

Supplementary Information: DNA viruses are constrained to ecological niches and share similar environmental adaptations with hosts

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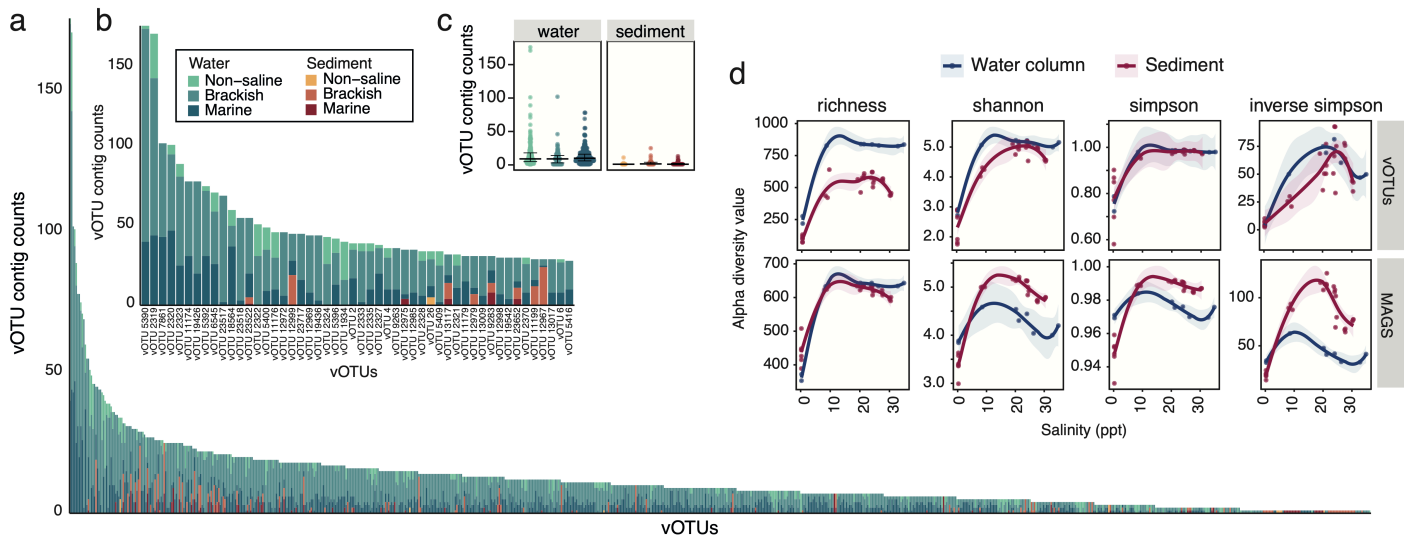
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Short title: DNA virus niche partitioning and adaptation

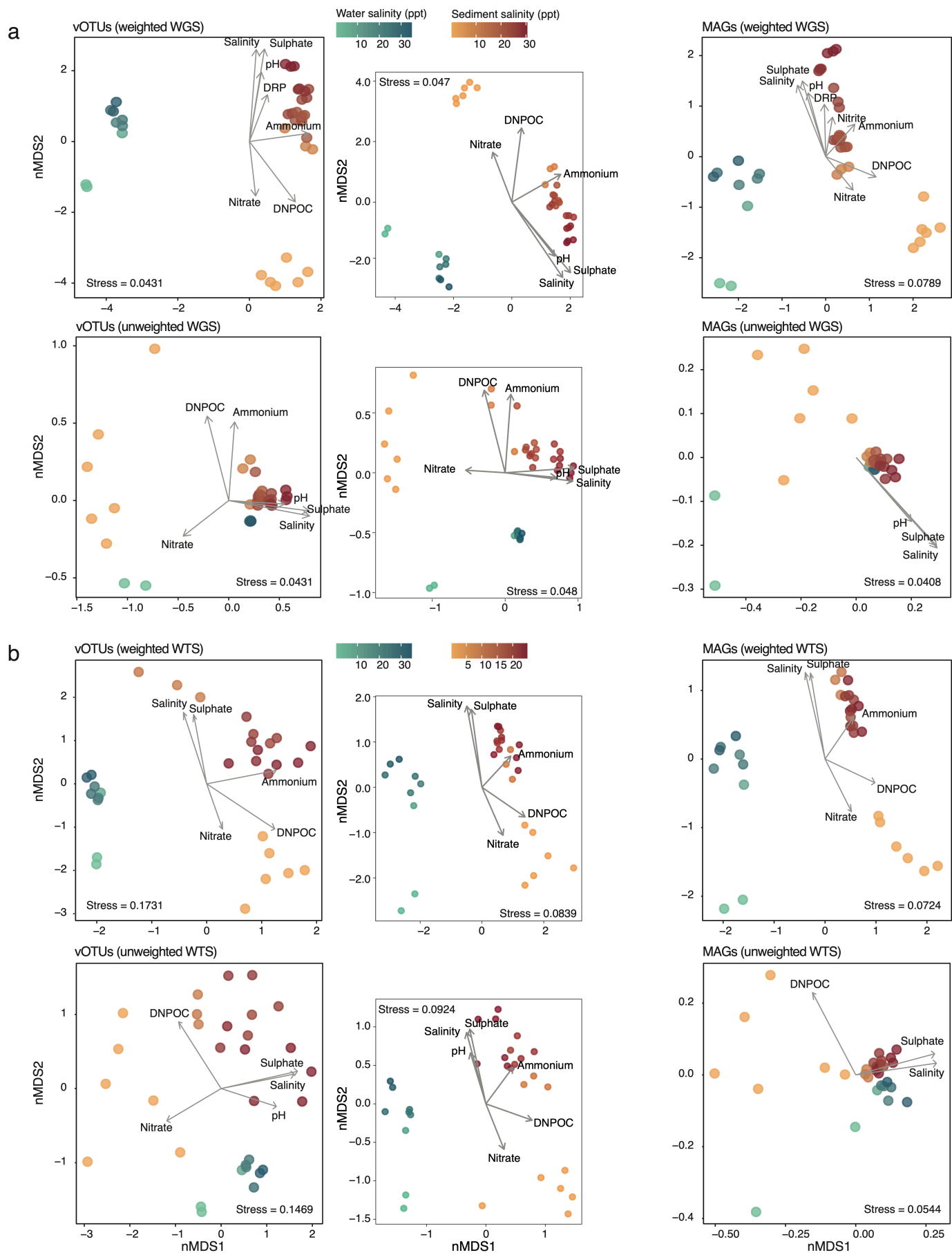
Supplementary Information

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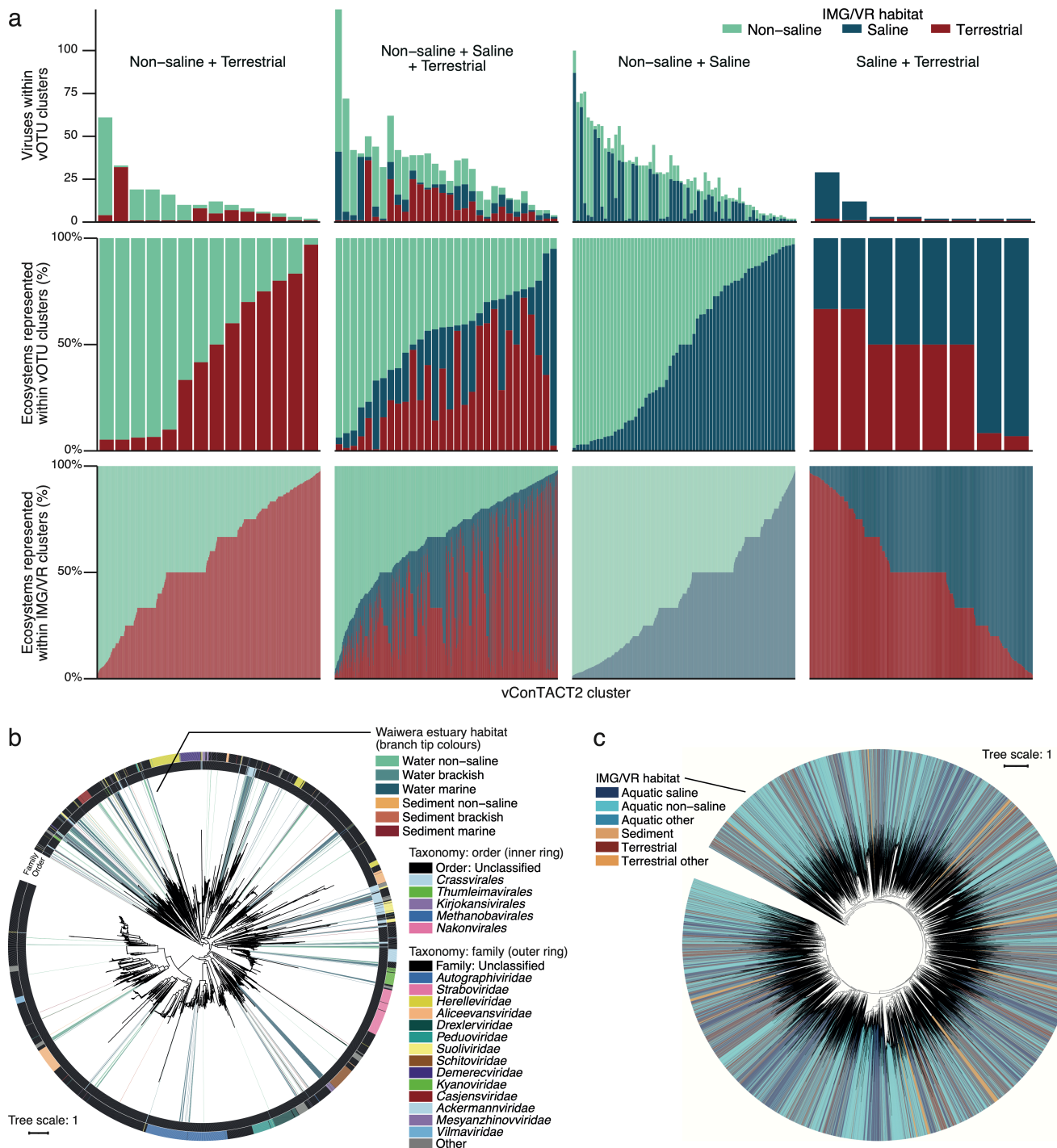
1. SUPPLEMENTARY FIGURES 1-13



