Improving bacterial metagenomic research through long read sequencing

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Abstract

Metagenomic sequencing analysis is central to investigating microbial communities in clinical and environmental studies. Short read sequencing remains the primary data type for metagenomic research. However, long read sequencing promises the advantages of improved metagenomic assembly and resolved taxonomic identification. To assess the comparative performance of short and long read sequencing data for metagenomic analysis, we simulated short and long read datasets using increasingly complex metagenomes of 10, 20, and 50 microbial taxa. In addition, an empirical dataset of paired short and long read data from mouse fecal pellets was generated to assess feasibility. We compared metagenomic assembly quality, taxonomic classification capabilities, and metagenome-assembled genome recovery rates for both simulated and real metagenomic sequence data. We show that long read sequencing data significantly outperforms short read sequencing. For simulated long read datasets, metagenomic assemblies were completer and more contiguous with higher rates of metagenome-assembled genome recovery. This resulted in more precise taxonomic classifications and increased accuracy of relative abundance estimates. Analysis of empirical data demonstrated that sequencing technology directly affects compositional results. Overall, we highlight strengths of long read sequencing for metagenomic research of microbial communities over traditional short read approaches.

Keywords:
Metagenomics, Shotgun Metagenomic Sequencing, Next-generation Sequencing, Third generation sequencing, Taxonomic classification, Metagenome-assembled genomes, Simulation

Introduction

Metagenomics has enabled the study of microbial communities in agricultural, ecological, and clinical environments without the need for isolation or lab cultivation of bacteria (Chen and Pachter 2005; Cheng et al. 2019; Song et al. 2022; Suttner et al. 2020). Advancement in the field has been driven by the continued improvement of sequencing technologies, allowing for high-quality and low-cost genomic data for studying microbial populations with greater resolution (Wallen et al. 2022). Currently, the most widely used next-generation sequencing (NGS) technology are short-read sequencers, such as those from Illumina™. Short-read sequencing platforms generate 75-300 bp single or paired-end reads with per-base accuracy estimated at 99.9% (Latorre-Pérez et al. 2020). Before the recent reduction in cost attributed to the widespread availability of NGS technology, so-called microbiome studies often employed metabarcoding, where small portions of the 16S rRNA gene is sequenced for taxonomic classification (Begmatov et al. 2022; Oyserman et al. 2022; Sun et al. 2022). Although effective for genus level classification, metabarcoding provides limited species level resolution (Gupta et al. 2019). Where metabarcoding uses small regions of a single gene, shotgun metagenomics involves the sequencing of whole genome fragments, which can be used to reconstruct partial or whole microbial genomes through reference-based or *de novo* assembly approaches. Offering greater taxonomic resolution, *de novo* assembly is particularly useful for the identification of organisms, such as those that are non-culturable, that do not have robust representation in genomic databases. Shotgun metagenomic sequencing can also inform differences in gene content, single nucleotide polymorphisms, and mobile genetic elements (Berglund et al. 2019; Sanderson et al. 2020).

In recent years, 3rd generation "long-read" sequencing platforms such as those from Oxford Nanopore Technologies™ (ONT) and PacBio have increasingly been applied to clinical and biomedical research (Charalampous et al. 2019; Sanderson et al. 2020). These platforms use a single-molecule sequencing approach that generates significantly longer DNA sequences (Petersen et al. 2019). A major strength of 3rd generation sequencers is their ability to span regions of genomes that are difficult for short-read sequencers to resolve, including common repetitive elements and other low complexity regions (Koren and Phillippy 2015). Functional analyses are further improved through the use of long reads as they can capture whole genes and preserve genetic structures (Singleton et al. 2021). Although there are numerous benefits, some notable drawbacks of long-read sequencing include lower per base accuracy. However, recent versions promise improvement of accuracy to 99% (Amarasinghe et al. 2020; Sereika et al. 2022). In addition, available tools for long-read data analysis are relatively limited when compared to tools for analysis of short-read data. More recently, the availability of tools has improved, with the number long-read sequencing programs more than quadrupling since 2018 (Amarasinghe et al., 2021, 2023).

Due to its high accuracy, wide-spread availability, and well-established published pipelines, short-read sequencing is typically employed over long read sequencing for metagenomic studies (Govender et al. 2021). However, the advantages afforded by long-read

sequencing makes it an approach particularly well-suited for metagenomic research. In this study, we sought to investigate the relative performance of short- and long-read sequencing for metagenomic analysis. To this end, we simulated metagenomic data from synthetic microbial communities of increasing complexity and measured precision, recall, and F-score. We then applied paired short- and long-read sequencing to mouse fecal samples, and empirically compared the relative performance.

Materials and Methods

Simulated metagenomes of synthetic microbial communities

Nine sets of simulated metagenomic sequencing data were generated (Figure 1). Each set consisted of 10 unique, simulated metagenomic samples of varying complexity. Complexity was defined by the number of comprising taxa, the relative abundance of organisms in a metagenome, and the presence or absence of simulated sequencing errors. The size of a simulated metagenome consisted of genomes from 10, 20, or 50 taxa. Simulated abundances were either evenly distributed or randomly generated for each taxon within a simulated metagenome. Simulated sequencing errors were present or absent. The three types of sequence data included “Perfect”, “Uneven”, and “True” datasets. Perfect datasets consisted of reads with no errors and even organism abundance distribution. Uneven datasets consisted of reads without errors and random abundance values for each organism. True datasets contained reads with simulated errors specific to each sequencing platform for a given sequence type and random abundance values for each organism. Simulated long-reads were generated to reflect sequence data from ONT’s MinION™ and short-reads mirrored sequence data from Illumina’s™ HiSeq™ sequencer.

Construction of synthetic metagenomes was performed using an assembly structure report downloaded from NCBI (Downloaded 04/18/2022; Figure S1). This report contained all bacteria present in RefSeq with the following criteria for inclusion: 1) Genomes must be part of the latest RefSeq database, 2) they must have a complete genome assembly, and 3) all anomalous assemblies were excluded.

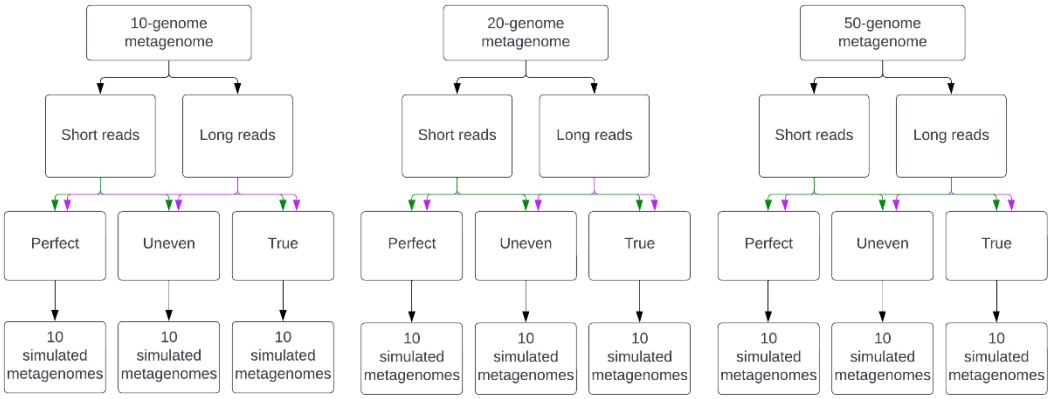


Figure 1. **Simulation of metagenomic datasets of increasing complexity.** Perfect metagenomic samples were simulated with no sequencing errors and an even abundance distribution of organisms. Uneven samples were simulated with no sequencing errors and randomly assigned abundance values for organisms in the metagenome. True datasets were simulated with sequencing errors specific to each read type based on the platform they would be generated from and randomly assigned abundance values for members of each metagenome

Simulation of ONT sequence data was performed using NanoSim version 3.0.0 (Yang et al. 2021). NanoSim input files (genome, abundance, and DNA lists) were created using a custom python script specifying parameters according to the level of metagenome complexity. The genome list (-gl) contained randomly selected taxa from the assembly structure report. The number of taxa selected was based on the desired size of the metagenome: 10, 20, or 50. Similar organisms could be present in a metagenome, such as different strains of the same species. The abundance list (-al) contained each organism and a value defining the abundance of that organism in the metagenome. This value was either equal across all organisms or randomly generated such that the total abundance of all organisms was 100%. The DNA list (-dl) contained all genetic molecules present in each organism's genome. This included an organism's chromosome and any plasmids, as well as whether the molecule was circular or linear. For simulating errors (-c), the pre-trained model 'metagenome_ERR3152364_Even' supplied by NanoSim was used. The basecaller (-b) specified was *guppy*. The '--perfect' option was specified for datasets without simulated errors, including "Perfect" and "Uneven" datasets. This option was not used for "True" datasets. All simulated reads were generated in FASTQ format (--fastq). The total number of reads generated per metagenomes was 650,000, with reads having an N50 of approximately 5.2 Kbps. Total bases for each sample were approximately 2.67 Gbps. These values were selected to mimic potential, realistic outputs from nanopore sequencing. For long reads, filtering was performed using Filtlong version 0.2.1 (R. Wick 2021). Long reads were filtered by minimum read length of 1000 bp (--min_length 1000).

Simulation of short read sequence data from an Illumina™ HighSeq™ was done using the randomreads.sh script from BBMap version 37.93 (Bushnell 2020). Because randomreads.sh uses a per-reference approach when determining coverage, bacterial genome files had sequences fused using the fuse.sh script with default parameters including 'pad=0' to prevent incorporation of Ns as spacers. Chosen reference genomes were then concatenated into a single file for simulation. For all datasets, the reference file used was the concatenated file described

previously (ref=<file>). The number of reads generated was set to 10 million (reads=10m), the length of reads was 150 bp (len=150 bp), for a total of 3Gbps, roughly the same as in long read datasets. As with simulated nanopore data, these quantities were chosen to mimic potential, realistic outputs. The option paired was specified to denote reads are paired-end (paired), both 'simplenames' and 'illuminanames' were specified to make potential debugging easier, coverage for each metagenome was set to 200X (coverage=200), 'superflat' was specified to create a flat insert size distribution, and the q-score was set to 36 (q=36). For simulation of datasets with uneven abundance distributions, the option 'metagenome' was included. For datasets with simulated errors, the 'metagenome' option was included along with the following error rate options: Maximum SNPs of 3 (maxsnps=3), maximum number of insertions of 2 (maxinss=2), maximum number of deletions of 2 (maxdels=2), maximum number of substitutions of 2 (maxsubs=2), insert rate of 0.00000315 (insrate=0.00000315), deletion rate of 0.000005 (delrate=0.000005), substitution rate of 0.0033 (subrate=0.0033), and 'adderrors=t'. The chosen error rates are based off average error rates observed by Schrimer and colleagues in their paper on Illumina™ error profiles from metagenomic data (Schirmer et al. 2016). Because simulated short read sequences were generated in one file, the reformat.sh from BBMap was used to separate paired-end reads into R1 and R2 files. No filtering was performed on short reads as all reads possessed a q-score of 36.

Metagenome assembly and quality assessment

For long reads, assembly was performed using Flye version 2.9-b1768 (Kolmogorov et al. 2020). Default parameters for Flye were kept with the tag '--nano-raw' and '--meta' added to denote assembly of uncorrected reads from a metagenomic sample. SPAdes version 3.15.5 was used for assembling metagenomes from simulated short read data (Nurk et al., 2017). Default options for SPAdes were used except for the following: '--meta' was specified to indicate the sequence data were from a metagenomic sample, kmer sizes (-k) consisted of a list of integers: 21, 33, 55, 77, 99, 127, and '--phred-offset' was set to 33 due to simulated short reads not possessing defined signatures of the PHRED quality. Metagenomic assembly quality was evaluated using metaQUAST version 5.0.2 (Mikheenko, Saveliev, and Gurevich 2016). Default parameters were used except for supplying the directory containing the reference genomes for each metagenome to '-r'.

Metagenome-assembled genome recovery and taxonomic classification

Metagenome-assembled genomes (MAGs) were generated using the following steps. First, long reads were mapped back to the assemblies using minimap2 version 2.24-r1122. For long reads, the flag '-x' was given the 'map-ont' argument to optimize alignment parameters for nanopore sequence data (Li 2018). Short reads were mapped using bwa-mem2 version 2.2.1 by first creating an index from a metagenome's assembly file using 'bwa-mem2 index', and then mapping the reads back using 'bwa-mem2 mem'. Each alignment process produced an unsorted SAM file which was then converted into sorted BAM files using Samtools version 1.16.1 with options 'samtools view -b' for creating the BAM file, and 'samtools sort' for sorting the BAM file (Danecek et al. 2021). A coverage file was generated for each read type using CoverM version 0.6.1 using the default '-m mean' for coverage estimation (Woodcroft 2021). For long reads, the mapper option for CoverM was set to '-p minimap2-ont', and for short reads it was '-p

bwa-mem'. Each coverage file was used to bin each read type's respective assemblies using MetaBAT 2 version 2.15 with default parameters except for the addition of the '--cvExt' argument to denote coverage files originated from a third-party tool (Kang et al. 2019). Binned contigs were then assessed for completeness and contamination using CheckM2 version 1.0.1 with default parameters (Chklovski et al. 2022). Taxonomic classification was performed using Kraken2 version 2.1.2 for both short and long read metagenomic assemblies (Wood, Lu, and Langmead 2019). Default parameters were used with the '--use-names' flag included. The kraken2 report and output files were kept for further analysis.

Result evaluation

For visual assessment of metagenome assembly quality, assembly graphs were visualized using Bandage version 0.8.1 (R. R. Wick et al. 2015). For quantitative comparison of genome fraction, NGA50, and misassembly counts reported by metaQUAST, a Mann-Whitney U test with Bonferroni correction was applied using the *statannotations* python package (Charlier et al., 2022). A p-value threshold of 0.05 was used to determine significance.

Performance evaluation of taxonomic classification from reads and assemblies was accomplished by calculating precision, recall, and F-score. Precision was defined as the rate of successful taxonomic assignment of a read/contig to the correct taxon within the metagenome over the total number of taxonomic assignments. Also referred to as the false positive rate, the equation used was $Precision = \frac{TP}{(TP+FP)}$ where TP is the true positive rate and FP is the false positive rate, or an incorrect classification of a read/contig at a given taxonomic rank. Recall, also defined as the rate of false negatives, was determined using the equation $Recall = \frac{TP}{total}$ where 'total' is the expected number of distinct organisms at a specified taxonomic rank. F-score, or the harmonic mean, generates an overall score factoring in both precision and recall. F-score was calculated using the equation $F1 = \frac{2*(precision*recall)}{(precision+recall)}$. A statistical comparison of precision, recall, and F-score was performed using a one-way ANOVA for assemblies and a Mann-Whitney U test with Bonferroni correction for reads with the *statannotations* python package. A p-value threshold of 0.05 was used to determine significance.

Estimation of relative abundance of taxa at the genus and species level was carried out using Bracken version 2.8 (Lu et al., 2017). Short and long reads were processed through Kraken2 to generate report files that were then used as inputs for Bracken. Bracken then returns a report file containing estimates of relative abundance. The absolute difference between the expected and estimated abundance from each read type was calculated to determine which read type produced the closest estimation. A result of short or long was stored based on which read type produced the smallest difference from the expected abundance of each taxon in a metagenome. Results of this analysis were plotted using overlapping boxplots and stripplots. Read types were compared using a Mann-Whtiney U test with Bonferroni correction using the *statannotations* package. A p-value threshold of 0.05 was used to determine significance.

MAG recovery rates were determined by comparing the total number of MAGs recovered by the binning of contigs from short and long read metagenomic assemblies. A MAG's quality is

determined by the completeness and contamination values of contigs assigned to a bin by MetaBAT2 and assessed by CheckM2. CheckM2 defines completeness as the percentage of universal, bacteria-specific gene markers present in a given bin. Contamination for CheckM2 is based on how many of these markers exist in multiple copies which indicates the presence of contigs that do not belong in a particular bin. For the minimum threshold of a bin to be considered a MAG, the cutoffs were defined as a completeness ≥50% and contamination ≤10% (A. Murat Eren 2016). For each of the 9 sets of metagenomes, the average number of MAGs recovered from short and long read assemblies was recorded. A Mann-Whitney U Test with Bonferroni correction was applied using the *statannotations* python package to compare differences in MAG recovery rates between short and long read assemblies. A p-value threshold of 0.05 was used to determine significance. Bins determined to be MAGs were also evaluated on quality which was defined by completeness only. High quality MAGs had a completeness ≥90%, medium quality MAGs had a completeness ≥70% and <90%, and low-quality MAGs had a completeness ≥50% and <70%, respectively.

