeq taxon which were determined to be first neighbors of fumarole viruses with Cytoscape plugin NetworkAnalyzer. Outmost ring refers to the temperature range of the sample from which the virus was metagenomically assembled. The middle ring refers to the sample site from which the corresponding sample was taken. All annotations of TerL in RefSeq and fumarole viruses were made with HHblits using parameters described in Methods.

Figure 03. A) Subcluster of viral genomes within the family Microviridae. Network constructed using vConTACT3 and visualized in Cytoscape. Reference nodes are colored by genus (RefSeq and vConTACT3 predictions), while fumarole viruses are colored by sample site. Edges represent gene-sharing strength. The blue node (phiX174) forms a weakly resolved clade with fumarole viruses in the maximum-likelihood phylogeny based on major capsid protein amino acid sequences. **B)** Pairwise genome comparisons of RefSeq and fumarole Microviridae using local alignment (MMseqs2). The top row and left column indicate predicted genus assignments (vConTACT3) for fumarole and RefSeq viruses. The top-left quadrant shows RefSeq–RefSeq comparisons; the top-right shows fumarole–RefSeq comparisons (RefSeq colored by genus). The second row (genome source) is colored by fumarole sample site. The star denotes comparisons involving the phiX174 RefSeq genome.

Alpha Diversity of Fumarole Viruses

Figure 04. A) Richness (Chao1) and evenness (Pielou) of fumarole vOTUs calculated from RPKM-based relative abundances (Supplemental Material S6). Each point represents a sample. Shape indicates sample site, and color indicates temperature at time of collection. **B)** Richness and evenness of microbial ASV relative abundances. Each point represents a sample; color and shape denote sample characteristics as described in (A). **C)** Relative abundance of the most abundant bacterial taxa across samples based on ASVs. The top five phyla per sample are shown and used for visualization. The second row indicates sample site, and the bottom row indicates temperature at collection. The phylogram shows spatial relationships among sites: the steam pit and steam wall are ~100 m apart, while the Kīlauea caldera cave is ~35 km from both. **D)** Sample temperature versus vOTU richness (Chao1). Each point represents a sample, colored by site. vOTU richness follows a temperature-dependent pattern modeled by a polynomial (quadratic) regression. Richness increases with temperature up to ~50°C and declines thereafter.

Figure 05. A) Counts of viral accessory genes in individual viral genomes. Columns represent viral metabolic gene counts per contig. The top row indicates vConTACT3 viral cluster (VC) assignments, with spacing grouping viruses within each VC; singletons are shown as individual VCs. Heatmap colors denote gene counts as shown in the legend. **B)** Gene diagrams of viral metabolic gene distribution across five recovered genomes from vOTU 158, enriched in putative AMGs. Genomes were inspected for flanking viral genes and AMG annotation coverage/identity. Arrows denote genes, and colors indicate AMG categories. The full curated AMG list is provided in Supplemental Material S8.

Supplemental Figures

Supplemental Figure 01. Upset plot of vOTUs identified by each tool. Bars (with counts above) show the number of vOTUs shared across tool combinations indicated by connected, filled points below. The left panel shows total vOTUs identified per tool.

Order-level Caudoviricetes Clades

Supplemental Figure 02. Maximum likelihood tree of Caudoviricetes (TerL) amino acid sequences showing two previously undescribed order-level clades. Clade 1 (Kilaueavirales) is highlighted in green, with green taxon labels indicating fumarole-derived viruses and black taxon labels representing RefSeq viruses (first neighbors). Clade 2 (Pahoavirales) is highlighted in red, with red taxon labels indicating fumarole-derived viruses and black taxon labels representing RefSeq viruses. Node support values are shown and available in the IQ-TREE files on Zenodo.