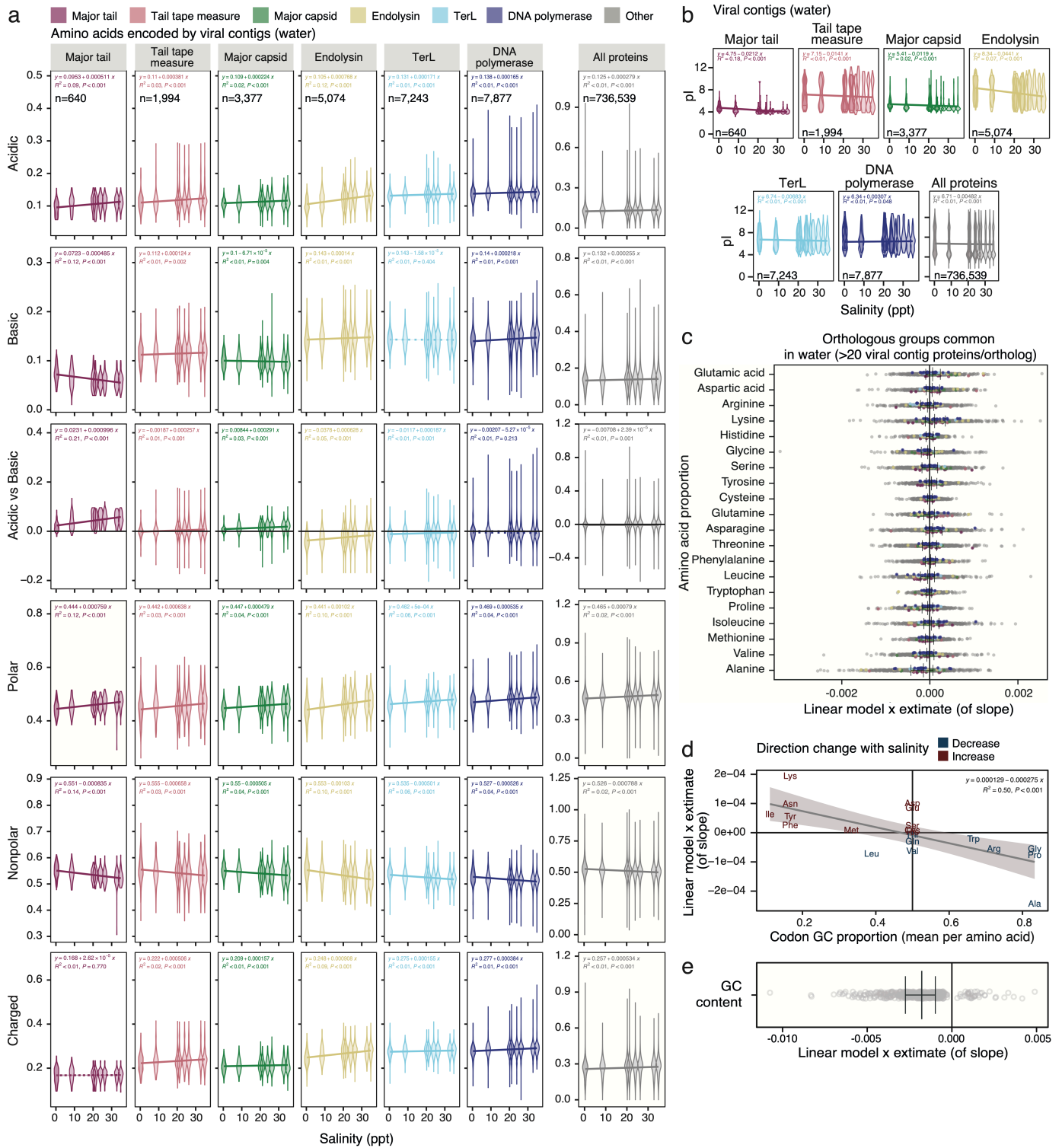
Supplementary Figure 1. Rank abundance plots of within-vOTU microdiversity for (a) all vOTUs and (b, inset) the 50 most diverse vOTUs sampled from the Waiwera estuary. Bars are coloured by the estuary zone that contigs within vOTUs were derived from. (c, inset) Beeswarm plots of within-vOTU microdiversity, with vOTUs categorised by the estuary zone in which they were most abundant. (d) Dot plots of alpha diversity indices (richness, Shannon (base = natural logarithm), Simpson, and inverse Simpson) against sample salinity for vOTUs (top row; $n=858$) and prokaryote MAGs (bottom row; $n=646$). Lines (with 95% confidence interval shaded) calculated via loess local smoothing.



