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The selection of shotgun metagenomics software introduces biases in microbial profiling and pathogen detection

**Abbreviated running headline**

Metagenomics software selection biases

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**Abstract**

**Aim**

Shotgun metagenomic sequencing analysis is widely used for microbial profiling of biological specimens and pathogen detection. However, very little is known about the technical biases caused by the choice of analysis databases and software. In this study, we evaluated popular shotgun metagenomics taxonomical profiling software to characterize the microbial compositions of wildly collected rodent samples.

**Method and Results**

We analyzed shotgun metagenomic sequence data from three sets of wild rodent tissue samples collected from St.Kitts using four different databases and nine of the most widely used metagenomics software. We demonstrated the discrepancies in results when different databases and software were used, which cause significant variation in microbial communintiy characterizations. Our analysis also showed that these software differed in their ability to identify the presence of *Leptospira,* a major zoonotic pathogen of one health importance in comparison to traditional methods.

**Conclusions**

Significant differences in compositional profiles for the same dataset while using different databases and software may lead to divergent biological conclusions in microbial profiling and zoonotic pathogens detection.

**Significance and Impact of Study**

This study highlights the importance to warrant caution while using shotgun metagenomics for pathogen detection and interpretation of taxonomical profiling analyses.

**Keywords**

shotgun metagenomic sequencing, pathogen detection, *Leptospira*, next-generation sequencing, software, databases, taxonomical profiles

**Introduction**

Studies analyzing the composition of microbial communities have been utilized in diverse study fields, such as ecology (Galbraith *et al.*, 2018; Grossart *et al.*, 2020), agriculture (Mashiane *et al.*, 2017; Granjou and Phillips, 2019), human and animal health (Chen *et al.*, 2019; Zhong *et al.*, 2019), and pharmacology (Chavira *et al.*, 2019; Wang *et al.*, 2019), as well as in zoonotic agent detection (Tun *et al.*, 2012). Zoonotic origin pathogens are responsible for over 60% of the infectious diseases identified in humans and can cause significant social and economic burdens (Karesh *et al.*, 2012; Cuervo-Soto, López-Pazos and Batista-García, 2018). Traditional methods used to identify the microbial agents within a biological specimen have relied on different laboratory techniques, including culture (Handelsman, 2004), antigen detection (Desmonts and Remington, 1980; Lequin, 2005), and nucleic acid marker detection (Yang and Rothman, 2004; Driscoll, 2009) protocols. However, these laboratory methods are limited to studying a single pathogen of interest and lack the ability to scrutinize the community of microorganisms potentially present in a sample. Next-Generation Sequencing (NGS) technologies have provided researchers with a set of culture-independent tools that identify pathogens directly from DNA sequences (Ghosh, Mehta and Khan, 2019), and characterize the diversity and abundance of microbial populations in biological specimens. These characteristics have led to the emergence of NGS technologies as popular tools for microbial profiling and pathogen detection (Tun *et al.*, 2012; Skarżyńska *et al.*, 2020; Grützke *et al.*, 2021).

Taxonomical profiling analysis in the metagenomics discipline utilizes two popular approaches: the 16S rRNA and the shotgun metagenomic sequencing-based approach (Jovel *et al.*, 2016). The 16S rRNA sequencing-based method uses polymerase chain reaction (PCR) to amplify hypervariable regions of bacterial 16S rRNA gene and compares these regions to a 16S reference database (DB) (Johnson *et al.*, 2019). In contrast, the shotgun metagenomic sequencing-based approach sequences all given DNA present in a sample (Sharpton, 2014). Although lower in cost (Breitwieser, Lu and Salzberg, 2019), 16S rRNA markers are only available in the genomes of most bacteria and archaea, and they suffer from primer amplification biases (Woese, Kandlert and Wheelis, 1990; Janda and Abbott, 2007). On the other hand, the taxonomical profiling of shotgun metagenomics sequencing data is done by comparison with a reference whole-genome DB. Since the data contain all genetic information present in the sample, this approach avoids the amplification biases observed in 16S rRNA sequencing (Fouhy *et al.*, 2016; Ranjan *et al.*, 2016) and increases the resolution of microbial identification (Durazzi *et al.*, 2021). Most importantly, it has broader applications such as functional profiling and identification of viruses and other microorganisms with simple genomes (Clark and Pazdernik, 2016).