Supplemental Figure 03. Maximum likelihood tree of Caudoviricetes (TerL) amino acid sequences showing relationships between fumarole viruses and previously described viral families. Node label colors (outer ring) indicate viral family classification (many lacking order-level assignment); grey denotes unassigned viruses, and red denotes steam vent virus TerL. Clade 1 (Kilaueavirales) groups with RefSeq genomes from two families lacking prior order assignment—Casjensviridae and Herelleviridae—and includes 30 fumarole vOTUs and 17 unassigned RefSeq viruses. Clade 2 (Pahoavirales) includes 56 fumarole vOTUs and places Mesyanzhinovviridae as more recently diverged relative to some fumarole viruses. Additional fumarole viruses show close phylogenetic similarity to incertae sedis families, including Peduoviridae, Zobellviridae, and Saffermanviridae.

Supplemental Figure 04. **A)** Maximum likelihood class-level tree of Tectiviricetes packaging ATPase amino acid sequences (annotated with HHblits). Red labels indicate fumarole-derived viruses, while Tectiviricetes RefSeq viruses are highlighted with genus-specific colors as described in the figure legend. Alphatectivirus is the only genus within Tectiviricetes containing plasmid-dependent phages, and all five genera are included as references. **B)** Maximum likelihood class-level tree of Laserviricetes packaging ATPase amino acid sequences (annotated with HHblits). RefSeq viruses are colored by genus as described in the figure legend, and the red label denotes a fumarole-derived virus closely related to Hukuchivirus, a genus of thermophilic Thermus phages.

Supplemental Figure 05. Maximum likelihood class-level tree of Malgrandaviricetes major capsid protein (MCP) amino acid sequences. RefSeq viruses from a shared cluster in the gene-sharing network (whole genomes) were used as references for phylogenetic estimation. Red labels indicate fumarole viruses. The outer ring denotes the temperature range of the source sample, and the middle ring indicates the corresponding sampling site. Fumarole viruses form a poorly resolved clade with phiX174.

Supplemental Figure 06. Whole-genome comparative map of Microviridae viruses within the Bullavirinae (phiX-like) subfamily. Genome names are shown to the left below each gene map, with corresponding NCBI accessions when available. Genes are depicted as blocks, with shared colors indicating conserved proteins. Conserved and variable genes are identified using a pairwise proteome composition distance matrix (LoVis4u), and syntenic rearrangements are indicated by linkages between genomes. The bar beneath each genome shows functional annotations from LoVis4u based on local alignments (MMSeq2). Compared to RefSeq Bullavirinae (phiX-like viruses), fumarole Microviridae display greater syntenic variation and distinct genomic differences both from RefSeq genomes and among themselves.

Temperature and vOTU Diversity
(Chao1 Richness/ Pielou Evenness)

Supplemental Figure 07. A) Diagnostic plots for the non-linear regression of temperature and vOTU richness (Chao1), including density, QQ, and variance plots of residuals. Residuals show a bimodal distribution in the density and QQ-plots, with constant variance. This supports the use of ordinary least squares (OLS) regression to assess the relationship between temperature and vOTU richness. B) Diagnostic plots for the non-linear regression of temperature and vOTU evenness (Pielou), including density, QQ, and variance plots of residuals. Residuals appear normally distributed with heteroscedasticity, indicating non-constant variance; thus, least squares regression is not sufficient to model the relationship between temperature and vOTU evenness.

Supplemental Figure 08. Comparative genomic map of three fumarole vOTUs within a vConTACT3 genus-level cluster. These vOTUs were detected in high abundance only at the steam pit site; two vOTUs show CRISPR-spacer matches to Gloeobacter kilaueensis, representing the first report of a virus infecting this genus. Genome names are shown to the left below each map, with NCBI accessions where available. Genes are depicted as blocks with shared colors indicating conserved proteins. Conserved and variable genes were identified using a pairwise proteome composition distance matrix (LoVis4u), with syntenic rearrangements indicated by linkages. The bar beneath each genome shows functional annotations from LoVis4u based on local alignments (MMSeq2).