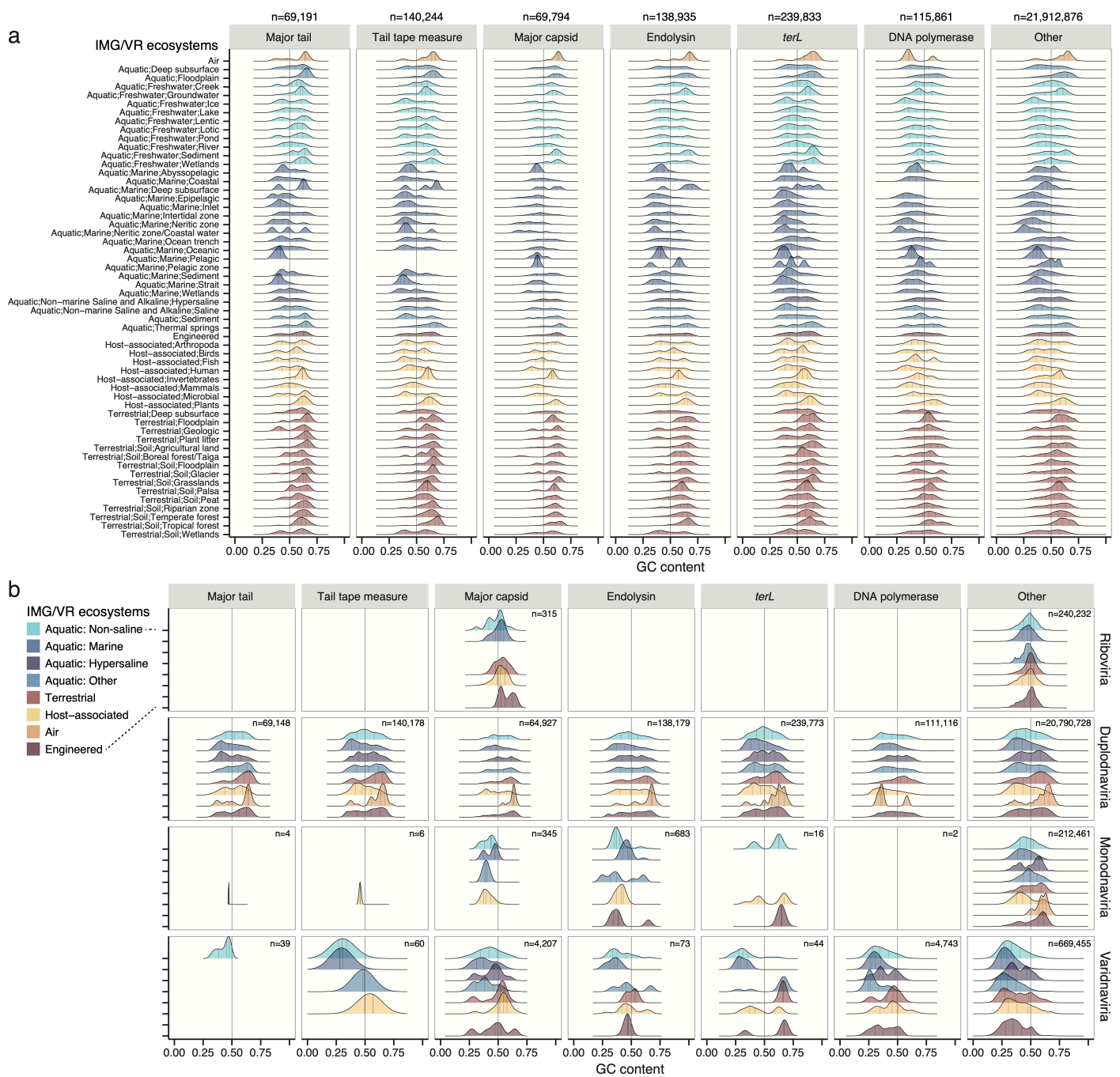
Supplementary Figure 2. vOTU and prokaryote MAG distributions throughout the Waiwera estuary. Non-metric Multidimensional Scaling (nMDS) ordinations comparing the composition of vOTUs (>50% complete, left column [$n=858$]; all vOTUs, centre column [$n=31,711$]) and MAGs (rightmost plots, $n=646$) per sample. The ordinations are based on weighted and unweighted Bray-Curtis dissimilarities using (a) whole genome sequencing data (genome relative abundance) and (b) transcriptome sequencing data. Arrows represent fitting of environmental parameters.



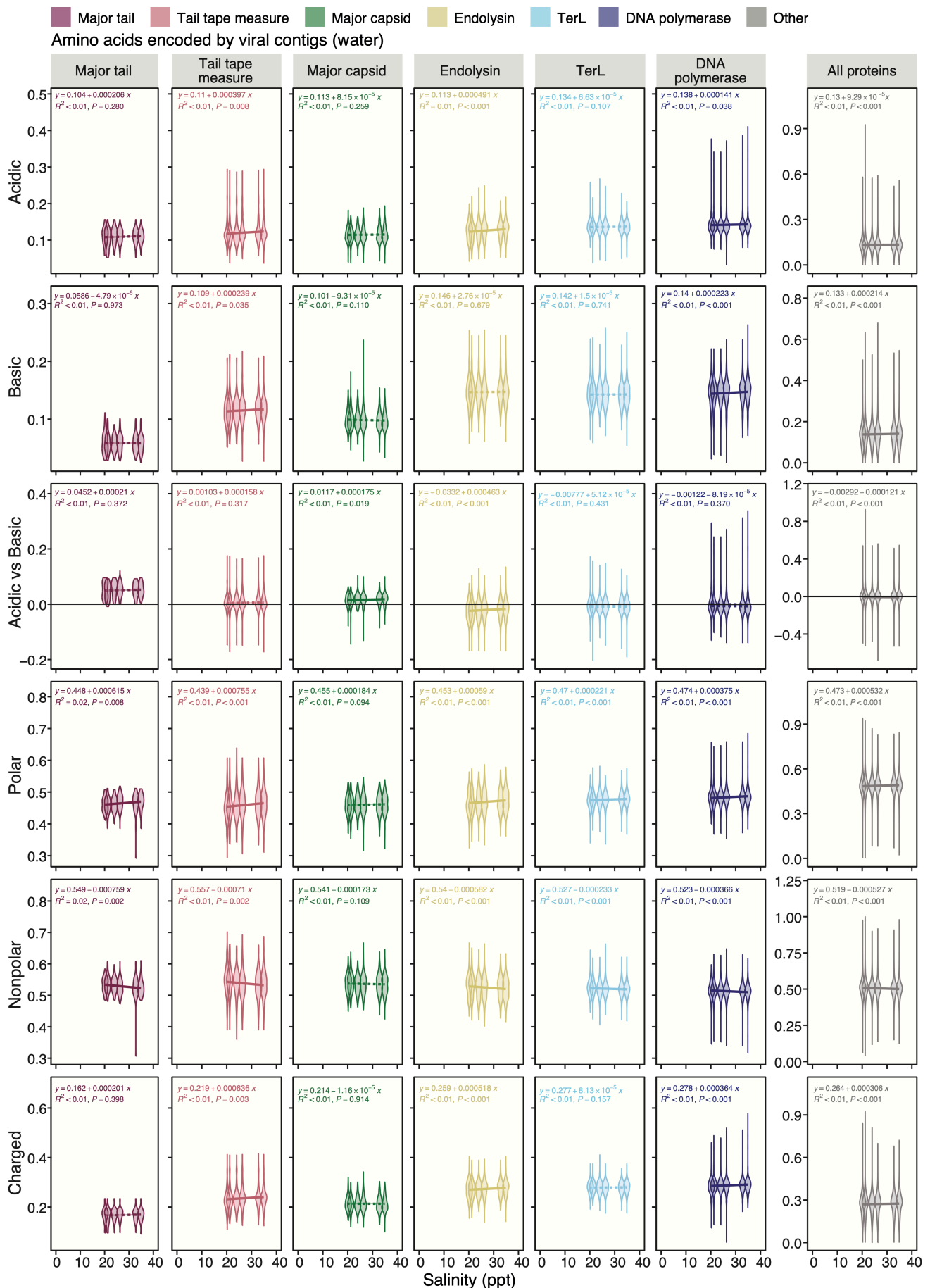
Supplementary Figure 3. Viral clusters and phylogeny by habitat. (a) Clusters generated by vConTACT2 containing viruses from more than one ecosystem type (non-saline, saline, or terrestrial). Counts (top row) and percentages (middle and bottom rows) of viruses recovered from these ecosystems. Clusters contain viruses from IMG/VR (all rows), and at least one Waiwera vOTU (top and middle rows). Cluster counts, percentage of total clusters (parentheses), and virus sequence counts per group: Non-saline+Terrestrial clusters=14 (5.0%), sequences=214; Non-saline+Saline+Terrestrial clusters=30 (10.7%), sequences=1,001; Non-saline+Saline clusters=65 (23.2%), sequences=1,895; Saline+Terrestrial clusters=8 (2.9%), sequences=55. All mixed clusters (including Waiwera vOTUs and high-quality IMG/VR sequences) are presented in the bottom row. Cluster counts (and percentage of total clusters) and virus sequence counts per group: Non-saline+Terrestrial clusters=1,379 (5.6%), sequences=14,945; Non-saline+Saline+Terrestrial clusters=713 (2.9%), sequences=13,759; Non-saline+Saline clusters=1,746 (7.1%), sequences=24,692; Saline+Terrestrial clusters=504 (2.0%), sequences=4,834. (b) Inferred *Caudoviricetes* phylogeny for vOTUs (this study) and viralRefSeq references based on TerL protein alignment. Lines extending from branch tips for Waiwera vOTUs are coloured by estuary zone. Estuary sample type represents the estuary zone where the vOTU was most abundant. The outer ring is coloured by predicted vOTU and RefSeq taxonomy. (c) Inferred *Caudoviricetes* phylogeny for high-quality environmental viruses from the IMG/VR database based on concatenated alignment of predicted core genes. Putative core genes were identified using high-quality IMG/VR *Caudoviricetes* viruses as the initial reference set. The number of concatenated proteins used was 13. Lines extending from branch tips are coloured by habitat. (b,c) Trees were generated via IQ-TREE using ModelFinder (with VT+F+I+G4 selected). The scale bars represent amino acid substitutions per site.