Currently developed shotgun metagenome sequencing-based taxonomical profiling software can be separated into two groups: the alignment-based and the alignment-free software. Alignment-based software, including BLASTN (Altschul *et al.*, 1990; Johnson *et al.*, 2008; Camacho *et al.*, 2009), which aligns sequences at the nucleotide level, and Diamond (Buchfink, Xie and Huson, 2015), which aligns sequences at the protein level, have high sensitivity and have been used as the standard for metagenomics profiling. However, these software require large amount of time and computational resources to build genome alignements for the high number of sequences usually involved in metagenomics profiling studies (Cannings, 2004; Zielezinski *et al.*, 2017). Furthermore, recent investigations in alignment-based methods have reported that alignment-based software’ decrease in sensitivity with the use of mosaic genomes (ex. viruses) (Zielezinski *et al.*, 2017). To overcome these limitations, multiple software have been developed using alignment-free algorithms. For example: 1) Kraken2 (Wood, Lu and Langmead, 2019, p. 2) and CLARK (Ounit *et al.*, 2015) were designed with k-mer matching algorithms, where only subtrings of sequences were matched (Healy and Chambers, 2014); 2) Metaphlan3 (Truong *et al.*, 2015; Beghini *et al.*, 2021) was designed to identify unique genetic markers within each microbial taxon; and 3) Centrifuge (Kim *et al.*, 2016) and Kaiju (Menzel, Ng and Krogh, 2016) wew designed to optimize the time and resources of profiling by compressing the reference microbial genomes into the index structures for storaging and searching (Burrows and Wheeler, 1994), at the nucleotide and protein levels, respectively. In addition to the software mentioned above, some software were developed to improve the results of existing software, such as Bracken (Lu *et al.*, 2017) that improves Kraken2’s output by eliminating false positive assignments using a Bayesian framework, and CLARK-s (Ounit and Lonardi, 2016) improves the sensitivity of CLARK with the use of spaced Kmers. Previous benchmarks on shotgun metagenomic sequencing taxonomical profiling software have evaluated the performances of these software using either in silico or in vitro datasets (Peabody *et al.*, 2015; Escobar-Zepeda *et al.*, 2018; Ye *et al.*, 2019). The advantage of using these artificial datasets is that their performances can be evaluated by comparing their microbial profiles with the known composition of the artificial datasets. However, the performance of these software to analyze the microbial profiling and diagnostic applications of biological specimens has been less studied. For samples collected from wild animals, the microbiome compositions are unknown and potentially contain taxa that do not have genomes available in the reference DB . These situations can become a potential source of technical errors for accurate detection and profiling a sample's microbiome.

In this study, we compare the microbial profiles of tissue samples from two species of *Rattus* (*Rattus rattus* and *Rattus norvegicus*) using different metagenomic software and DBs . We especially address how the use of different DBs and software influence the profiling of the rat samples and subsequent downstream analyses. We also test how well these methods detect the zoonotic pathogen *Leptospira* in rat kidneys. Specifically, the objectives of the current study are to 1) compare the taxonomical profiles classified by four DBs and nine metagenomics profiling software introduced above; 2) demonstrate the effect of 1) in the downstream analyses; and 3) identify the presence of potential zoonotic pathogens such as *Leptospira* from each software’s profiling results. Our results show that there are significant differences among the characterizations of the microbial communities analyzed from the microbial profiles obtained using different DB and software and that caution should be taken in the choice of the appropriate software to answer a specific metagenomic question. This study highlights the potential biases introduced by metagenomic profiling software for microbial community characterization and the limitation of using shotgun metagenomics as a tool for pathogen detection.

**Materials and Methods**

**Samples.** Tissue samples from kidney (K), spleen (S), and lung (L) were obtained from four rats from two different species, *Rattus rattus* (R28) and *Rattus* *norvegicus* (R22, R26, and R27). Rats were captured from the island of Saint Kitts (longitude 17.3434° N and latitude – 62.7559°W) following protocols approved by the Ross University School of Veterinary Medicine (RUSVM) IACUC (approval # 17-01-04). DNA was extracted from samples using DNeasy Blood and Tissue Kits (QIAGEN Scientific Inc., MD, USA), following the manufacturer's protocol.

**Metagenomic shotgun sequencing.** DNA sample quality was assessed via analysis of the DNA purity and integrity with the agarose gel. DNA purity (OD260/OD280) and concentration were measured using the Nanodrop and Qubit 2.0. The library for metagenomic sequences was constructed with 1 μg DNA per sample. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina following manufacturer's instructions. The DNA sample was fragmented (350 bp), end-polished, A-tailed, ligated with Illumina sequencing adaptor and amplified with the PCR technique. The PCR products were then purified for sequencing. Before sequencing, samples were clustered on a cBot Cluster Generation System, then sequenced on an Illumina HiSeq platform for paired-end reads.