Comparison of experimental metagenomic data

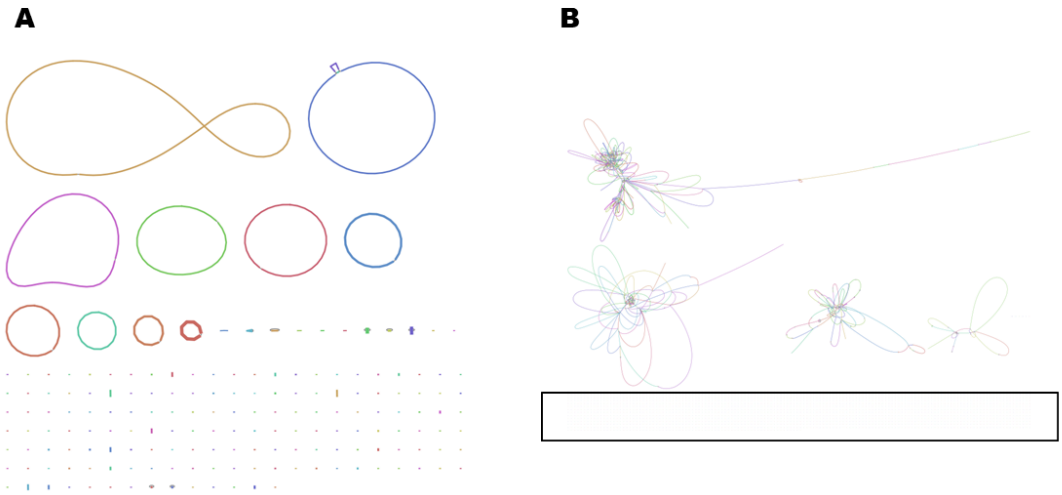
To examine whether read type affects assessment of microbial composition, we generated short- and long-read sequencing data from 19 mouse fecal pellets collected during ongoing experimental studies. Long-read sequencing data were generated using the ONT GridION. Prior to DNA extraction, host DNA was depleted using an enzymatic approach as described previously with some modifications (Charalampous et al., 2019). Each sample extraction used 1-2 mouse fecal pellets. Fecal pellets were homogenized in 1 mL of InhibitEX buffer (Cat. No./ID: 19593, Qiagen, Hilden, Germany). After pelleting the homogenate and aspirating off the liquid, 250 µL of 1X PBS and 250 µL 4.4% saponin was added and mixed thoroughly before resting at room temperature for 10 minutes. This detergent weakens eukaryotic cell membranes while not affecting bacteria due to their cell walls. Nuclease-free water (350 µL) along with 12 µL of 5M NaCl were added to induce osmotic lysis. After pelleting, the liquid was again aspirated off and the pellet was resuspended in 100 µL of 1X PBS. One-hundred microliters of HL-SAN enzyme buffer added (5.5 M NaCl and 100 mM MgCl2 in nuclease-free water), followed by 10 µL of HL-SAN endonuclease (Article No. 70910-202, ArcticZymes, Tromsø, Norway) before being incubated on a shaking incubator at 37°C for 30 minutes at 1000 rpm. Two final washes of the pellet were performed using 800 and 1000 µL of 1X PBS before proceeding to extraction. Genomic DNA (gDNA) was extracted and purified using the New England Biolabs Monarch® Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA) following manufacturer instructions. Quality and concentration of gDNA was assessed using Agilent 4200 TapeStation System and Qubit 4 Fluorometer. Sequencing libraries for nanopore sequencing were prepared using the Rapid PCR Barcoding Kit (SQK-RPB004). Using an Oxford Nanopore GridION™, libraries were sequence on a R9.4.1 flow cell generating an average of 1.84 Gbps of sequence data with a mean read length of 3,491.62 bps and a mean read quality of 12.9. The longest read produced was 42,266 bps. Short read sequencing was performed by SeqCenter LLC (SeqCenter, Pittsburgh, PA) using an Illumina™ NovaSeq™ with Nextera Flex Library Preparation Kit® and V3 flow cell chemistry to produce an average of 2.53 Gbps of paired-end 2x150 bp reads across

all samples. Illumina data were filtered using Trimmomatic v0.39 for paired-end reads with the following trimming settings: SLIDINGWINDOW:4:20 MINLEN:100.

Analysis followed the methodological approaches described previously for short- and long-reads, respectively. Reads were first classified using Kraken2 and relative abundances were estimated using Bracken. Before analysis, bracken reports were trimmed to remove all taxonomic identifications where the number of reads assigned to an organism fell below a given threshold of 1/20,000 (0.00005). To our knowledge, no studies using shotgun metagenomic sequencing have given the expected taxonomic diversity of the mouse gut microbiome at the genus or species level. The Murine Microbiome Database consists of 1732 genera and 4703 species, however these data come from 16S sequence analysis (Yang et al., 2019). An alternative database, the Comprehensive Mouse Microbiota Genome (CMMG), which derived 1,573 species from mouse gut metagenomes combined with known reference metagenome-assembled genomes at the time of its creation (Kieser et al., 2022). In this study, we identified a cutoff where the count of unique taxa was closer to that of the CMMG, as 16S sequence often reports higher diversity. Initial plotting of the number unique taxa showed counts of over 1750 genera and over 6000 species for both short- and long-read data when no abundance cutoff was applied. Using a cutoff of 1/20,000 yielded unique taxa counts for species similar to that of the CMMG. Short- and long-read data for a group were then compared to each other using the beta_diversity.py script from the KrakenTools suite. Qualitative assessment was performed by generating a hierarchically clustered heatmap of the resulting Bray-Curtis Dissimilarity matrix. For quantitative analysis, a principal component analysis (PCA) was performed. First, data were transformed using a center-log ratio transformation (Aitchison, 1986). Then, PCA was run using the PCA function from scikit-learn's decomposition library (Pedregosa et al., 2011). Briefly, sample IDs were separated as features and abundance values for each taxa at the genus and species level were retained. Abundance values for unique taxa were then given to the PCA object which created a fit and subsequently applied dimensional reduction to the abundance data. The resulting principal components were kept in a table from which the top two principal components were plotted.

Results

Comparison of metagenomic assembly completeness



Supplemental Figure 1. **Assembly graphs from a "True" 10-genome metagenome visualized by Bandage.** 2A. Bandage graph generated from metagenomic assembly of long read sequence data. 2B. Bandage graph generated from metagenomic assembly of short read sequence data. The black box highlights small, disconnected contigs.

Metagenomic assemblies for each synthetic dataset were qualitatively assessed through visualization (Figure 2). Long read assemblies produced graphs with greater contiguity and often resulted in circularization of unitigs, an indication they represented complete chromosome or, in the case of smaller, circularized contigs, plasmids.

Assemblies from long reads demonstrated significantly higher genome fraction coverage than their short read counterparts (Figure 3A, D, and G; Supplementary Table S1). This was observed across all metagenome sets, regardless of complexity, with the exception of the synthetic dataset composed of 50 organisms with perfect reads (Figure 3A). Similar results were observed when comparing assemblies by NGA50 values, with long read data showing significantly higher scores (Figure 3B, E, and H; Supplementary Table S1). Assemblies generated from short read data resulted in significantly fewer misassemblies than those constructed from long read data (Figure 3C, F, and I; Supplementary Table S1). An exception was with 50-genome metagenomes where abundance was varied and reads contained no errors and therefore no significant differences were observed (Figure 3F).

Table S1. Results of Mann-Whitney U testing for genome fraction, NGA50, and number of misassemblies.

[1] "Perfect" indicates reads had no errors and abundances were evenly distributed. "Uneven" denotes reads had no errors, but variable abundances for each organism. "True" reads had simulated sequencing errors based on the read type and variable abundances for the organisms.

[2]P-value from Mann-Whitney U test with Bonferroni correction applied

simulated metagenome	complexity[1]	metaQUAST metric	statistic	p-value[2]
10-genome metagenome	perfect	genome fraction	7600.5	5.37E-10
10-genome metagenome	perfect	NGA50	8465	7.67E-17
10-genome metagenome	perfect	number of misassemblies	6473	1.64E-07
20-genome metagenome	perfect	genome fraction	28270	2.61E-11
20-genome metagenome	perfect	NGA50	31239.5	2.47E-20
20-genome metagenome	perfect	number of misassemblies	30033.5	1.65E-26
50-genome metagenome	perfect	genome fraction	137999.5	5.19E-02
50-genome metagenome	perfect	NGA50	160049	2.56E-12
50-genome metagenome	perfect	number of misassemblies	197850	7.26E-68
10-genome metagenome	uneven	genome fraction	8322	1.24E-15
10-genome metagenome	uneven	NGA50	8937	1.17E-21
10-genome metagenome	uneven	number of misassemblies	3919	1.75E-02
20-genome metagenome	uneven	genome fraction	34421	6.37E-34
20-genome metagenome	uneven	NGA50	35711	3.30E-41
20-genome metagenome	uneven	number of misassemblies	12878.5	1.27E-10
50-genome metagenome	uneven	genome fraction	177734.5	5.14E-30
50-genome metagenome	uneven	NGA50	187183.5	5.78E-43
50-genome metagenome	uneven	number of misassemblies	114691	5.66E-02
10-genome metagenome	true	genome fraction	8895	1.55E-19
10-genome metagenome	true	NGA50	9245	2.90E-23
10-genome metagenome	true	number of misassemblies	3629	4.66E-04
20-genome metagenome	true	genome fraction	32609	2.74E-27
20-genome metagenome	true	NGA50	33525	2.19E-32
20-genome metagenome	true	number of misassemblies	12834	2.74E-10
50-genome metagenome	true	genome fraction	180443	6.14E-32
50-genome metagenome	true	NGA50	189300	2.18E-44
50-genome metagenome	true	number of misassemblies	110455	1.49E-03

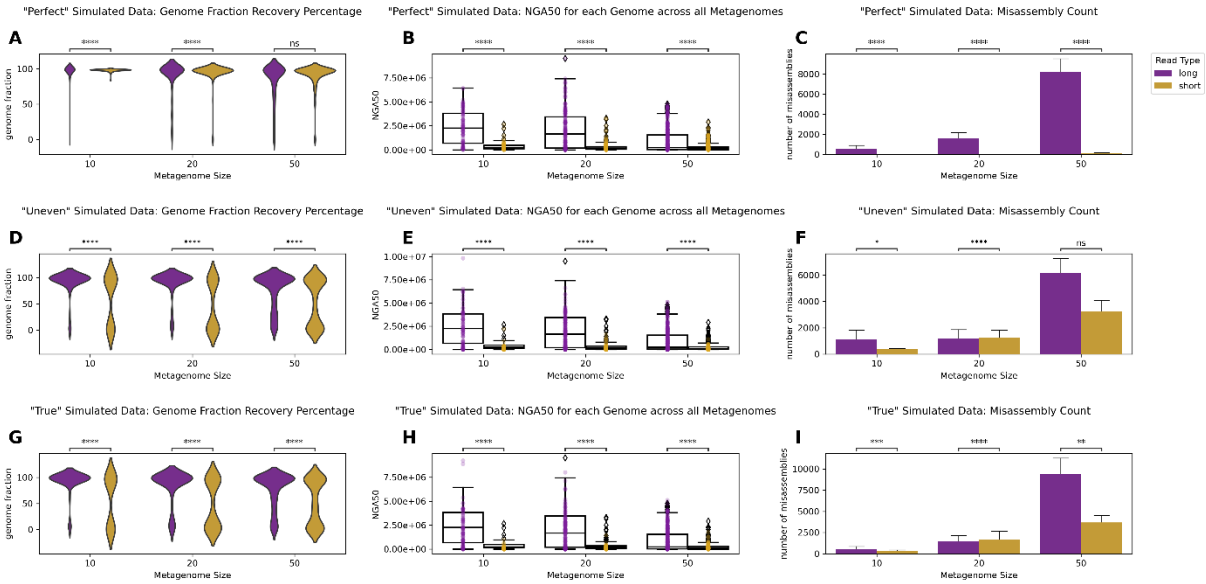


Figure 2. Comparisons of assembly quality metrics. *Violin plots depict the differences in genome fraction values from all genomes in a set of metagenomes (A, D, G). Stripplots depict the differences in NGA50 across all genomes in a set of metagenomes (B, E, H). Barplots depict total misassembly counts across all genomes in a set of metagenomes (C, F, I). "Perfect" denotes simulated data have no errors and abundances are evenly distributed for each organism in the metagenome. "Uneven" denotes simulated data have no errors, but variable abundances for each organism. "True" denotes simulated data have simulated sequencing errors based on the read type, as well as variable abundances for each organism. Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$*

Evaluating taxonomic classification

Evaluation of taxonomic classifications at the genus and species-level was assessed for precision, recall, and F-score. Using recall to measure genus and species-level classification, short and long reads did not significantly differ (Figure 4B, E, and H; Figure 5B, E, and H). However, long reads significantly outperformed short reads in precision across almost all levels of metagenome complexity at both the genus and species level (Figure 4A, D, and G; Figure 5A, D, and G). The F-score, or harmonic mean of the precision and recall, was calculated to measure the accuracy of classification results using either short or long reads. Long reads demonstrated significantly higher F-scores than short reads, indicating that long reads provide greater accuracy for taxonomic classification.

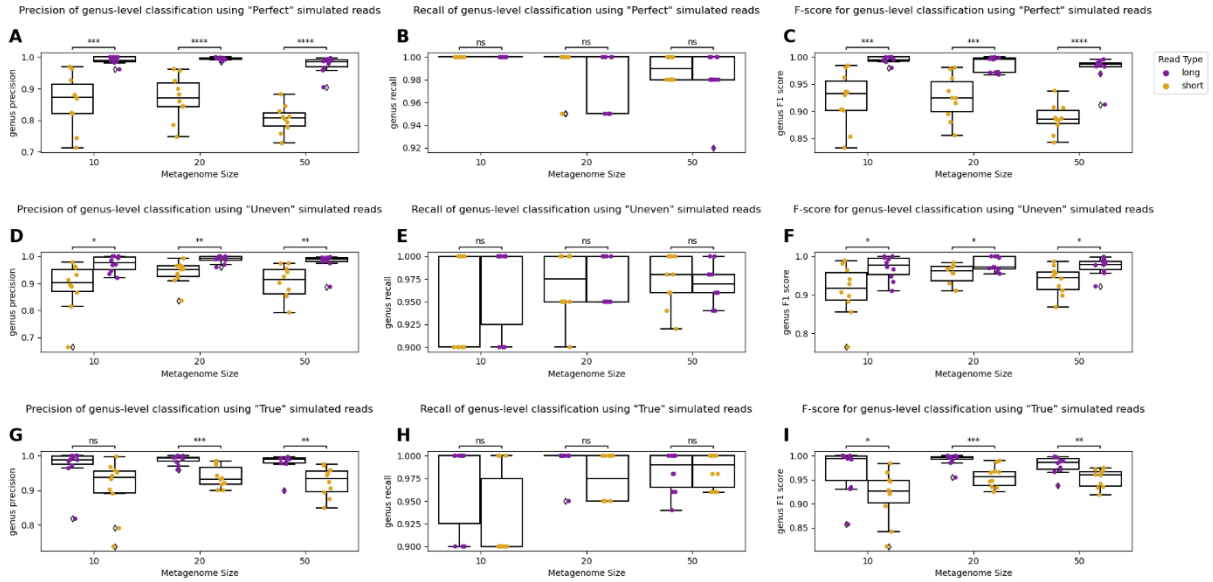


Figure 3. **Performance evaluation of genus-level classification on metagenomic assemblies using short or long reads.** Stripplots and boxplots show precision, recall, and F-score metrics for metagenomes of varying size and complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a One-way ANOVA. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