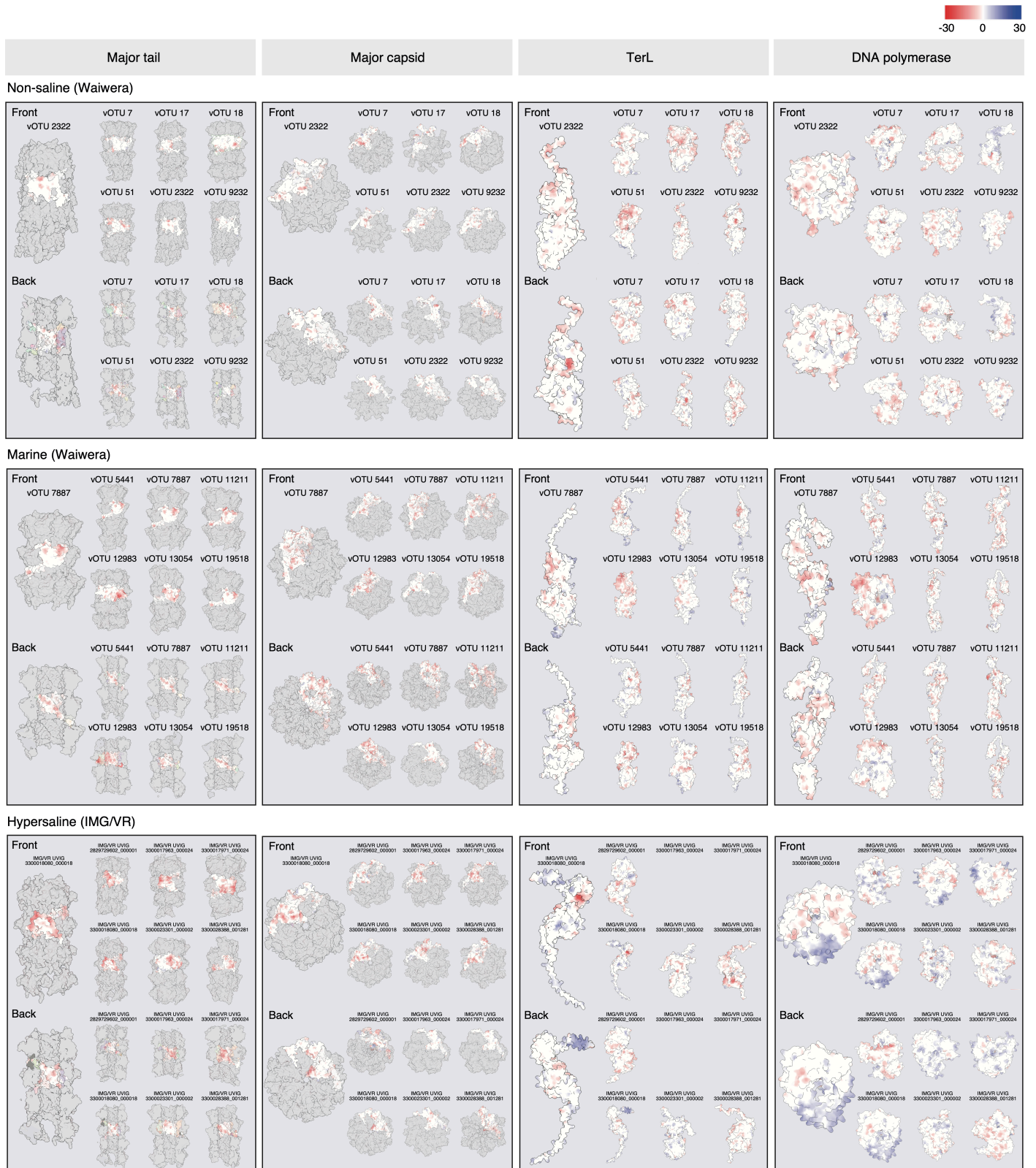
Supplementary Figure 4. Molecular signatures of environmental adaptation in DNA viruses from the Waiwera estuary. (a) Proportions of acidic, basic, polar, nonpolar, and charged amino acids, and the difference between the proportion of acidic-basic amino acids, in predicted viral contig protein subsets and all combined proteins in the estuary water column. Linear modelling equation and statistics were calculated via the *stat_poly_line()* and *stat_poly_eq()* functions from the *ggpmisc* package in R. (b) Predicted protein isoelectric points against salinity for predicted viral contig protein subsets and all combined proteins in the estuary water column. (c) x-estimates from linear models showing the trend of changes in individual amino acid proportions with respect to increases in salinity across the estuary in water, with bars representing median and interquartile range. Plots represent orthologous groups per protein type with a minimum of 20 sequences/ortholog. Numbers of orthologous groups (and sequences) per protein type: major tail = 7 (364); tail tape measure = 4 (201); major capsid = 10 (3,272); endolysin = 9 (2,131); TerL = 7 (2,874); DNA polymerase = 17 (4,958); and total = 1,230 (184,695). (d) Mean GC content of amino acid codons against x-estimates from linear models showing the trend of changes in amino acid proportions with respect to increases in salinity across the estuary in water. (e) x-estimates from linear models showing the trend of changes in viral contig gene GC content for individual orthologous groups (>20 sequences/ortholog) with respect to increases in salinity across the estuary in water, with bars representing median and interquartile range. VOGs with *p*-values <0.05 in individual linear models are shown.



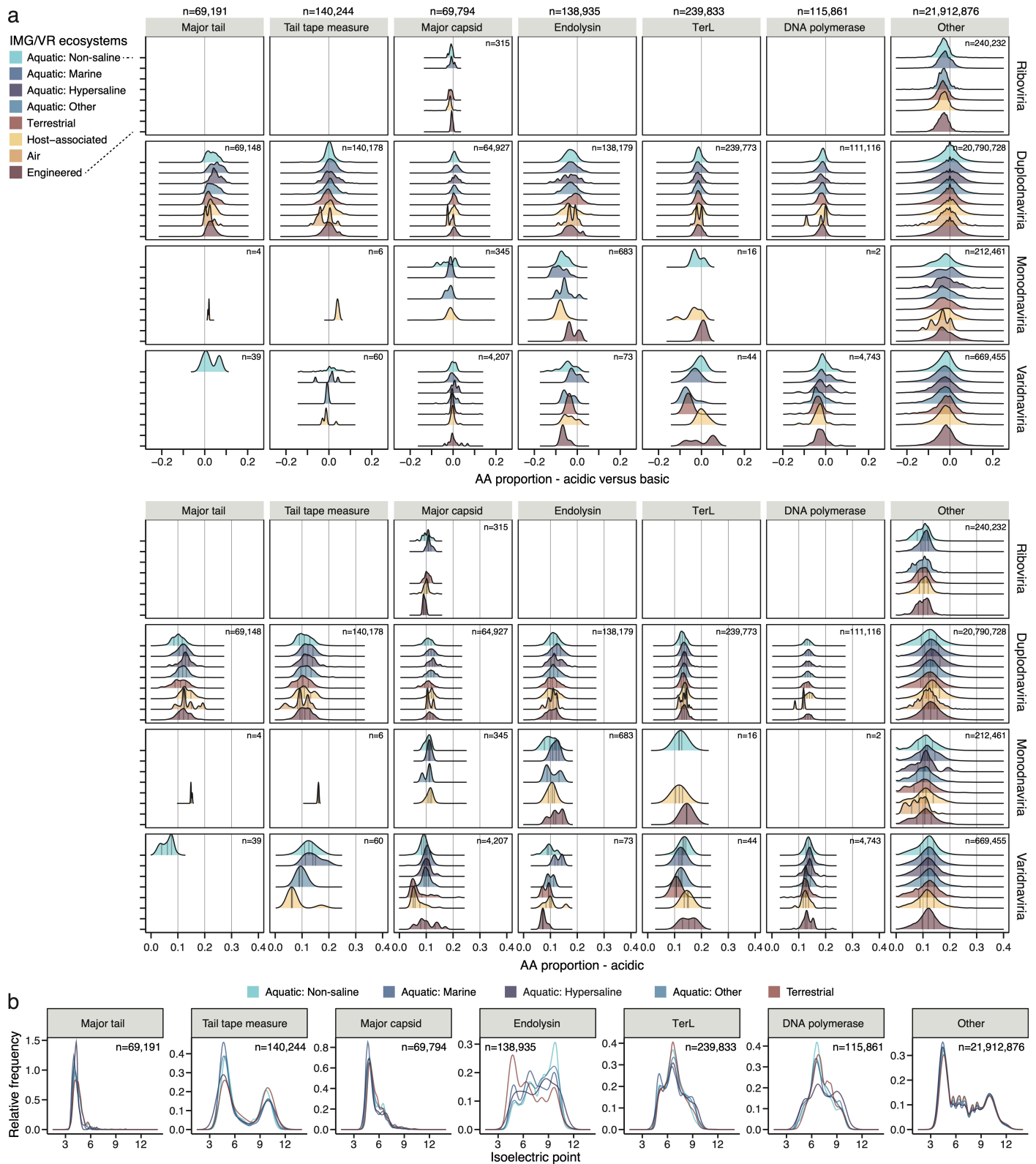
Supplementary Figure 5. Molecular signatures of environmental adaptation in DNA viruses from the IMG/VR database. (a) GC content per functional category per ecosystem type for high-quality IMG/VR viruses. (b) GC content per ecosystem type (summarised into broad ecosystem categories) split by functional category and viral realm for high-quality IMG/VR viruses.



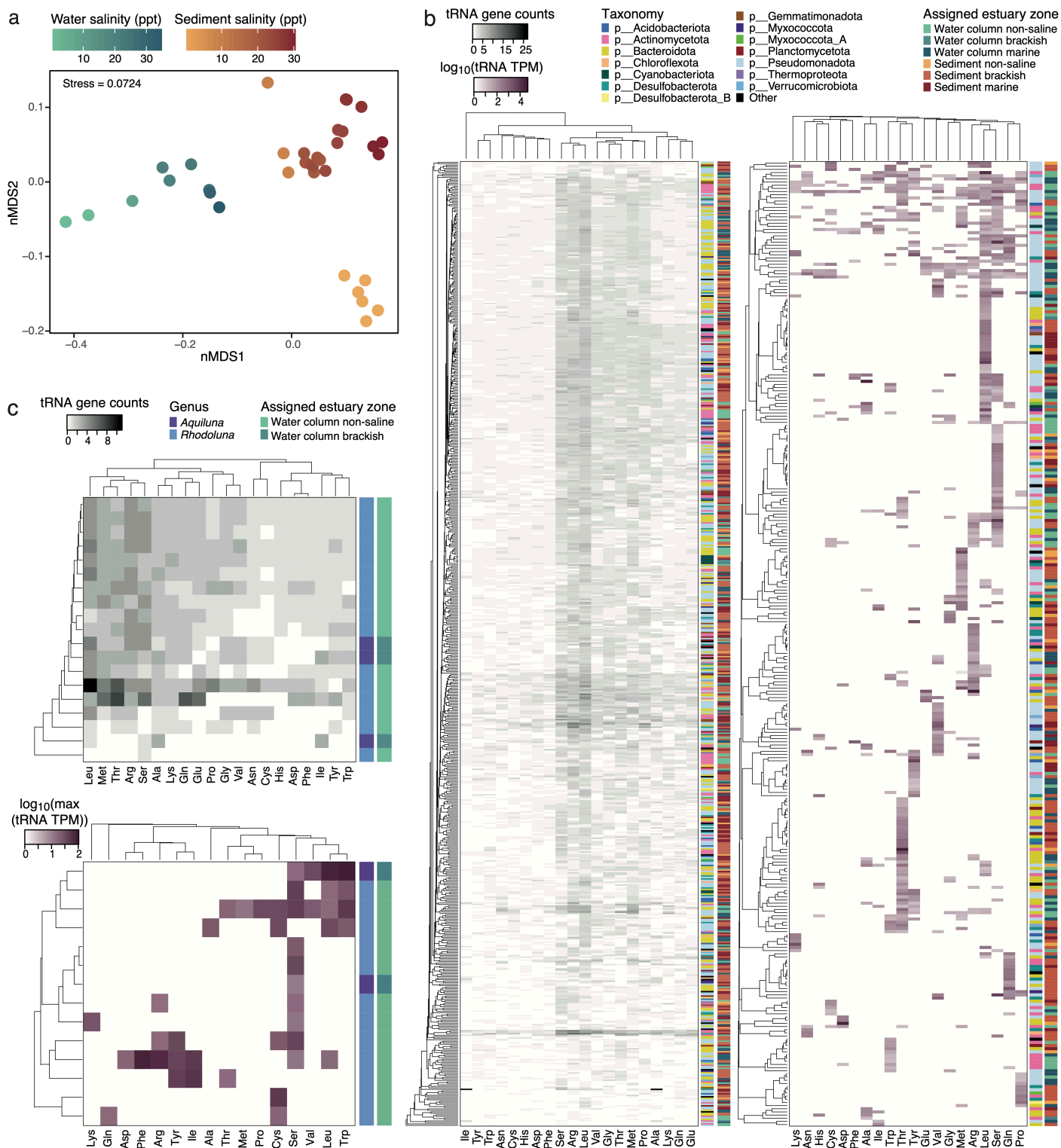
Supplementary Figure 6. Amino acid trends in DNA viruses from only saline parts of the Waiwera estuary. Proportions of acidic, basic, polar, nonpolar, and charged amino acids, and the difference between the proportion of acidic-basic amino acids, in predicted viral contig protein subsets and all combined proteins in the estuary water column. Linear modelling equation and statistics were calculated via the *stat_poly_line()* and *stat_poly_eq()* functions from the *ggpmisc* package in R.



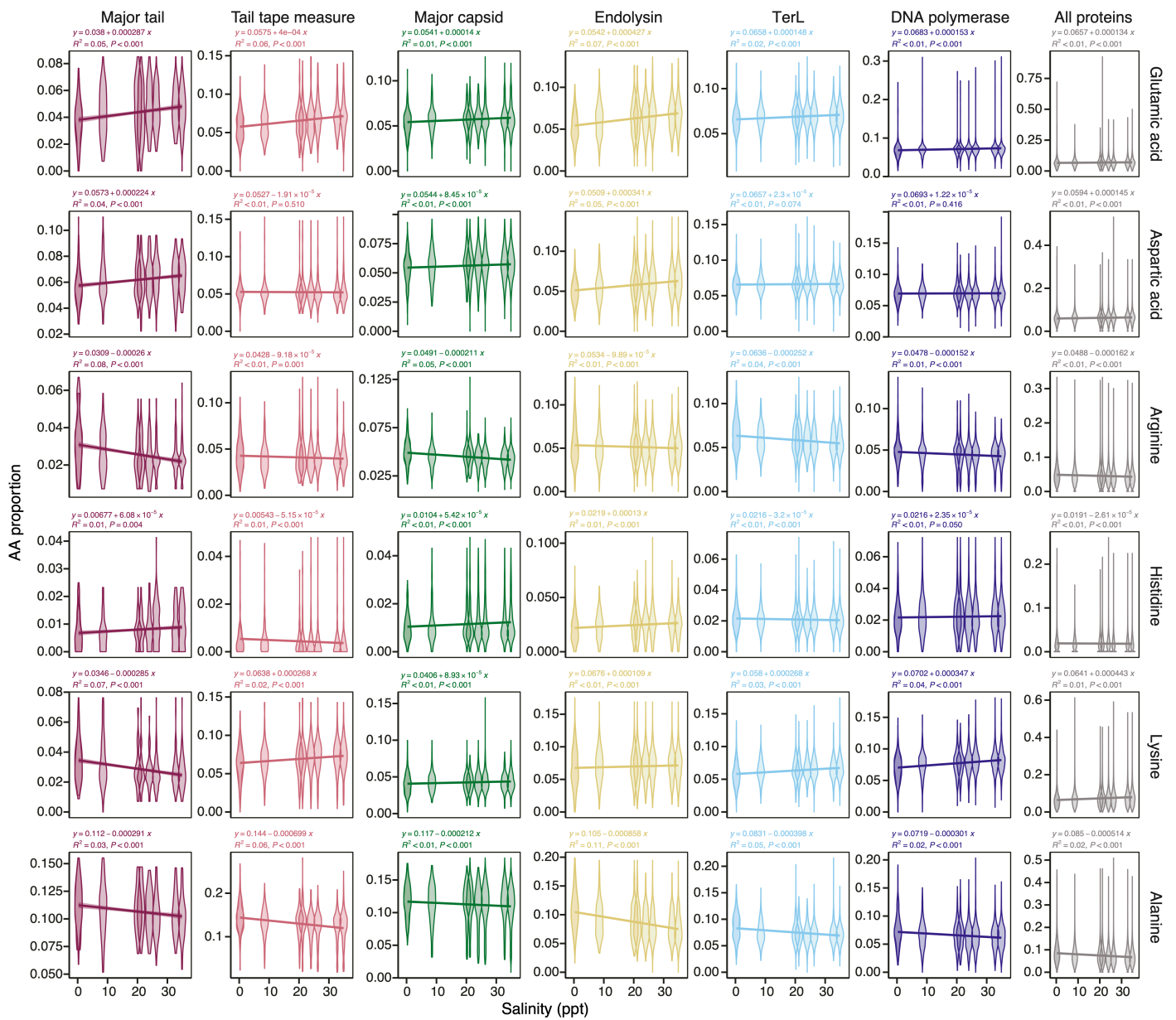
Supplementary Figure 7. Front and back views of three-dimensional structural predictions of viral protein complexes (major tail and major capsid) and individual proteins (large terminase and DNA polymerase) colored by surface electrostatic potentials (ESP; blue to red surface coloring) for six representatives each from non-saline, marine, and hypersaline environments. Proteins from non-saline and marine habitats were from the Waiwera estuary. Those from hypersaline habitats were from IMG/VR. Protein structures were predicted from protein sequences via AlphaFold 3 server, with visualisation and ESP calculation in ChimeraX. ESP was calculated via the *coulombic* function with a standardised range (coulombic range -30,30). Two putative large terminase proteins were excluded during analyses due to poor structural predictions, resulting in 70 proteins in total.