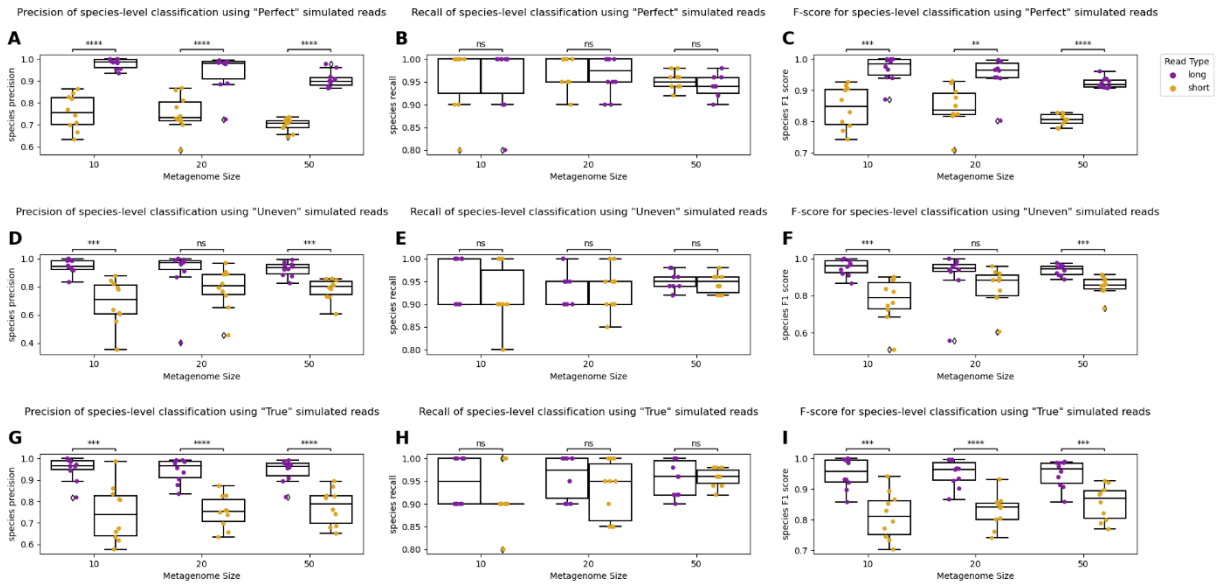
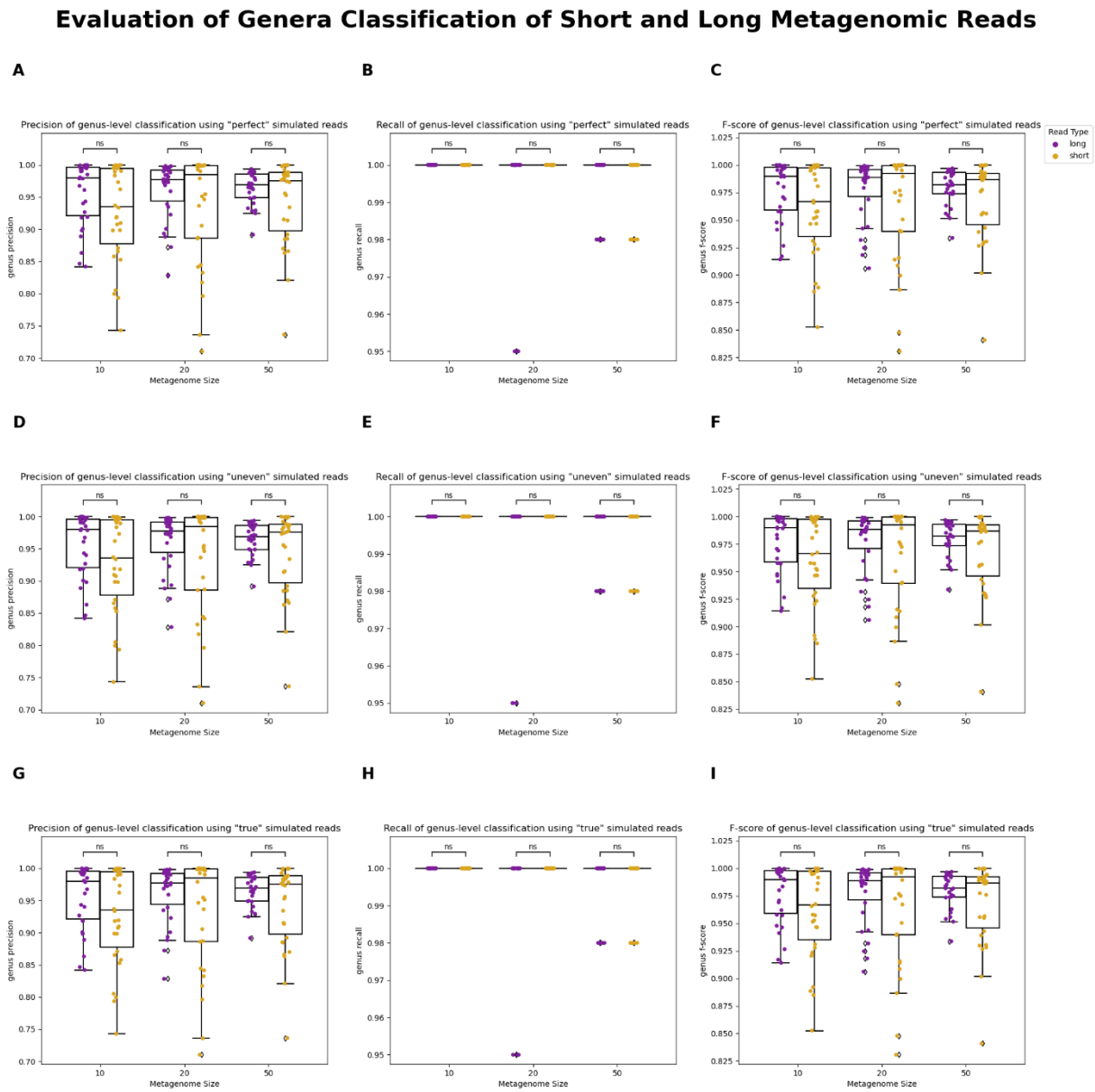


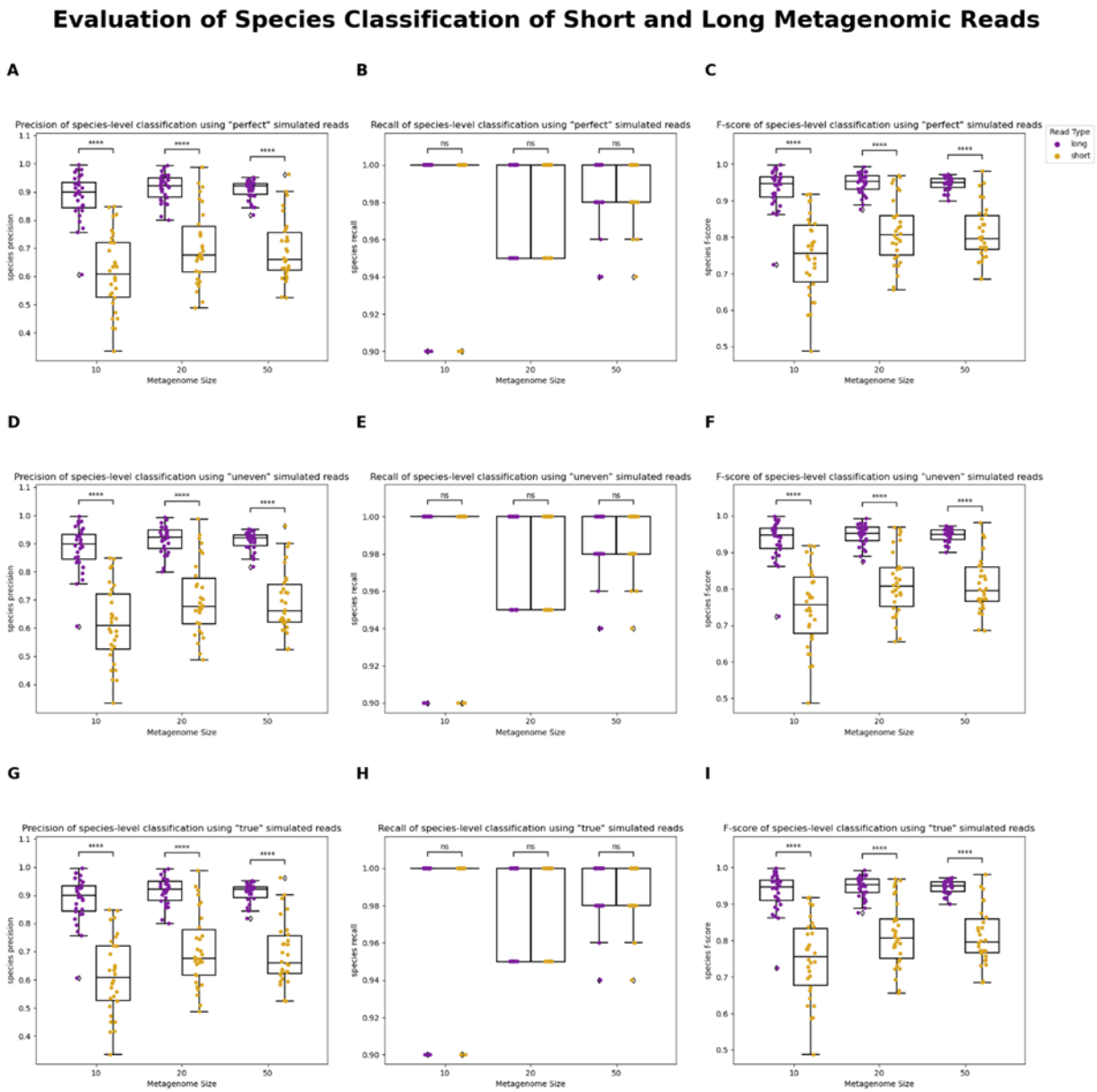
Figure 4. **Performance evaluation of species-level classification on metagenomic assemblies using short or long reads.** Stripplots and boxplots show precision, recall, and F-score metrics for metagenomes of varying size and complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a One-way ANOVA. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Estimating relative abundance using short and long read data

Before analyzing the performance of each read type when used for relative abundance estimation, the classification capabilities of the reads were first assessed. Precision, recall, and F-score of genus-level classification were not significantly different between read types, which differed from classification using metagenomic assemblies (Supplementary Figure S1). However, when examining the performance for each read type on species-level classification, long reads outperformed short reads in a similar way as observed when comparing classification of assemblies. Both precision and F-score were higher for classifications using long reads than short reads (Supplementary Figure S2). Regardless of metagenomic complexity, long reads provided a more accurate assessment of the relative abundance of taxa present in a metagenome than long reads at both genus and species-level classification (Figure 6).



Supplemental Figure 2. **Performance evaluation of genus-level classification using short and long reads.** Overlapping stripplots and boxplots show precision, recall, and F-score metrics for metagenomes of varying complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a Mann-Whitney U test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$



Supplemental Figure 3. **Performance evaluation of species-level classification using short and long reads.** Overlapping stripplots and boxplots showing (precision, recall, and F-score metrics for metagenomes of varying complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a Mann-Whitney U test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

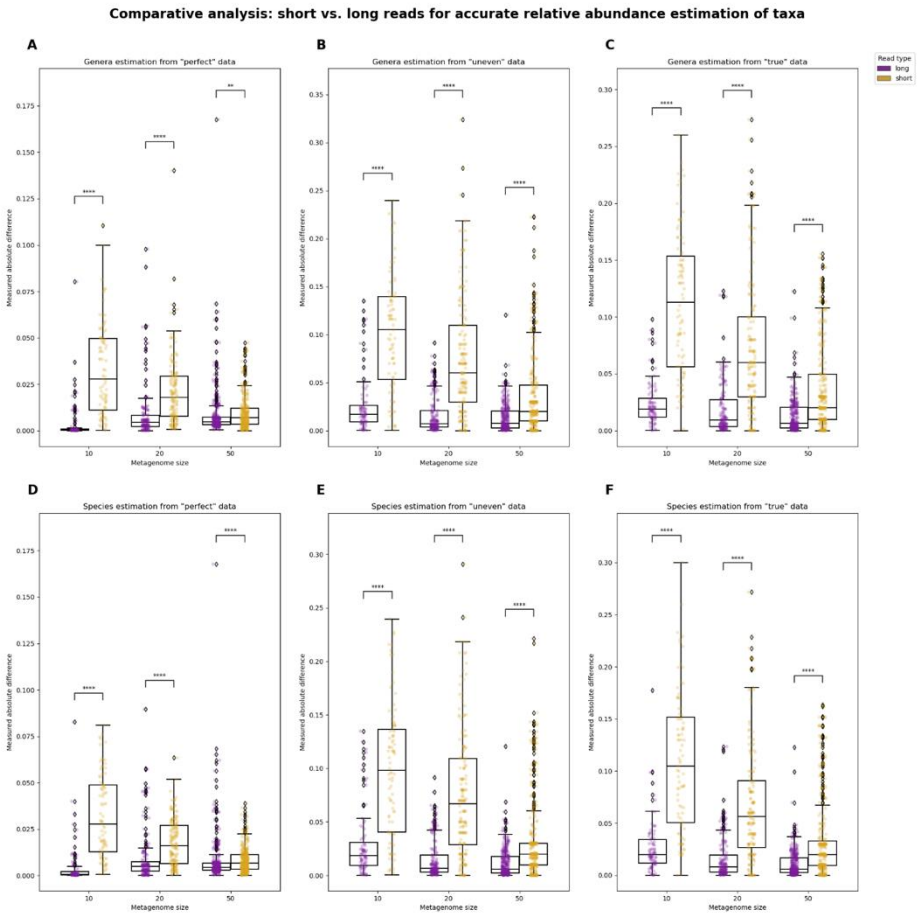


Figure 5. **Comparison of short and long read capacity for relative abundance estimation.** Overlapping stripplots and boxplots showing differences in relative abundance estimation of organisms present in metagenomic samples when using short or long reads. Read types were compared across metagenomes of varying complexity at both the genus and species level. "Perfect" datasets consisted of reads without sequencing errors and organism abundance was evenly distributed (6A, 6D), "Uneven" consisted of reads without sequencing errors and randomly distributed abundances of organisms (6B, 6E), and "True" consisted of reads with simulated errors and randomly distributed abundances of organisms (6C, 6F). Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Metagenome-assembled genome (MAG) recovery from short- and long-read data

Simulated data from the "Perfect" datasets showed no significant difference in the number of MAGs recovered from assemblies generated from either read type. Among more complex datasets, assemblies using long reads produced significantly more MAGs, regardless of the size of the metagenome (Figure 7). It was found that the binning of contigs from long read assemblies produced more total bins than assemblies produced from short reads (Supplementary Table S2). Following this observation, the quality of MAGs recovered were evaluated. Bins were assigned a quality of high, medium, or low, depending on their completeness. In most cases, bins of contigs from long read assemblies produced significantly more high-quality MAGs than bins from short read assemblies (Supplementary Figure 3).

Table S2. **Bin and metagenome-assembled genome (MAG) counts from metagenomic samples.** Total bins recovered for each metagenomic sample and counts of MAGs identified from those bins. MAGs were further separated by quality as described in the methods section.

[1]Samples consist of the 10 metagenomes simulated for a given complexity.

[2]A metagenome set is the given complexity level for a collection of simulated metagenomes.