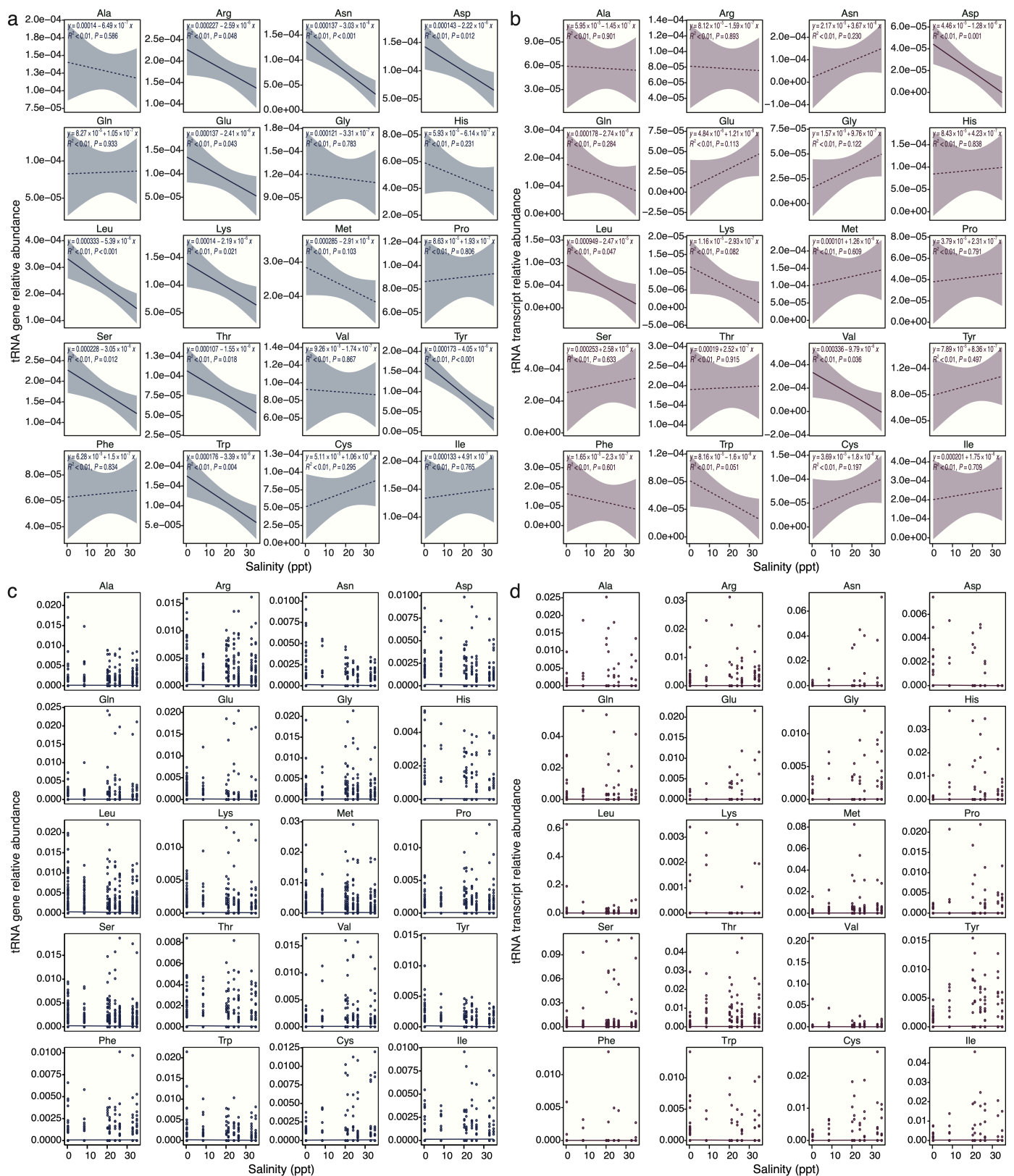
Supplementary Figure 8. Molecular signatures of environmental adaptation in DNA viruses from the IMG/VR database. Amino acid composition (a) and isoelectric points (pI) (b) of predicted proteins per ecosystem type split by functional category (a and b) and viral realm (a only) for high-quality IMG/VR viruses. Number of predicted genes per functional category per realm are shown on plots ($n=$).



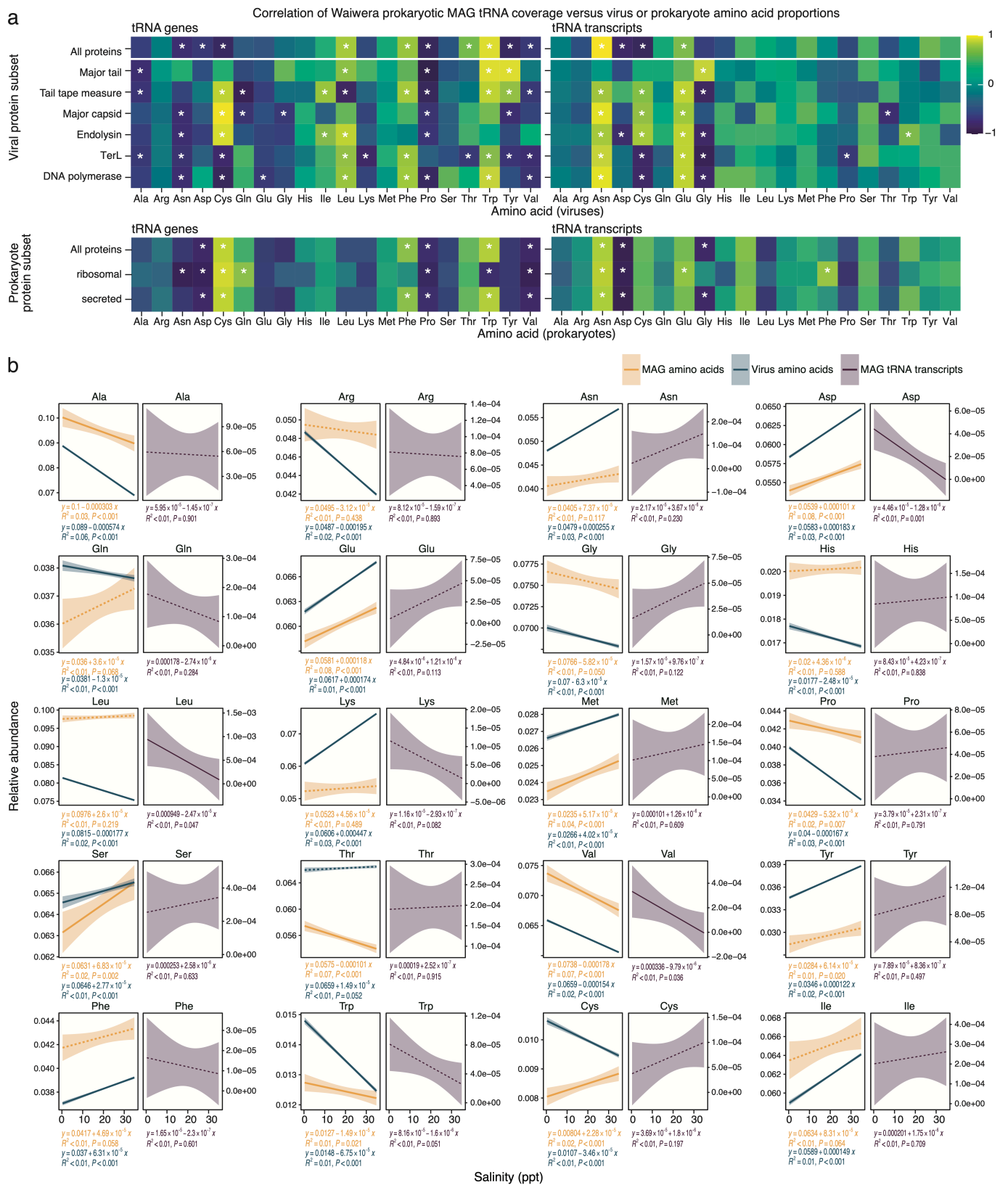
Supplementary Figure 9. Prokaryote phylogeny and tRNA gene and transcript counts based on MAGs from the Waiwera estuary. (a) Non-Metric Multidimensional Scaling ordination comparing samples based on UniFrac distances calculated from prokaryote MAG core gene alignments. (b-c) Counts of tRNA genes and gene transcripts for each amino acid in all prokaryote MAGs ($n=646$) (b), and in Luna cluster prokaryote MAGs ($n=19$) (c). Inner colour bars represent taxonomy. Outer colour bars represent assigned estuary zone based on the site where the MAG was most abundant.



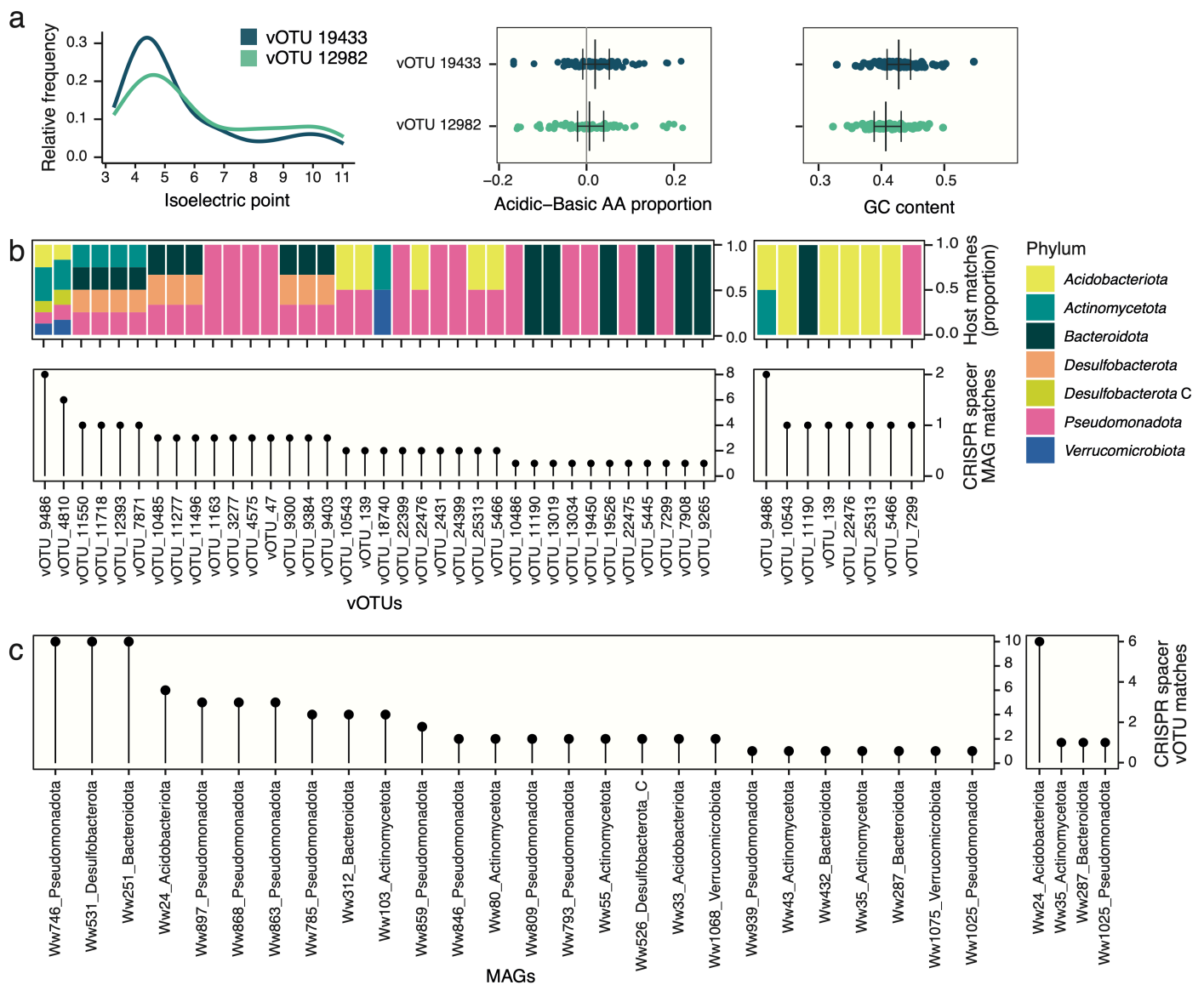
Supplementary Figure 10. Molecular signatures of environmental adaptation in DNA viruses from the Waiwera estuary. Proportions of glutamic acid, aspartic acid, arginine, histidine, lysine, and alanine in predicted viral contig protein subsets and all combined proteins in the estuary water column. Linear modelling equation and statistics were calculated via the *stat_poly_line()* and *stat_poly_eq()* functions from the *ggpmisc* package in R.



Supplementary Figure 11. Proportions of prokaryote tRNA gene (a,c) and transcript (b,d) abundances in each MAG against salinity in the Waiwera estuary water column for all amino acids. (a,b) Linear modelling equations and statistics were calculated via the *stat_poly_line()* and *stat_poly_eq()* functions from the *ggpmisc* package in R, with line shading representing 95% confidence interval. (c,d) Individual data points show per MAG tRNA gene and transcript proportions for each amino acid type.



Supplementary Figure 12. Comparisons between amino acid proportions and prokaryote tRNA gene and transcript abundances. (a) Spearman's correlations comparing predicted virus (top row) and prokaryote (bottom row) protein amino acid and cognate prokaryote tRNA gene (left panels) and transcript (right panels) relative proportions across the Waiwera estuary water samples for protein subsets and all proteins combined. * = Spearman's p -value ≤ 0.05 . (b) Linear modelling comparing trends across the estuary for mean amino acid proportions per virus or prokaryote (MAG) genome (left panel per amino acid pair) and relative transcription of prokaryote tRNAs for each amino acid (right panel per amino acid pair); reproduced from **Supplementary Figure 11b**. Linear modelling equations and statistics were calculated via the *stat_poly_line()* and *stat_poly_eq()* functions from the *ggpmisc* package in R, with line shading representing 95% confidence interval. Line is solid (versus dashed) for significant correlations.



Supplementary Figure 13. Traits of closely related vOTUs and host predictions. (a) Comparison of genes/proteins in common (>40% AA identity) for the two closely related vOTUs with complete genomes that collectively span the salinity divide. Comparisons are based on protein isoelectric points, the difference between encoding of acidic-basic amino acids, and gene GC content. Bars in dot plots represent the median and interquartile range. Each dot represents values for individual genes or proteins. (b-c) vOTU-prokaryote host (MAG) matches based on CRISPR spacer blast searches at 100% identity. (b) Counts and taxonomy (phylum) of MAG matches per vOTU. (c) Counts of vOTU matches per prokaryote MAG. Smaller plots are after filtering for MAGs with CRISPR regions further verified via CRISPRDetect.

2. SUPPLEMENTARY METHODS

Data acquisition

Waiwera estuary metagenomics and metatranscriptomics

Metagenomic and metatranscriptomic Illumina sequencing data previously generated from filtered water and triplicate sediment samples from nine sites spanning the salinity gradient and large differences in nutrient availability (water $n = 9$ samples; sediment $n = 9 \times 3 = 27$ samples)¹ were reanalysed for this study. In brief, filtered water and triplicate sediment samples were collected in November 2018 during low tide from nine sites spanning the salinity gradient (0.2 to 35 ppt) of the Waiwera river and estuary, Aotearoa New Zealand (36° 33' 02.4" S, 174° 39' 08.1" E). Water samples (~10L) were sequentially filtered through sterile 1.2 µm and 0.22 µm mixed cellulose ester filters (Merck Millipore, MA, USA). The viral fraction of these samples includes those associated with cells or biofilms on particles trapped on 0.22 micron filters. Triplicate wet-sieved (1 mm mesh sieve) sediment samples ~5 m apart were collected to a depth of 2 cm at each of the nine sites. Samples were placed in sterile 50 mL Falcon tubes, preserved in LifeGuard Soil Preservation Solution (Qiagen, MD, USA), and stored at -80 °C. Additional water and sediment samples were also collected for analyses of physicochemical and nutrient composition as previously described¹.