[3a]Sum of all bins predicted by MetaBAT2 from long-read assemblies

[3b]Sum of all bins predicted by MetaBAT2 from short-read assemblies.

sample[1]	metagenome set[2]	bin count (long)[3a]	bin count (short)[3b]	MAG count (long)	MAG count (short)	High quality MAG count (long)	High quality MAG count (short)	Medium quality MAG count (long)	Medium quality MAG count (short)	Low quality MAG count (long)	Low quality MAG count (short)
mock_metagenome_1	10_genomes_perfect	20	14	10	10	8	10	2	0	0	0
mock_metagenome_10	10_genomes_perfect	17	15	10	9	8	8	2	1	0	0
mock_metagenome_2	10_genomes_perfect	12	13	9	9	6	8	3	0	0	1
mock_metagenome_3	10_genomes_perfect	11	13	10	10	10	10	0	0	0	0
mock_metagenome_4	10_genomes_perfect	13	12	9	9	7	7	1	2	1	0
mock_metagenome_5	10_genomes_perfect	19	12	9	7	4	6	5	1	0	0
mock_metagenome_6	10_genomes_perfect	18	11	8	8	7	8	1	0	0	0
mock_metagenome_7	10_genomes_perfect	13	12	10	8	8	8	2	0	0	0
mock_metagenome_8	10_genomes_perfect	11	12	9	9	8	8	1	1	0	0
mock_metagenome_9	10_genomes_perfect	16	13	9	9	6	8	1	1	2	0
mock_metagenome_1	10_genomes_true	13	9	9	6	8	6	0	0	1	0
mock_metagenome_10	10_genomes_true	16	8	9	6	7	6	1	0	1	0
mock_metagenome_2	10_genomes_true	13	8	9	6	8	6	1	0	0	0
mock_metagenome_3	10_genomes_true	12	8	7	6	6	5	1	1	0	0
mock_metagenome_4	10_genomes_true	14	8	9	6	8	5	0	1	1	0
mock_metagenome_5	10_genomes_true	13	8	7	5	6	4	1	0	0	1
mock_metagenome_6	10_genomes_true	14	9	7	6	6	6	1	0	0	0
mock_metagenome_7	10_genomes_true	14	8	7	4	5	4	2	0	0	0
mock_metagenome_8	10_genomes_true	17	8	9	6	7	6	1	0	1	0
mock_metagenome_9	10_genomes_true	11	9	7	6	5	5	1	1	1	0
mock_metagenome_1	10_genomes_uneven	14	9	9	5	8	3	1	2	0	0
mock_metagenome_10	10_genomes_uneven	11	8	10	6	10	5	0	1	0	0
mock_metagenome_2	10_genomes_uneven	16	8	9	6	6	6	2	0	1	0
mock_metagenome_3	10_genomes_uneven	12	10	9	5	8	4	1	0	0	1
mock_metagenome_4	10_genomes_uneven	12	9	9	6	8	4	1	2	0	0
mock_metagenome_5	10_genomes_uneven	14	9	8	6	7	3	0	1	1	2
mock_metagenome_6	10_genomes_uneven	17	9	7	4	6	3	0	0	1	1
mock_metagenome_7	10_genomes_uneven	20	11	7	6	3	5	2	1	2	0
mock_metagenome_8	10_genomes_uneven	17	10	9	6	6	5	1	1	2	0
mock_metagenome_9	10_genomes_uneven	11	9	7	4	7	4	0	0	0	0
mock_metagenome_1	20_genomes_perfect	28	20	18	13	14	13	4	0	0	0
mock_metagenome_10	20_genomes_perfect	23	23	18	16	14	15	4	1	0	0
mock_metagenome_2	20_genomes_perfect	24	20	17	15	14	14	0	1	3	0
mock_metagenome_3	20_genomes_perfect	26	21	17	14	13	13	3	1	1	0
mock_metagenome_4	20_genomes_perfect	21	22	18	17	16	15	2	0	0	2
mock_metagenome_5	20_genomes_perfect	24	23	19	17	17	16	1	1	1	0
mock_metagenome_6	20_genomes_perfect	28	20	14	13	10	12	2	1	2	0
mock_metagenome_7	20_genomes_perfect	35	34	11	13	10	10	1	3	0	0
mock_metagenome_8	20_genomes_perfect	24	24	14	13	10	12	3	0	1	1
mock_metagenome_9	20_genomes_perfect	28	19	19	13	14	13	4	0	1	0
mock_metagenome_1	20_genomes_true	19	11	11	8	11	8	0	0	0	0
mock_metagenome_10	20_genomes_true	23	11	19	8	14	6	3	2	2	0
mock_metagenome_2	20_genomes_true	26	12	14	8	12	7	1	1	1	0
mock_metagenome_3	20_genomes_true	25	12	14	6	12	6	1	0	1	0
mock_metagenome_4	20_genomes_true	26	11	16	9	12	7	3	2	1	0
mock_metagenome_5	20_genomes_true	26	11	14	6	14	5	0	1	0	0
mock_metagenome_6	20_genomes_true	28	13	14	9	7	8	5	1	2	0
mock_metagenome_7	20_genomes_true	17	12	9	7	7	6	2	0	0	1
mock_metagenome_8	20_genomes_true	21	12	12	8	9	7	3	1	0	0
mock_metagenome_9	20_genomes_true	25	10	14	8	10	6	2	2	2	0
mock_metagenome_1	20_genomes_uneven	27	12	18	10	14	9	3	0	1	1
mock_metagenome_10	20_genomes_uneven	21	12	15	9	12	8	3	0	0	1
mock_metagenome_2	20_genomes_uneven	22	10	16	7	12	7	3	0	1	0
mock_metagenome_3	20_genomes_uneven	25	12	16	9	14	7	2	2	0	0
mock_metagenome_4	20_genomes_uneven	21	10	16	7	13	7	3	0	0	0
mock_metagenome_5	20_genomes_uneven	28	12	19	9	15	7	3	1	1	1
mock_metagenome_6	20_genomes_uneven	28	13	18	9	17	7	1	0	0	2
mock_metagenome_7	20_genomes_uneven	25	11	16	9	13	8	3	0	0	1
mock_metagenome_8	20_genomes_uneven	24	12	19	10	14	6	4	2	1	2
mock_metagenome_9	20_genomes_uneven	27	11	15	7	10	5	4	2	1	0
mock_metagenome_1	50_genomes_perfect	59	51	33	30	19	22	9	5	5	3
mock_metagenome_10	50_genomes_perfect	56	45	33	27	28	24	1	3	4	0
mock_metagenome_2	50_genomes_perfect	49	50	35	36	28	31	6	4	1	1
mock_metagenome_3	50_genomes_perfect	53	45	32	32	22	28	5	3	5	1
mock_metagenome_4	50_genomes_perfect	52	52	29	31	19	27	6	2	4	2
mock_metagenome_5	50_genomes_perfect	58	43	30	33	21	29	3	3	6	1
mock_metagenome_6	50_genomes_perfect	49	48	33	32	23	28	5	4	5	0
mock_metagenome_7	50_genomes_perfect	50	59	33	30	24	27	4	2	5	1
mock_metagenome_8	50_genomes_perfect	51	46	26	30	17	25	6	5	3	0
mock_metagenome_9	50_genomes_perfect	46	46	34	33	28	28	4	3	2	2
mock_metagenome_1	50_genomes_true	54	30	26	21	16	18	4	2	6	1
mock_metagenome_10	50_genomes_true	48	27	23	21	20	16	2	5	1	0
mock_metagenome_2	50_genomes_true	52	28	28	21	19	17	4	4	5	0
mock_metagenome_3	50_genomes_true	54	26	25	18	18	16	3	2	4	0
mock_metagenome_4	50_genomes_true	58	24	26	21	14	19	5	0	7	2
mock_metagenome_5	50_genomes_true	52	22	23	18	16	11	2	7	5	0
mock_metagenome_6	50_genomes_true	53	26	22	19	18	14	4	4	0	1
mock_metagenome_7	50_genomes_true	54	25	24	18	18	14	3	4	3	0
mock_metagenome_8	50_genomes_true	43	25	24	19	16	15	6	4	2	0
mock_metagenome_9	50_genomes_true	41	26	25	18	13	16	8	2	4	0
mock_metagenome_1	50_genomes_uneven	50	28	31	20	22	18	6	2	3	0
mock_metagenome_10	50_genomes_uneven	63	28	38	23	26	17	7	4	5	2
mock_metagenome_2	50_genomes_uneven	46	26	28	17	19	14	6	1	3	2
mock_metagenome_3	50_genomes_uneven	49	23	33	21	20	18	8	2	5	1
mock_metagenome_4	50_genomes_uneven	54	26	33	15	22	8	10	4	1	3
mock_metagenome_5	50_genomes_uneven	41	27	26	20	22	16	2	3	2	1
mock_metagenome_6	50_genomes_uneven	58	30	32	18	16	16	12	1	4	1
mock_metagenome_7	50_genomes_uneven	51	25	32	19	23	18	6	1	3	0
mock_metagenome_8	50_genomes_uneven	55	26	32	19	19	14	7	5	6	0
mock_metagenome_9	50_genomes_uneven	50	25	28	22	23	19	3	3	2	0

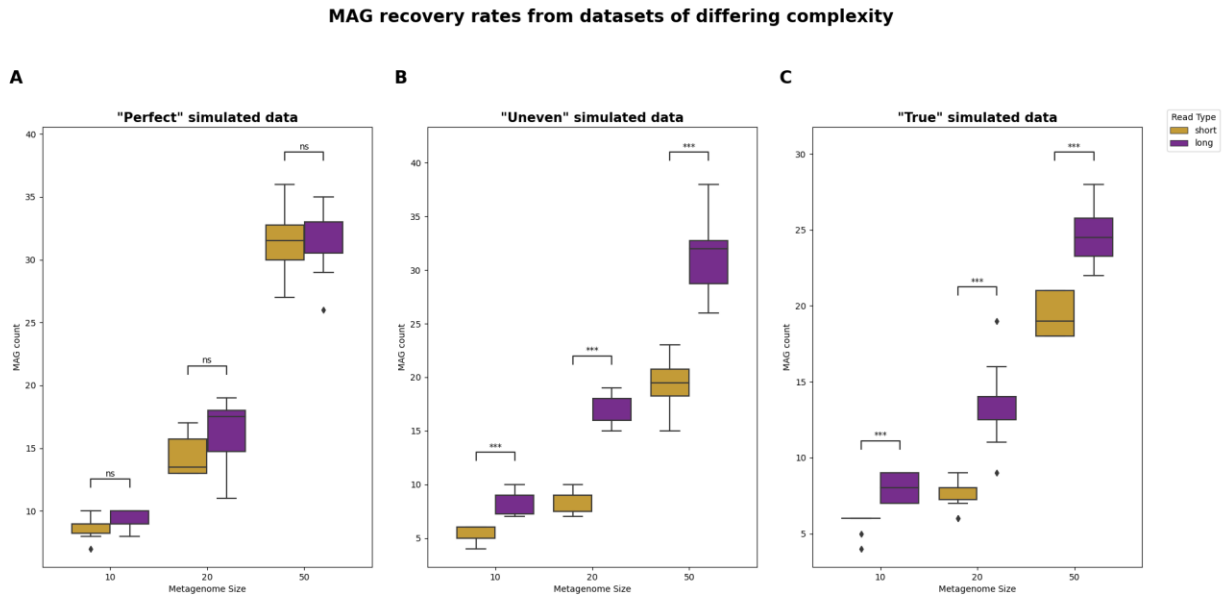
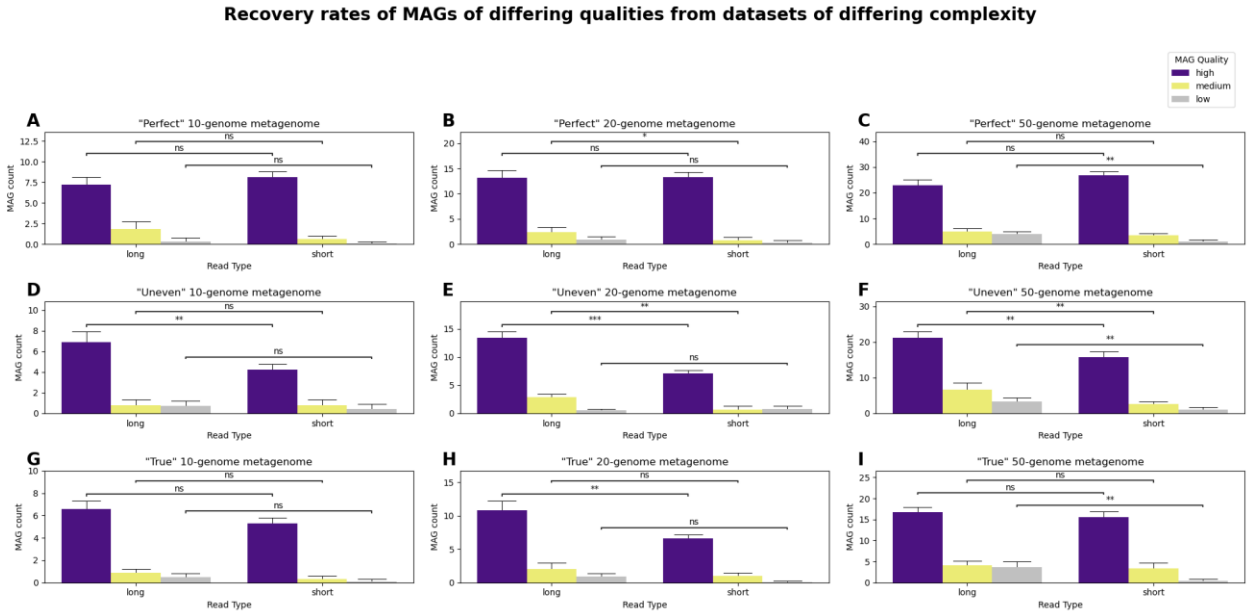


Figure 6. **Comparison of metagenome-assembled genome (MAG) recovery capabilities from assemblies produced using short or long reads.** Data depicted show the total number of MAGs recovered from assemblies of simulated short or long read sequence data. "Perfect" datasets consisted of reads without sequencing errors and organism abundance being evenly distributed (7A), "Uneven" consisted of reads without sequencing errors and organism abundance being randomly assigned (7B), and "True" consisted of reads with simulated sequencing errors and organism abundance being randomly assigned (7C). Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

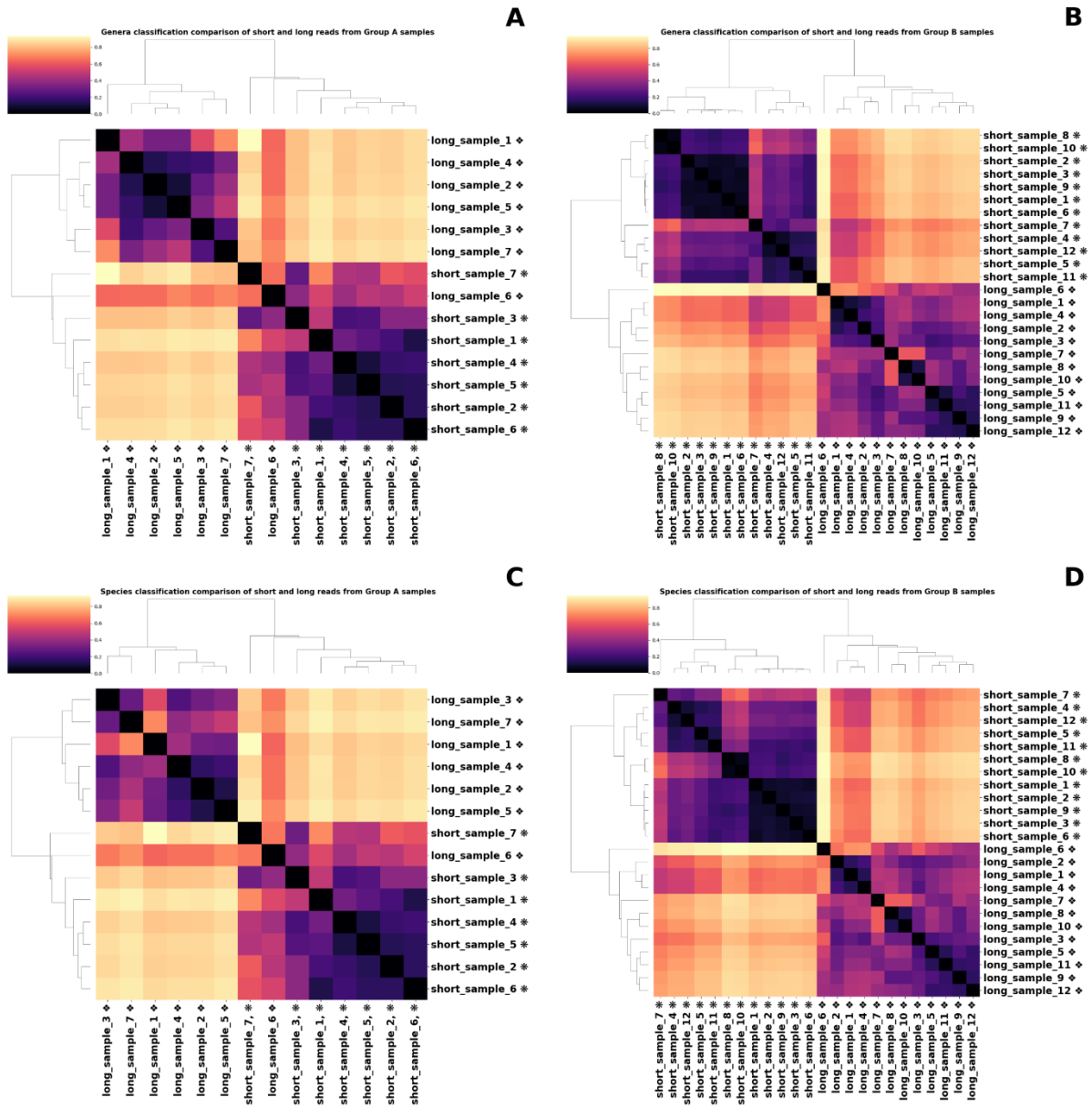


Supplemental Figure 4. **Comparison of metagenome-assembled genome (MAG) counts recovered from short and long read assemblies by quality.** Barplots depict the total number of MAGs of a given quality recovered from both short and long read assemblies. "Perfect" denotes simulated data have no errors and abundances are evenly distributed for each organism in the metagenome. "Uneven" denotes simulated data have no errors, but variable abundances for each organism. "True" denotes simulated data have simulated sequencing errors based on the read type, as well as variable abundances for each organism.

Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Microbial compositional differences between short and long reads

Using experimental data obtained from mouse fecal pellets, we examined whether read type significantly altered inferences of microbial composition for a metagenomic sample. Qualitative assessment showed clustering of results around similar read types instead of around sample types (Figure S4). This trend was also observed when results were run through a PCA where samples clustered around similar read types instead of around similar sample types (Figure 8).



Supplemental Figure 5. **Hierarchically-clustered heatmap of Bray-Curtis Dissimilarity.** Heatmap visualizing the dissimilarity of samples based on the read type used in beta-diversity assessment. Brighter values indicate lower similarity (or higher Bray-

Curtis dissimilarity), darker values indicate higher similarity (or lower Bray-Curtis dissimilarity). ❖: Samples consist of long read data. ❋: Samples consist of short read data

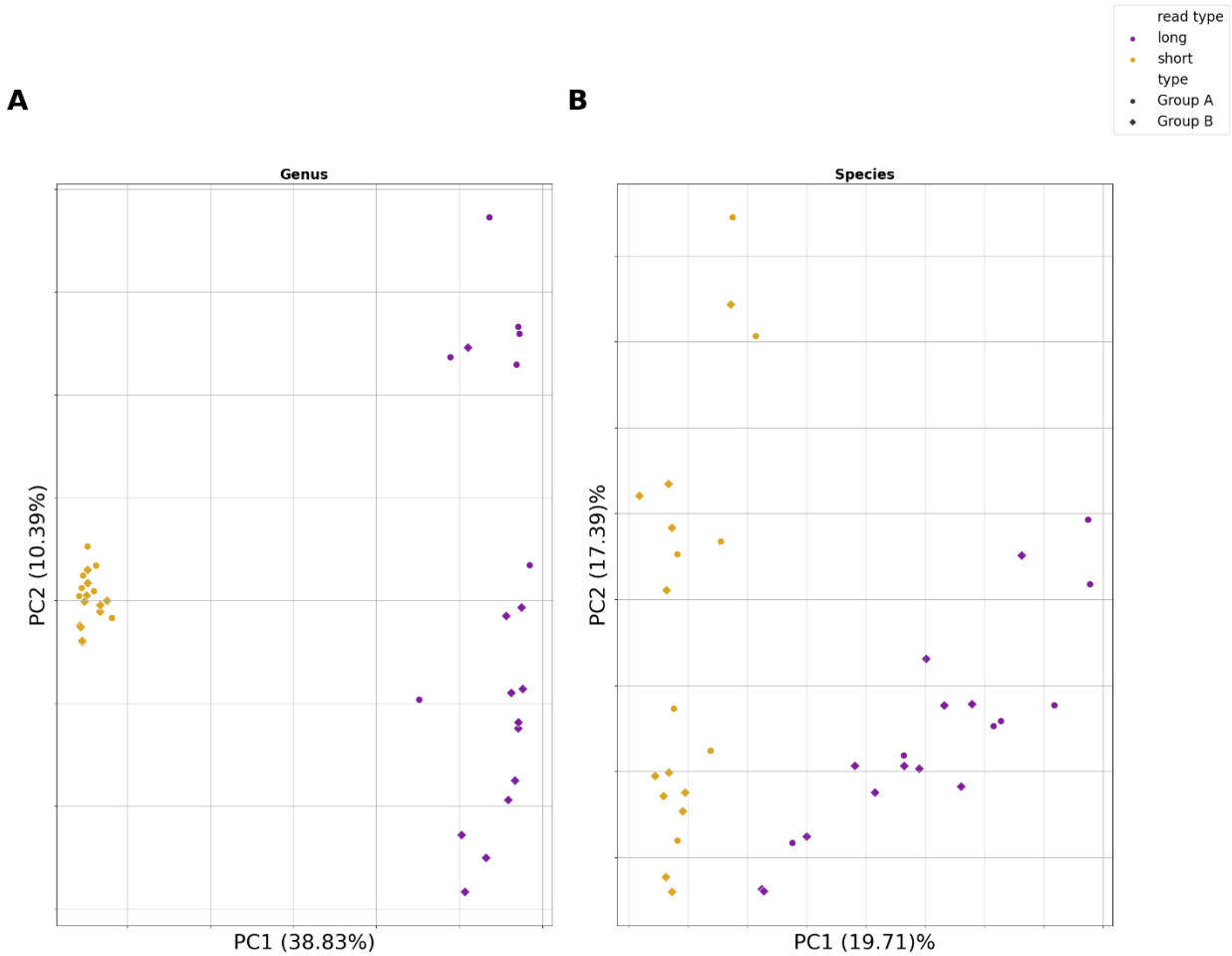


Figure 7. **Principal Component Analysis (PCA) results from short and long read sequence data analysis of empirical metagenomic samples from mouse fecal pellets.** PCA plot depicting the distribution of metagenomic samples according to their two main principal components. Coloration indicates the read type: purple for long read data and yellow for short read data. Shape indicates the sample type. Principal components 1 and 2 are on the x and y-axis respectively with their explained variance ratios written as a percentage

Discussion

For metagenomic studies of microbial communities, long-read sequencing platforms potentially offer numerous advantages, yet short read sequencing remains the most commonly applied technology (Watson et al. 2021). We sought to quantitatively assess the use long-read sequence data by using simulated metagenomes with increasing complexity. We found long reads produced higher quality assemblies that more accurately depicted simulated metagenomes when compared to results from short reads. Analyses that rely on reads instead of contigs, including relative abundance estimation, were also improved by using long read data. We then used paired long and short read metagenomic data from murine fecal samples to assess the generalizability of our findings. This showed that long reads perform better with real-world applications. Overall,

we highlight that choice of sequence type has a direct impact on the ability to accurately identify comprising members of a diverse microbial community.

For optimal performance, sequencing data must be capable of producing high quality assemblies for taxonomic classification, relative abundance estimation, functional annotation, and other related analyses. We show that in simulated long read assemblies had significantly higher percent genome coverages than short read assemblies. Analyses that rely on examining contiguous sequences, such as gene annotation, would thus benefit from assemblies generated using long-read data. Short reads are known to struggle with repetitive regions that are common in many bacterial genomes (Hu et al., 2021), which likely explains the lower genome coverages and greater discontiguity observed from short read assemblies. Our results also suggest long reads would perform better at recovering novel genomes; however, that was not directly tested here. Higher NGA50 values indicate greater contiguity, further supporting the findings highlighted by higher genome fraction observed; however, long-read assemblies presented significantly more misassemblies (Figure 2C, F, I). This observation may be associated with lower per-base accuracy of long-read sequencers, as simulated in "True" datasets. For example, the accuracy of ONT sequencing, until recent advances with V14 kit chemistry, was approximately 90-95%, whereas Illumina™ short read accuracy is 99.99%. Interestingly, a high rate of misassemblies was also observed among long-read assemblies with no simulated errors. As such, an alternative explanation is that metagenomic assemblers, in attempt to balance accuracy and contiguity, increase the possibility of misassemblies (Kolmogorov et al., 2020).

Taxonomic classification is a central to metagenomic studies and several tools exist for use on filtered reads or assembled contigs. For long-read assemblies, we found that classifications demonstrated significantly higher precision at both the genus and species level. When classifying filtered reads at the genus level, short and long reads did not significantly differ (Supplementary Figure 2). However, at the species level, long reads retained higher precision, indicating a lower rate of false positive classifications. For assemblies or filtered reads, we observed no difference in sensitivity. These findings are relevant for the potential application of long-read metagenomic sequencing to infectious disease diagnostics (Bastida et al., 2021; Hoang et al., 2022; Huang et al., 2019). For example, the ability of ONT sequencing platforms to generate sequencing reads in real time, which can be used for taxonomic classification without sacrificing precision or sensitivity, is invaluable and avoids lengthy culturing, isolation, and molecular diagnostics.

In addition to taxonomic classification, assessment of differences in the relative abundance of comprising species is of great interest. This generally involves alignment of reads to a reference genome database and subsequent estimation of abundances from the coverage, which can be performed using both short and long reads (Verma & Sharma, 2020; Warwick-Dugdale et al., 2019). Our results show that long reads provide better estimation of the true abundances of each simulated genome in a metagenome, regardless of metagenome complexity (Figure 6). The improved performance likely results from Bracken's utilization of Kraken2 reports for calculation of relative abundance, whereby having more accurate classifications leads to better estimations. False positive identifications would remove reads from being assigned to

organisms present in a metagenome, thus skewing estimated abundances for all organisms. Studies that derive conclusions based on significant shifts in abundances of either whole taxa or specific organisms can thus benefit from reliance on abundance estimation. Also, in clinical diagnostics, accurate estimation of abundance may enable clinicians to identify the etiologic microbe associated with the disease of interest (Wen et al., 2017).

A considerable challenge in microbial research is that most bacteria are non-culturable, which makes identification of organisms of potential importance difficult. Using shotgun sequencing and *de novo* metagenomic assembly, it is possible to recover both known and novel genomes and infer the identity and functional capacities of the organisms (Parks et al. 2017). Assemblies produced from long read data resulted in recovery of significantly more MAGs in both "uneven" and "true" datasets, regardless of the number of organisms in each metagenome (Figure 7). This was likely due to the ability of longer reads to capture more genomic information, thus enabling binning tools to separate genetically similar organisms more accurately. We observed that long read assemblies produced more total bins than short read assemblies, which may have contributed to the greater recovery rate since total MAG recovery ignored MAG quality. When examining the quality of MAGs recovered, long reads often produced significantly higher quality MAGs than their short read counterparts. Quality assessment relies on predictions of whether contigs in a bin likely belong to the same organism. This likely explains why discontiguous assemblies from short reads did not produce as many high-quality MAGs as more small contigs may have been misassigned.

Recently, it was found that the choice of sequencing platform can directly impact the taxa reported during analysis (Stevens et al., 2023). However, this study relied on taxonomic classification using partial/total 16S sequences and not shotgun metagenomic sequencing. In the present study, bacterial metagenomic DNA extracted from murine fecal samples were sequenced using both short- and long-read sequencing platforms. Qualitative assessment measuring the beta diversity between samples of the same type, but different sequence data, revealed samples clustered by sequence type, not sample type (Supplementary Figure 4). Comparisons using PCA revealed the same trend of samples clustering by sequence type, rather than by sample type (Figure 8). This is likely to be due to an inflation of misclassified taxa in short read assemblies, causing clustering to be due to those unique taxa. This is consistent with our findings that simulated short read data produced more false positives than long reads. This, in turn, artificially increased the diversity of the sample and explains clustering due to read type instead of sample type. These results support the previous findings that the choice of sequencing platform directly impacts the taxa observed (Stevens et al., 2023).

Taken together, our results show that long reads produce the more accurate representation of a metagenome, along with providing high quality MAGs that facilitate downstream metagenomic analysis. While this study provides evidence supporting the use of long read sequencing for metagenomic research, it does have some limitations. The most complex metagenomes that were simulated consisted of 50 organisms with variable abundance and simulated sequencing errors specific to each read type, but empirical data may consist of hundreds or even thousands of unique organisms (Bastida et al. 2021). Future work could

employ simulations that mimic high diversity as well as abundance distributions observed in real metagenomic samples; however, we feel that increasing taxa would only increase the disparity between sequence data types. Another consideration is that pipelines for processing and analyzing metagenomic data can vary considerably. Various tools exist for read correction, assembly, polishing, taxonomic classification, and binning, all of which can affect results. Steps such as read error correction and assembly polishing are often used in metagenomic studies; however, we did not include them here to reduce computational requirement and to limit variables in the comparison, especially if they are unique to one read type. Another limitation was that binning algorithms are usually designed with short reads in mind, leveraging the higher coverage of data that accompanies short read sequencing. In recent years, tools such as a LRBinner have emerged which offer better binning for error-prone long reads (Wickramarachchi and Lin 2022), but those were not applied here. Overall, we aimed to use the most commonly employed tools used for these studies at the time of writing. Last, we used a limited number of real-world samples, which could be expanded in future studies.

Short and long read sequencing have their respective places in metagenomic research. Short read sequencing does have its strengths, especially with pipelines for analyzing short read data being more established. For studies concerned with misassemblies, such as those focused on specific species of interest, short reads produce significantly fewer and may be preferable. For studies concerned with microbial composition, long reads provided higher precision with similar sensitivity to short reads in spite of lower sequencing depths. Leveraging the strengths of both read types is also possible. Tools such as OPERA-MS combine long reads containing more genetic information per-read with high accuracy short reads (Bertrand et al., 2019). Other programs like PolyPolish enable the use of short reads for improving long read assembly quality (Wick & Holt, 2022). Further advancement may shift practice towards use of hybrid approaches as has occurred with genome sequencing. In conclusion, our results show long read sequence data outperforms short read sequence data when used for taxonomic classification, relative abundance estimation of microbial members, and MAG recovery from metagenomic samples. This work provides direct evidence for the consideration of long read sequencing for research focused on accurate reconstruction of microbial populations and its strength in clinical diagnostics.

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Figure Legends

Figure 1. Flow chart detailing the set up for simulated metagenomic datasets. Perfect metagenomic samples had simulated reads with no sequencing errors and an even abundance distribution of organisms. Uneven samples had simulated reads with no sequencing errors and randomly assigned abundance values for organisms in the metagenome. True datasets had simulated reads with simulated sequencing errors specific to each read type based on the platform they would be generated from and randomly assigned abundance values for members of each metagenome.

Figure 2. Assembly graphs from a “True” 10-genome metagenome visualized by Bandage. 2A. Bandage graph generated from metagenomic assembly of long read sequence data. 2B. Bandage graph generated from metagenomic assembly of short read sequence data. The black box highlights small, disconnected contigs.