Illumina HiSeq (2500) paired end 2x250 bp and 2x125 bp sequencing of extracted total DNA and RNA (RNeasy PowerSoil Total RNA and RNeasy PowerSoil DNA Elution kits (Qiagen, MD, USA)), respectively, was conducted by the Otago Genomics Facility (University of Otago, Dunedin, New Zealand). Metagenomics reads were quality trimmed and filtered with Trimmomatic (v0.38)² and assembled with metaSPAdes (v3.11.1)³ (**Supplementary Data 8**). Individual assemblies were generated for each sample type (water and sediment) and site ($n = 9$) from the estuary (triplicate sediment samples for each site were pooled prior to assembly), generating 18 metagenome assemblies.

Data processing

Inference of Caudoviricetes phylogeny via concatenated protein alignments of putative single copy core genes

Phylogeny of *Caudoviricetes* viruses (vOTUs, viralRefSeq, and IMG/VR sequences) was inferred via generating trees of concatenated protein alignments of putative single copy “core genes” broadly following methodology previously described⁴ (further details available in the online workflow [<https://zenodo.org/doi/10.5281/zenodo.19197341>]⁵). The VOG database has expanded since this previous work, and marker gene IDs identified there are no longer applicable. As such, the process of selecting core genes and generating a concatenated gene tree of alignments was reproduced here, with core genes re-identified based on *Caudoviricetes* reference sequences in the viralRefSeq database (**Supplementary Data 6**). Genomic sequences were downloaded from the viralRefSeq database v223⁶, filtered to retain putative *Caudoviricetes* sequences based on the provided sequence taxonomy and sequences with predicted genome completeness $\geq 95\%$ (based on CheckV analysis⁷), and genes predicted and annotated via DRAM-v v1.4.6⁸. Predicted proteins for all viralRefSeq reference genomes and vOTUs from this study were searched via hmmsearch (HMMR v3.3.2; hmmer.org) against profile hidden Markov models (HMMs) for viral proteins downloaded from the viral orthologous groups database v222 (VOGdb) with a threshold of $-E\ 1e-3$. For each geneID, VOGdb matches with the lowest full sequence evalue score were retained. Putative single copy core genes were identified based on the following criteria: 1. present in $\geq 10\%$ of reference genomes; 2. average copy number ≤ 1.2 ; and 3. average predicted protein length > 100 amino acid residues. For each core gene, matching proteins in viralRefSeq references and vOTUs (this study) were identified and predicted protein amino acid sequences aligned with Clustal Omega v1.2.4⁹. Sequences with predicted genome completeness $\leq 85\%$ (estimated via CheckV) were omitted to minimise false weighting from missing

genes due to genome sequence incompleteness. Aligned proteins were trimmed to remove columns (amino acid positions) represented in <50% of genomes prior to concatenation (with gaps introduced where proteins were missing from a genome). Finally, genomes with amino acid representation of <5% total concatenated alignment length were removed, resulting in a final concatenated protein alignment matrix of 13,415 columns (amino acid positions) x 4,481 genomes, with 85% missing data (missing data value comparable to that of Low and colleagues (84%)⁴). Phylogenetic trees based on the concatenated alignment were generated via IQ-TREE v2.2.2¹⁰ using ModelFinder (with VT+F+I+G4 selected)¹¹, and with ultrafast bootstrap (nmax = 1000 replicates)¹². Trees were visualised using iTOL¹³.

An alternative set of putative core genes were also identified for all high quality *Caudoviricetes* sequences (based on the provided taxonomy) in the IMG/VR database (**Supplementary Data 6**) following the method outlined above with the following modifications: IMG/VR sequences were partitioned 100 ways (partition.sh from BBMap suite of tools v38.95¹⁴), and genes predicted via prodigal-gv v2.9.0^{15,16}. For each partition, hmmsearch against HMM profiles from the VOGdb database was performed with -Z 24911692 (the count of all predicted proteins from the IMG/VR dataset across all partitions) and -E 1e-3. Separate concatenated protein alignments of core genes and phylogenetic trees were generated based on each of the RefSeq and IMG/VR core genes sets, as per the method outlined above.

Identification of putative prokaryote hosts

Putative prokaryote hosts for Waiwera estuary vOTUs were predicted using a combination of approaches, including CRISPR-spacer, tRNA, and genome homology via pairwise blast searches (BLAST v2.13.0)¹⁷, analysis of vOTUs co-binned with prokaryote MAGs, oligonucleotide frequency similarity via VirHostMatcher v1.0.0¹⁸, and machine learning-based methods RaFAH v0.3¹⁹ and HostG (accessed 06 Dec 2021)²⁰. These six methods were applied to the non-dereplicated MAG dataset and to all viral contigs, which were later summarised by vOTU cluster. CRISPR-spacers were extracted from filtered and trimmed metagenomics sequencing reads using crass v1.0.1²¹ with the default parameters. Spacer sequences were searched against all non-dereplicated prokaryote MAGs (pre-dRep MAGs; n = 905) and viral contigs using blastn with the parameters -dust no -word_size 7. Viral contigs and MAGs that shared perfect matches to two or more unique spacer sequences (zero mismatches or gaps) were considered as a putative virus-host match. To provide more confidence in virus-host predictions based on CRISPR matching, CRISPR regions were also searched within all MAGs via CRISPRDetect v2.4²² with -array_quality_score_cutoff 1. tRNA sequences were identified in prokaryote MAGs and vOTUs from the Waiwera estuary with Aragorn v1.2.38²³, followed by pairwise blastn with the parameters -num_alignments 5 -dust no, and filtering to retain the top three matches with $\geq 90\%$ nucleotide identity over $\geq 90\%$ sequence length. Genome homology between MAGs and vOTUs was compared via blastn with filtering to retain the top three matches with nucleotide identity $\geq 70\%$, bit score $\geq 50\%$, and evalue ≤ 0.001 . Contigs predicted to contain viral sequence were searched within prokaryote MAGs to identify any viruses that were co-binned with individual MAGs. Co-binned vOTUs also predicted to be prophage (based on trimming conducted by VIBRANT, VirSorter2, or CheckV) were identified as candidate integrated prophage within the host MAG. For VirHostMatcher, the Waiwera Estuary prokaryote MAG dataset (this study) was provided as the database of potential hosts, with filtering to retain the top five hits with dissimilarity score ≤ 0.25 . RaFAH and HostG were run using the default settings, with additional filtering for HostG to retain predictions with softmax scores ≥ 0.8 .

3. SUPPLEMENTARY NOTES

Host matching remains a significant challenge in viral metagenomics studies

We assessed the putative prokaryote host range of the vOTUs in our estuary data set using a combination of approaches, including CRISPR-spacer, tRNA, and genome pairwise blast searches, analysis of vOTUs co-binned with prokaryote MAGs, VirHostMatcher, and machine learning-based methods RaFAH and HostG. Inconsistent results, including up to the rank of phylum, were frequently observed among the different methods. Previous studies have applied a ranking system across multiple methods based on the reliability of the method, with, for example, CRISPR-spacer matches out-ranking matches based on tRNA or genome blast searches^{24,25}. However, the inconsistencies observed here for predicted viral host matches question how robust inferences are based on many of the currently applied approaches. As CRISPR-spacer matches are considered the most robust method in metagenomics-based phage host-matching²⁵, our remaining analyses were restricted to this subset of results. Based on CRISPR spacer analysis, 121 Waiwera vOTUs (4 vOTUs $\geq 50\%$ complete) were putatively matched to 28 prokaryote MAGs in this dataset, with viral sequences within individual vOTUs matching between 1 and 11 (mean = 3.1) prokaryote MAGs and individual MAGs matching between 1 and 90 vOTUs (**Supplementary Figure 13b,c; Supplementary Data 12**). CRISPR spacer matches for viral sequences within individual vOTUs also often spanned bacterial phyla, with *Pseudomonadota* returning the most frequent matches. Further filtering to retain putative hosts where CRISPR arrays were also identified using CRISPRDetect resulted in matches remaining for 8 vOTUs (1 vOTU $\geq 50\%$ complete) to 4 MAGs (**Supplementary Figure 13b,c**).

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