Figure 3. Comparisons of assembly quality from metaQUAST reports. Violin plots depict the differences in genome fraction values from all genomes in a set of metagenomes (A, D, G). Stripplots depict the differences in NGA50 across all genomes in a set of metagenomes (B, E, H). Barplots depict total misassembly counts across all genomes in a set of metagenomes (C, F, I). “Perfect” denotes simulated data have no errors and abundances are evenly distributed for each organism in the metagenome. “Uneven” denotes simulated data have no errors, but variable abundances for each organism. “True” denotes simulated data have simulated sequencing errors based on the read type, as well as variable abundances for each organism. Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure 4. Performance evaluation of genus-level classification on metagenomic assemblies using short or long reads. Stripplots and boxplots were used to visualize the performance metrics (precision, recall, and F-score) on metagenomes of varying size and complexity. “Perfect” simulated reads have no errors and even abundances across organisms (A, B, C). “Uneven” simulated reads have no errors and randomly varied abundances across organisms (D, E, F). “True” simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a One-way ANOVA. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure 5. Performance evaluation of species-level classification on metagenomic assemblies using short or long reads. Stripplots and boxplots were used to visualize the performance metrics (precision, recall, and F-score) on metagenomes of varying size and complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a One-way ANOVA. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure 6. Comparison of short and long read capacity for relative abundance estimation. Overlapping stripplots and boxplots were used to portray the differences in relative abundance estimation of organisms present in metagenomic samples when using short or long reads. Read types were compared across metagenomes of varying complexity at both the genus and species level. "Perfect" datasets consisted of reads without sequencing errors and organism abundance was evenly distributed (6A, 6D). "Uneven" datasets consisted of reads without sequencing errors and randomly distributed abundances of organisms (6B, 6E). "True" datasets consisted of reads with simulated errors and randomly distributed abundances of organisms (6C, 6F). Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure 7. Comparison of MAG recovery capabilities from assemblies produced using short or long reads. Data depicted show the total number of MAGs recovered from assemblies of simulated short or long read sequence data. "Perfect" datasets consisted of reads without sequencing errors and organism abundance being evenly distributed (7A). "Uneven" datasets consisted of reads without sequencing errors and organism abundance being randomly assigned (7B). "True" datasets consisted of reads with simulated sequencing errors and organism abundance being randomly assigned (7C). Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure 8. Principal Component Analysis results from short and long read sequence data analysis of real-world metagenomic samples. PCA plot depicting the distribution of metagenomic samples according to their two main principal components. Coloration indicates the read type, purple for results from long read data and yellow for results from short read data. Shape indicates the sample type, circles for control data and diamonds for experimental data. Principal components 1 and 2 are on the x and y-axis respectively with their explained variance ratios written as a percentage

Figure S1. Performance evaluation of genus-level classification using short and long reads. Overlapping stripplots and boxplots were used to visualize the performance metrics (precision, recall, and F-score) on metagenomes of varying complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I).

Significance was calculated using a Mann-Whitney U test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure S2. Performance evaluation of species-level classification using short and long reads. Overlapping stripplots and boxplots were used to visualize the performance metrics (precision, recall, and F-score) on metagenomes of varying complexity. "Perfect" simulated reads have no errors and even abundances across organisms (A, B, C). "Uneven" simulated reads have no errors and randomly varied abundances across organisms (D, E, F). "True" simulated reads have simulated errors specific to each read type and varied abundances across organisms (G, H, I). Significance was calculated using a One-way ANOVA. ns: non-significant p-value ($p > 0.05$; blank means $p = 1$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure S3. Comparison of MAG counts recovered from short and long read assemblies by quality. Barplots depict the total number of MAGs of a given quality recovered from both short and long read assemblies. "Perfect" denotes simulated data have no errors and abundances are evenly distributed for each organism in the metagenome. "Uneven" denotes simulated data have no errors, but variable abundances for each organism. "True" denotes simulated data have simulated sequencing errors based on the read type, as well as variable abundances for each organism. Significance was calculated using a Mann-Whitney U Test with Bonferroni correction. ns: non-significant p-value ($p > 0.05$), *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$

Figure S4. Hierarchically-clustered heatmap of Bray-Curtis Dissimilarity. Heatmap visualizing the dissimilarity of control and experimental samples based on the read type used in beta-diversity assessment. Brighter values indicate lower similarity, darker values indicate higher similarity.

Table S1. Results of Mann-Whitney U testing for genome fraction, NGA50, and number of misassembly values from metaQUAST.

[1]"Perfect" indicates reads had no errors and abundances were evenly distributed. "Uneven" denotes reads had no errors, but variable abundances for each organism. "True" reads had simulated sequencing errors based on the read type and variable abundances for the organisms.

[2]P-value from Mann-Whitney U test with Bonferroni correction applied

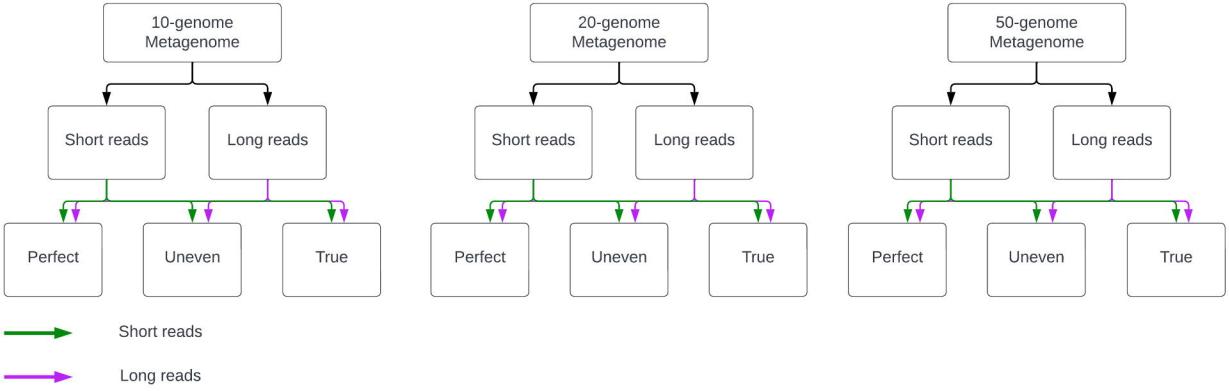
Table S2. Bin and MAG counts from metagenomic samples. Total bins recovered for each metagenomic sample and counts of MAGs identified from those bins. MAGs were further separated by quality as described in the methods section.

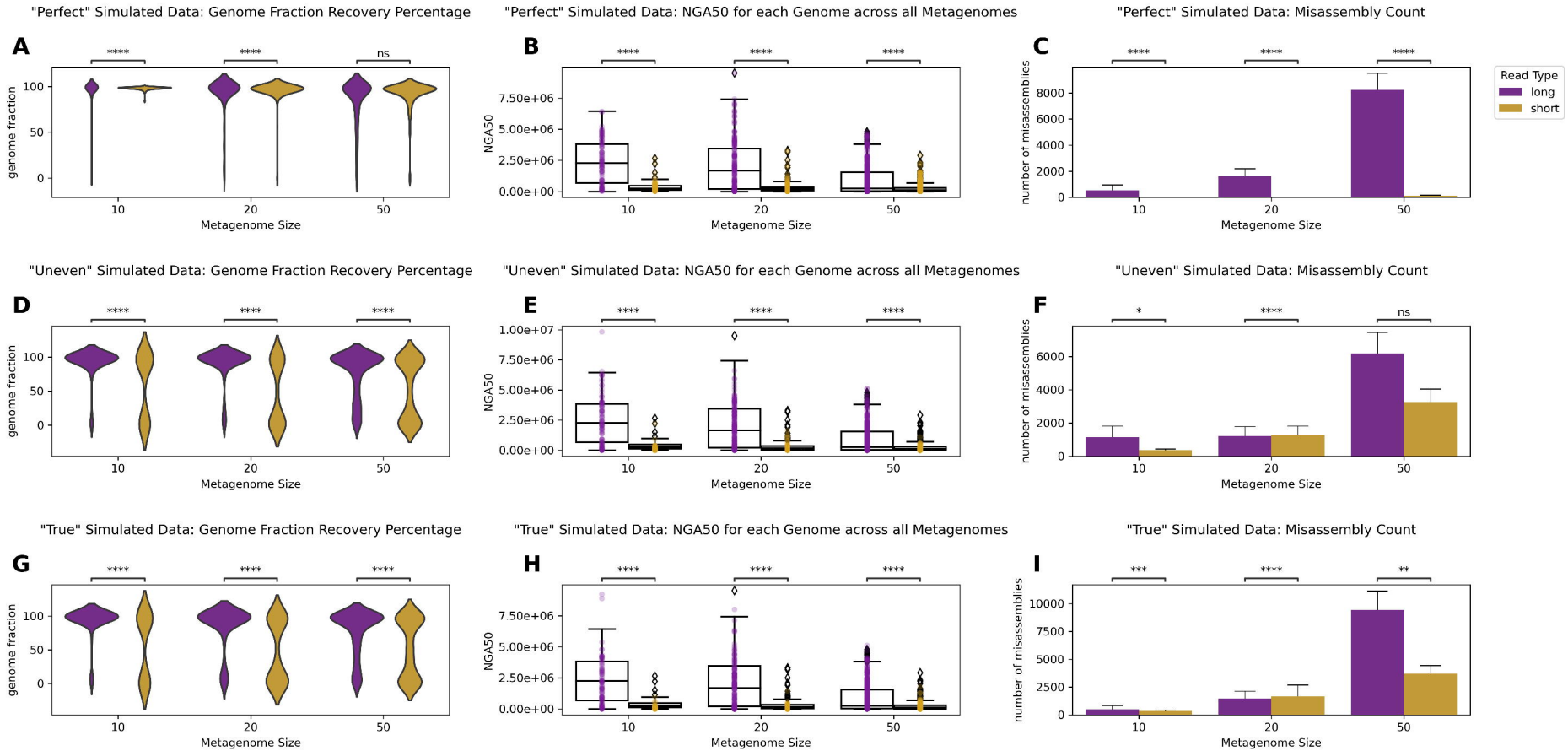
[1]Samples consist of the 10 metagenomes simulated for a given complexity

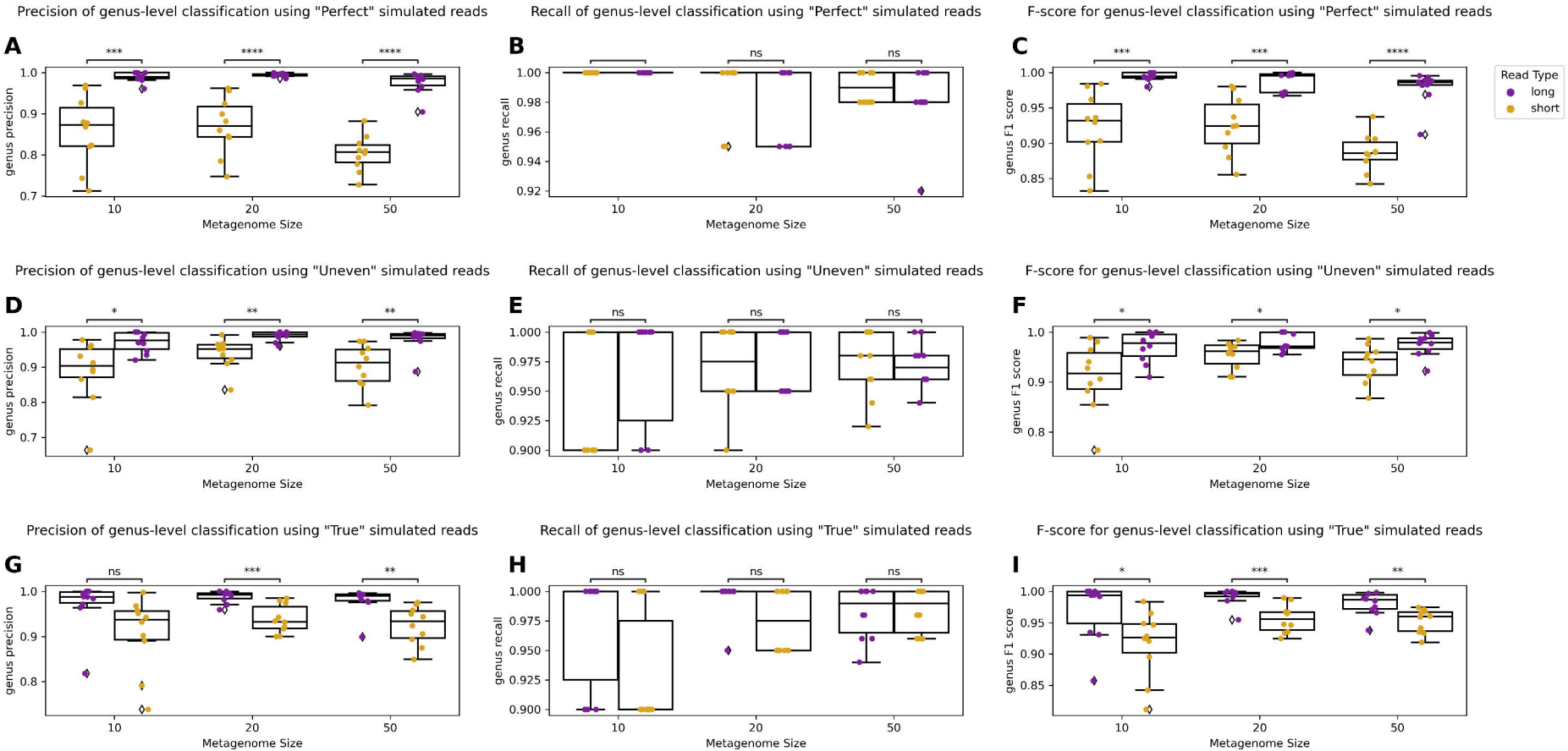
[2]A metagenome set is the given complexity level for a collection of simulated metagenomes

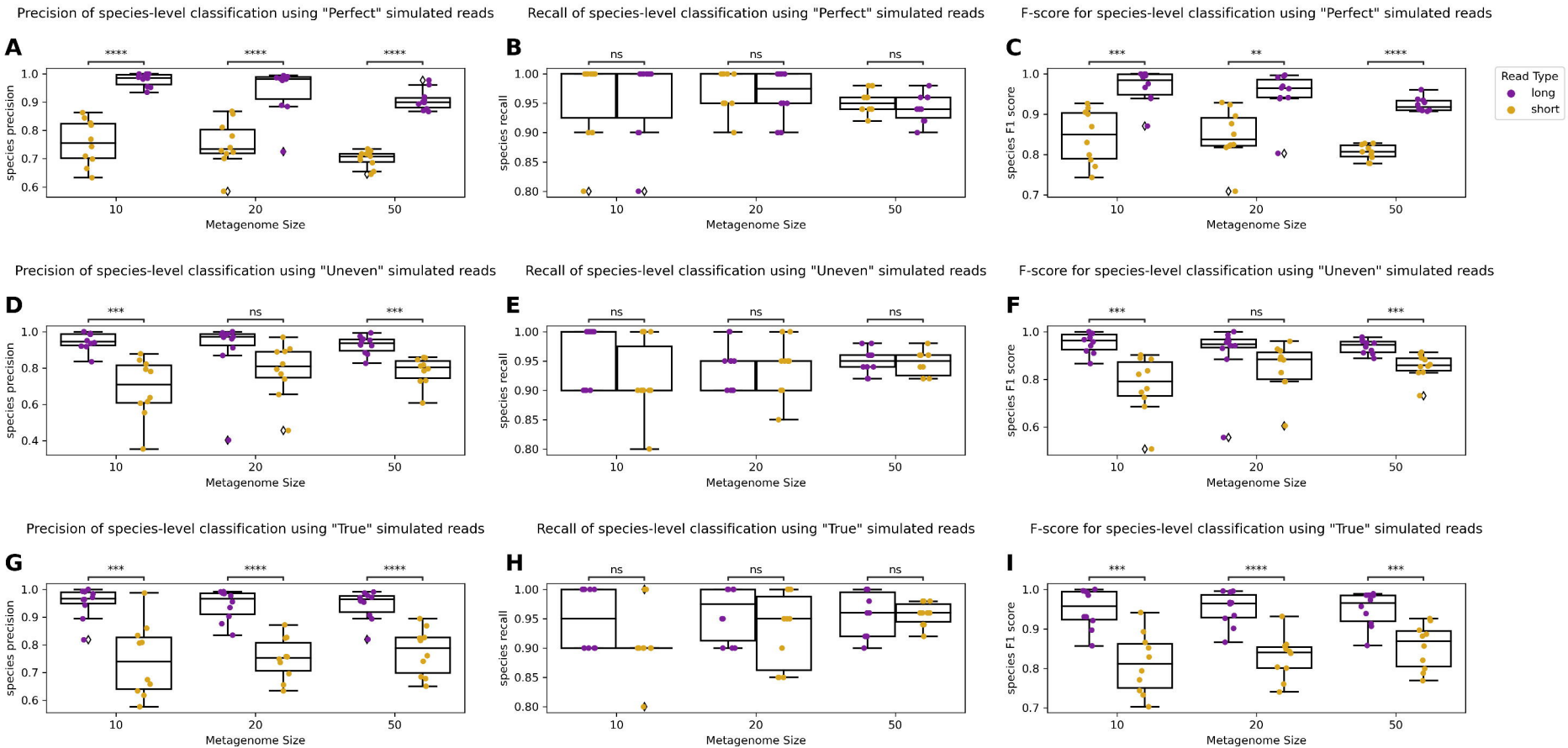
[3a]Sum of all bins predicted by MetaBAT2 from long-read assemblies

[3b]Sum of all bins predicted by MetaBAT2 from short-read assemblies

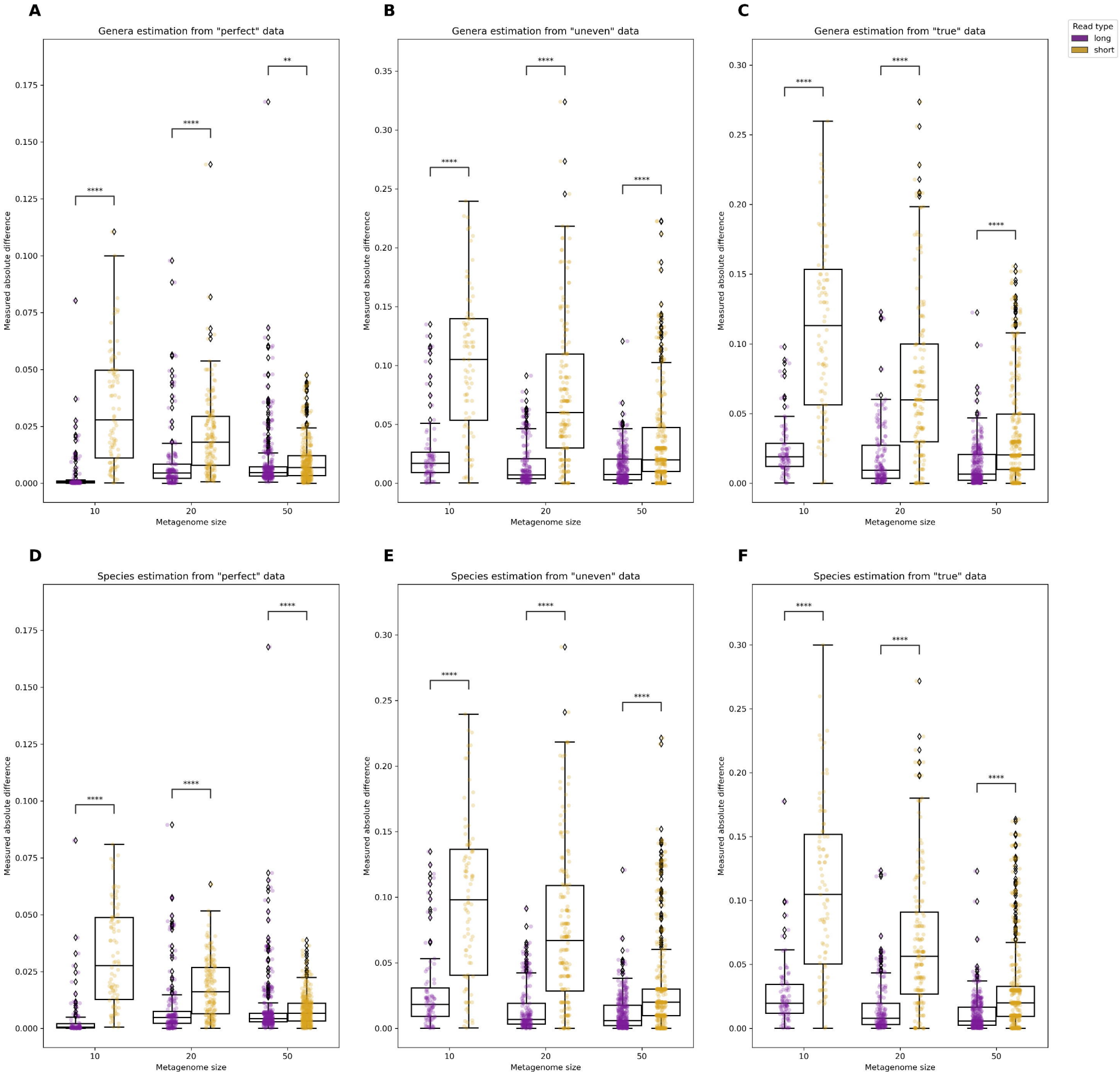

10-genome Metagenome
Short reads
Long reads
Perfect
Uneven
True
20-genome Metagenome
Short reads
Long reads
Perfect
Uneven
True
50-genome Metagenome
Short reads
Long reads
Perfect
Uneven
True
Short reads
Long reads


"Perfect" Simulated Data: Genome Fraction Recovery Percentage
A
genome fraction
Metagenome Size
"Perfect" Simulated Data: NGA50 for each Genome across all Metagenomes
B
NGA50
Metagenome Size
"Perfect" Simulated Data: Misassembly Count
C
number of misassemblies
Metagenome Size
Read Type
long
short
"Uneven" Simulated Data: Genome Fraction Recovery Percentage
D
genome fraction
Metagenome Size
"Uneven" Simulated Data: NGA50 for each Genome across all Metagenomes
E
NGA50
Metagenome Size
"Uneven" Simulated Data: Misassembly Count
F
number of misassemblies
Metagenome Size
"True" Simulated Data: Genome Fraction Recovery Percentage
G
genome fraction
Metagenome Size
"True" Simulated Data: NGA50 for each Genome across all Metagenomes
H
NGA50
Metagenome Size
"True" Simulated Data: Misassembly Count
I
number of misassemblies
Metagenome Size

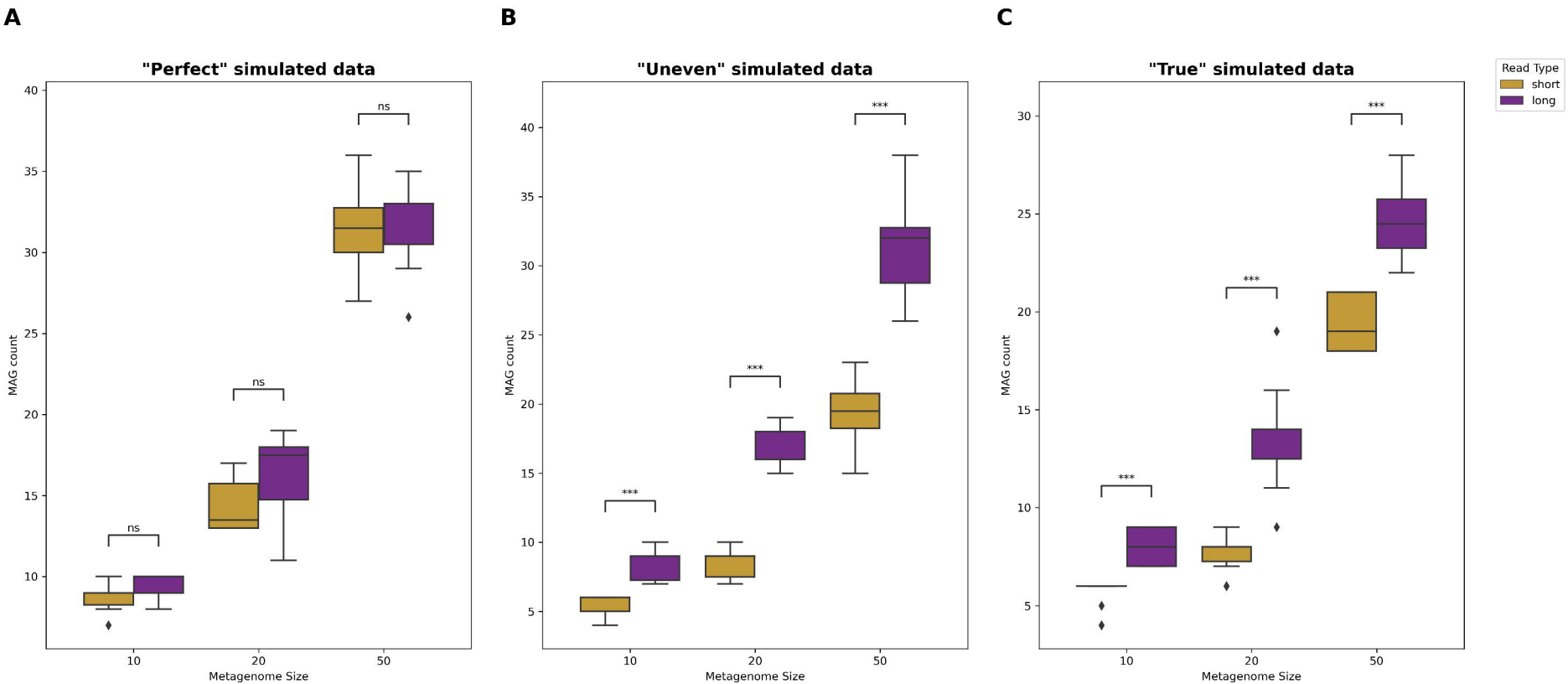

Precision of genus-level classification using "Perfect" simulated reads
Recall of genus-level classification using "Perfect" simulated reads
F-score for genus-level classification using "Perfect" simulated reads
Precision of genus-level classification using "Uneven" simulated reads
Recall of genus-level classification using "Uneven" simulated reads
F-score for genus-level classification using "Uneven" simulated reads
Precision of genus-level classification using "True" simulated reads
Recall of genus-level classification using "True" simulated reads
F-score for genus-level classification using "True" simulated reads
A
B
C
D
E
F
G
H
I
genus precision
genus recall
genus F1 score
Metagenome Size
10
20
50
Read Type
long
short

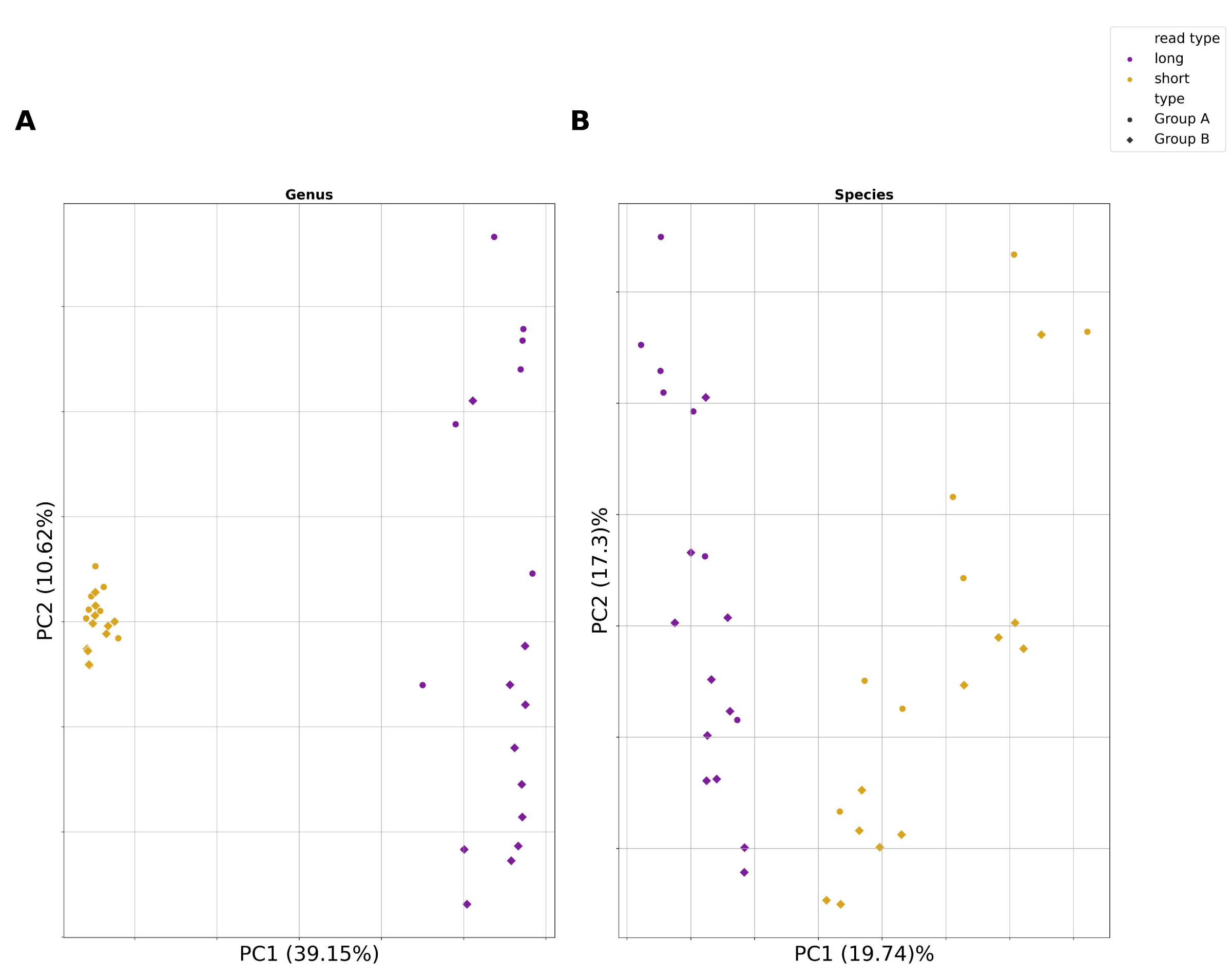

Precision of species-level classification using "Perfect" simulated reads
Recall of species-level classification using "Perfect" simulated reads
F-score for species-level classification using "Perfect" simulated reads
Precision of species-level classification using "Uneven" simulated reads
Recall of species-level classification using "Uneven" simulated reads
F-score for species-level classification using "Uneven" simulated reads
Precision of species-level classification using "True" simulated reads
Recall of species-level classification using "True" simulated reads
F-score for species-level classification using "True" simulated reads
A
B
C
D
E
F
G
H
I
species precision
species recall
species F1 score
Metagenome Size
10
20
50
****
***
**
ns
Read Type
long
short

Comparative analysis: short vs. long reads for accurate relative abundance estimation of taxa

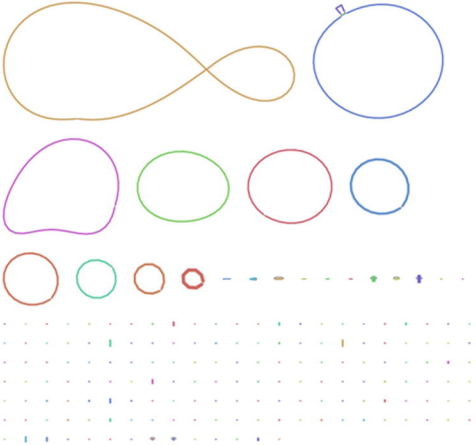


MAG recovery rates from datasets of differing complexity



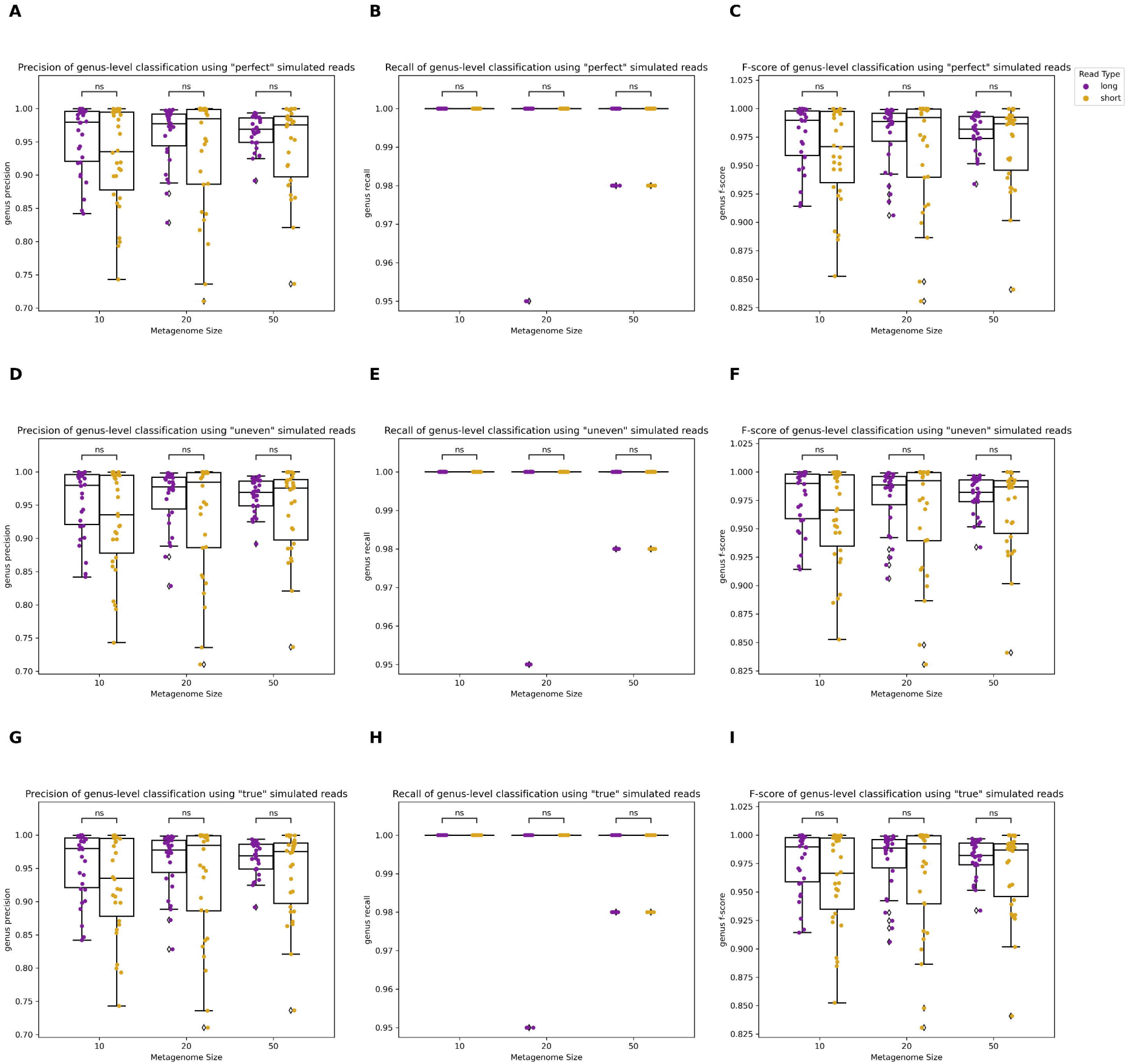

read type
long
short
type
Group A
Group B
A
B
Genus
Species
PC2 (10.62%)
PC1 (39.15%)
PC2 (17.3)%
PC1 (19.74)%

A

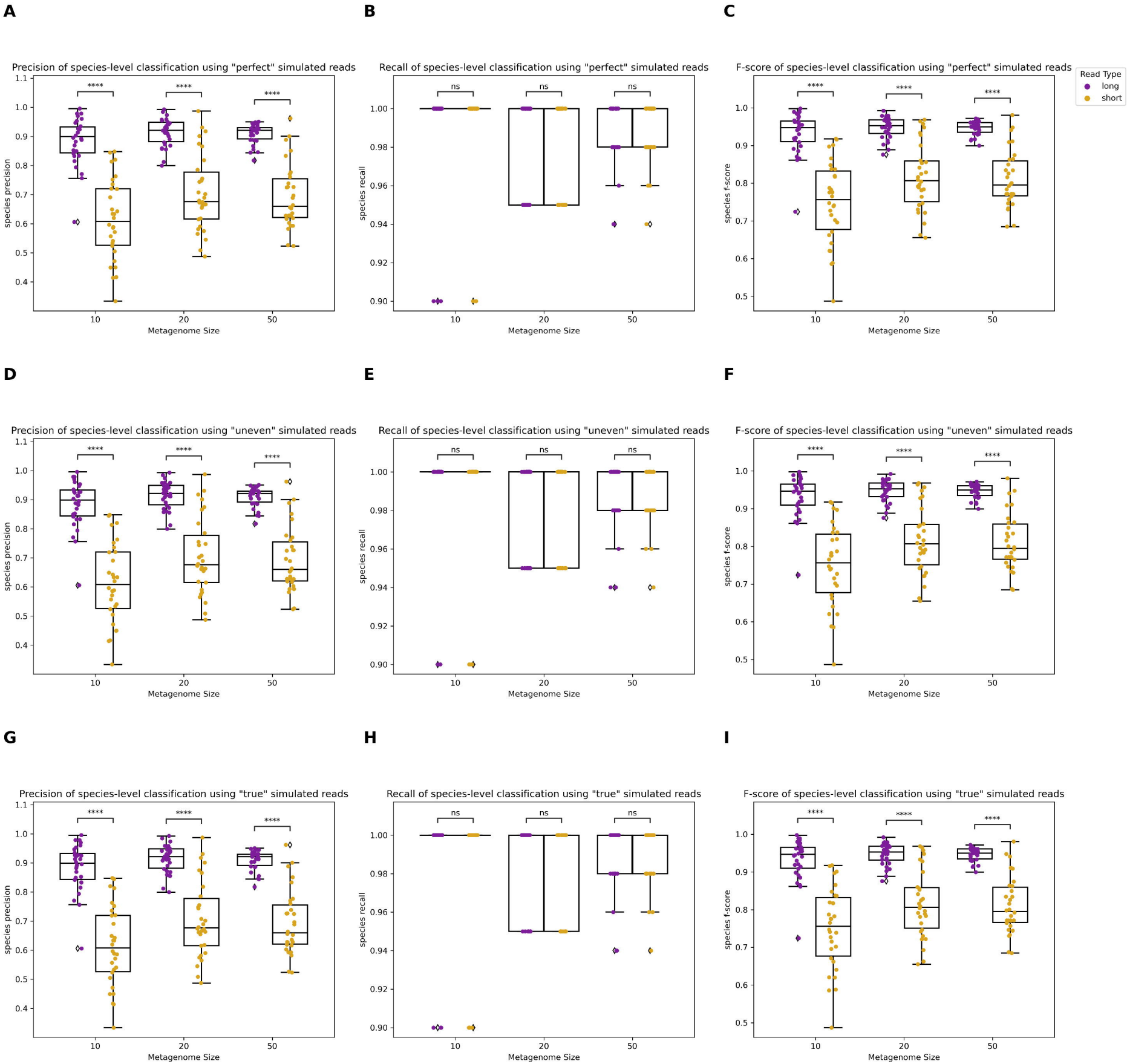


B

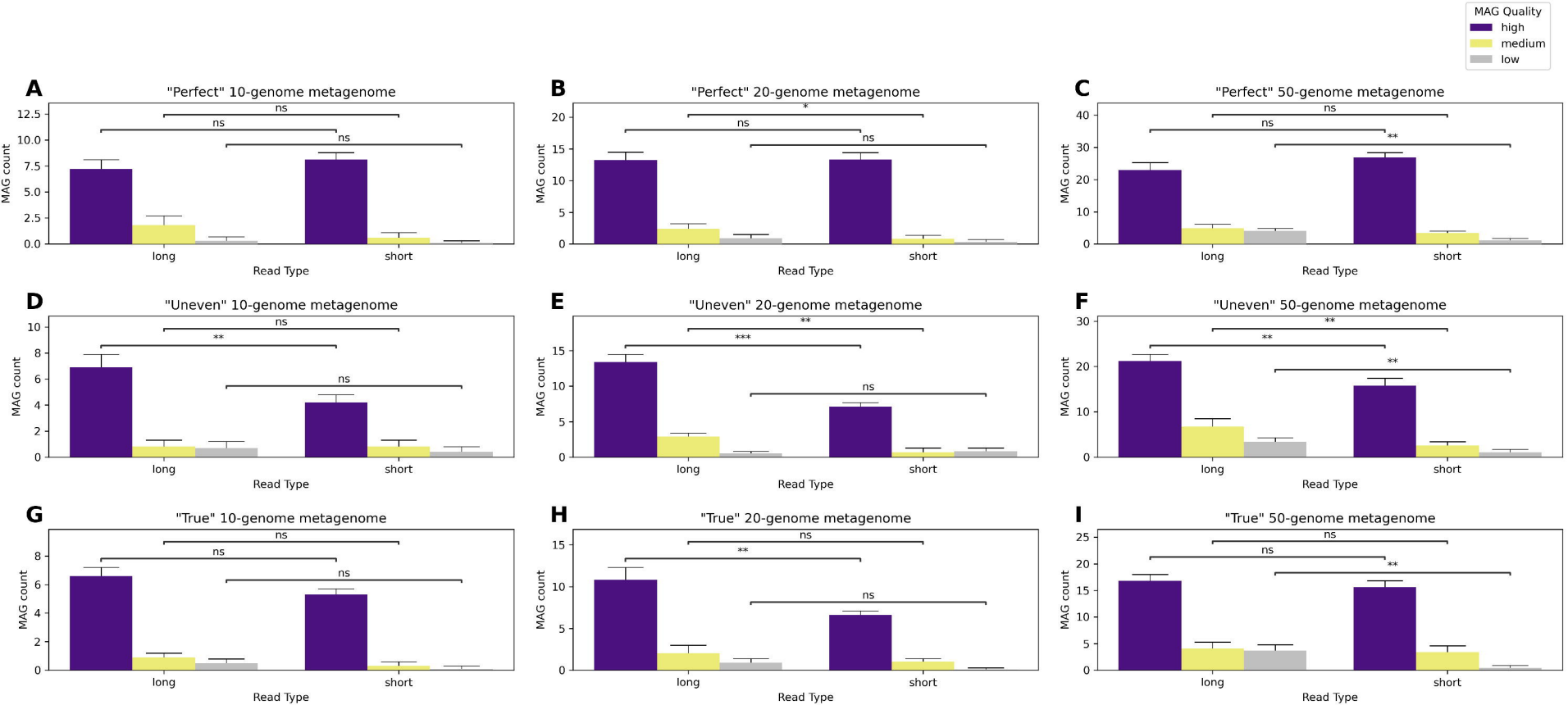
Evaluation of Genera Classification of Short and Long Metagenomic Reads

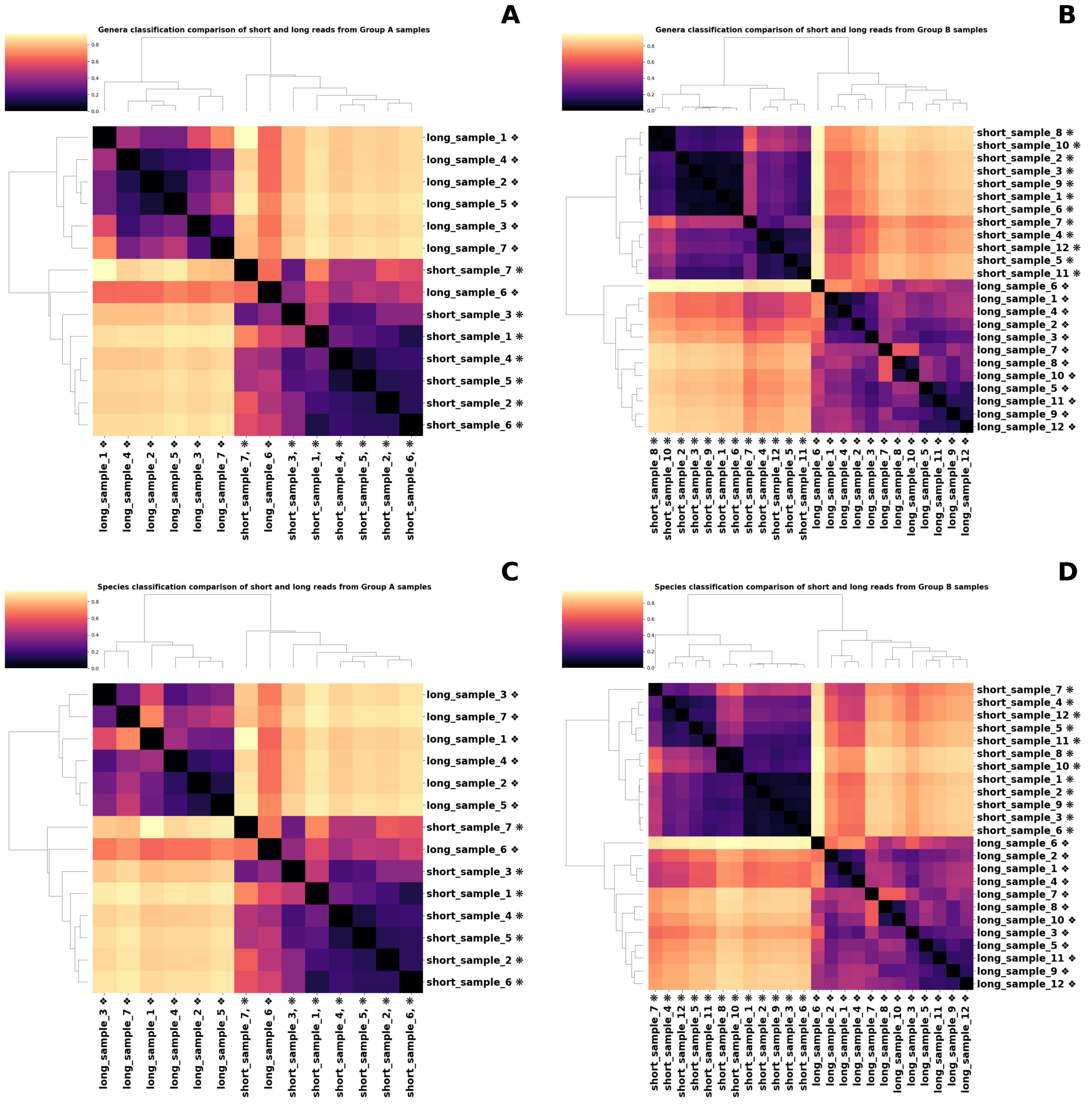


Evaluation of Species Classification of Short and Long Metagenomic Reads



Recovery rates of MAGs of differing qualities from datasets of differing complexity




A
Genera classification comparison of short and long reads from Group A samples
0.8
0.6
0.4
0.2
0.0
long_sample_1 ❖
long_sample_4 ❖
long_sample_2 ❖
long_sample_5 ❖
long_sample_3 ❖
long_sample_7 ❖
short_sample_7 ✳
long_sample_6 ❖
short_sample_3 ✳
short_sample_1 ✳
short_sample_4 ✳
short_sample_5 ✳
short_sample_2 ✳
short_sample_6 ✳
B
Genera classification comparison of short and long reads from Group B samples
0.8
0.6
0.4
0.2
0.0
short_sample_8 ✳
short_sample_10 ✳
short_sample_2 ✳
short_sample_3 ✳
short_sample_9 ✳
short_sample_1 ✳
short_sample_6 ✳
short_sample_7 ✳
short_sample_4 ✳
short_sample_12 ✳
short_sample_5 ✳
short_sample_11 ✳
long_sample_6 ❖
long_sample_1 ❖
long_sample_4 ❖
long_sample_2 ❖
long_sample_3 ❖
long_sample_7 ❖
long_sample_8 ❖
long_sample_10 ❖
long_sample_5 ❖
long_sample_11 ❖
long_sample_9 ❖
long_sample_12 ❖
C
Species classification comparison of short and long reads from Group A samples
0.8
0.6
0.4
0.2
0.0
long_sample_3 ❖
long_sample_7 ❖
long_sample_1 ❖
long_sample_4 ❖
long_sample_2 ❖
long_sample_5 ❖
short_sample_7 ✳
long_sample_6 ❖
short_sample_3 ✳
short_sample_1 ✳
short_sample_4 ✳
short_sample_5 ✳
short_sample_2 ✳
short_sample_6 ✳
D
Species classification comparison of short and long reads from Group B samples
0.8
0.6
0.4
0.2
0.0
short_sample_7 ✳
short_sample_4 ✳
short_sample_12 ✳
short_sample_5 ✳
short_sample_11 ✳
short_sample_8 ✳
short_sample_10 ✳
short_sample_1 ✳
short_sample_2 ✳
short_sample_9 ✳
short_sample_3 ✳
short_sample_6 ✳
long_sample_6 ❖
long_sample_2 ❖
long_sample_1 ❖
long_sample_4 ❖
long_sample_7 ❖
long_sample_8 ❖
long_sample_10 ❖
long_sample_3 ❖
long_sample_5 ❖
long_sample_11 ❖
long_sample_9 ❖
long_sample_12 ❖