**Data pre-processing**. Sequencing adapters, low-quality reads, and host DNA reads within the metagenomic samples were removed using the software KneadData (The Huttenhower Lab, no date) with the default Trimmomatic (Bolger, Lohse and Usadel, 2014) (version 0.33) settings (SLIDINGWINDOW:4:20 MINLEN:50) and the "—very-sensitive" Bowtie (Langmead *et al.*, 2019) (version 2.3) option. The hosts' reference sequences, which were used to separate host reads from the microbial reads, were downloaded from the NCBI's RefSeq DB (Human: GCA\_000001405.28\_GRCh38.p13; *R. norvegicus*: GCF\_015227675.2\_mRatBN7.2; *R. rattus*: GCF\_011064425.1\_Rrattus\_CSIRO\_v1).

**Metagenomic profiling.**

Software. Nine software (BLASTN, Diamond, Kraken2, Bracken, Centrifuge, CLARK, CLARK-s, Metaphlan3, and Kaiju) were chosen to determine the rats tissues' metagenomic profiles. All software were used with the default settings according to the instruction manuals provided by the developers.

Database building. If the software have pre-built DBs, these were downloaded directly from the software’ homepage (BLASTN, minikraken DB of Kraken2, Centrifuge, and Metaphlan3). Otherwise, DBs were built based on the standard instructions provided by the software’ manual (CLARK, CLARK-s, Diamond, and Kaiju), with the exception of of some software that had their DBs available online with the contribution of the science community (and based on the instruction manual of the corresponding software). In this case, the DBs were downloaded directly from the online resources (standard DB of Kraken2, maxikraken DB of Kraken2, and Bracken). Detailed information about DB building is available in Table I.

For this specific analysis, a custom Kraken2 DB was built following the manual’s instructions. All the libraries included in Kraken2’s standard DBs (which include NCBI RefSeq’s bacterial, archaeal, viral, human genome, and known vectors (UniVec\_Core) libraries) were included in the costumized DB, with the addition of the two genomes from the two rat species: *R. norvegicus* (GCF\_015227675.2\_mRatBN7.2) and *R. rattus* (GCF\_011064425.1\_Rrattus\_CSIRO\_v1).

**Statistical analysis**. Metagenomic profiles were loaded into R (R Core Team, 2020) for statistical analysis using the package “phyloseq”. Pairwise significant difference assessments were performed by Wilcoxon signed-rank test implemented in R’s “rstatix” package, which is a non-parametric statistical hypothesis test used for comparing repeated measurements on a single sample (R Core Team, 2020). Alpha (Shannon, 1948; Simpson, 1949) and beta diversity (Bray and Curtis, 1957) indices (Whittaker, 1960) were used to describe the relationship of the microbes within and between samples, respectively, and were calculated with the R package "vegan" (Oksanen *et al.*, 2013). The Differential Abundance taxa analyses between samples collected from two different tissues were done by using the R package "DeSeq2" using the "Wald” test (Love, Huber and Anders, 2014), which normalizes reads classified under each species taxon with the “poscounts” method. The data visualization for the metagenomics profiles was performed using the R package "ggplot2" (Ginestet, 2011). For all statistical analysis, p-values were adjusted with the Holm-Bonferroni method. Results with p-adjusted value (padj) < 0.05 were identified as significant.

**Results**

**Setting up DBs for Microbial Profiling**

To address the biases introduced from database and software selection during metagenomics profiling, four different Kraken2 databases (minikraken, standard, customized, and maxikraken) and nine different profiling software were used to classify the microbial communities of the wild rat samples. […] (Table 1). The analysis of Kraken2 loads selected DB into the workstation for every analysis the software performs, thus the memory resources utilized during Kraken2’s analyses are directly correlated with the choice of the databases. Four different DBs were selected to perform Kraken2’s analysis: the minikraken DB was built by the developer of Kraken2 and was distributed available on the software’s homepage; the standard and maxikraken DBs were built by the science community based on instructions on Kraken2’s manual, and was released online without charge; the customized DB was built in this study following the instruction of Kraken2’s manual, took into the consideration of the host genomes corresponding to this dataset. The maxikraken2 DBs, includes both complete and draft genomes of the microbe, requires over 150 GB memory available on the workstation for downstream analysis, while the standard requires only 53 GB and minikrakenV2 requires 8GB. Customized database (60 GB) was built with the same composition of the standard database, with the addition of the genomes of two Rattus species the dataset was collected from. With 12 threads of CPU used on UGA’s high memory computing node, the building of the customized database took ~15 hrs (Table I) to complete the building process. Time of the analyses also changes with the selection of the databases, but the differences is only in the range of minutes.

For rest of the software, pre-built DBs were chosen to perform the profiling, if these were provided by the software (Table I). CLARK, CLARK-s, Diamond, and Kaiju were the only four software in our study without a pre-built software provided. With 12 threads of CPU used on UGA’s high memory computing node, building of CLARK’s database took over 42 hours to complete, with over 400 GB memory utilized (Table I). CLARK-s database, required to build on top of the CLARK’s database, took around 40 additional hour to complete, with around 300 GB memory utilized (Table I). Building of Diamond’s database, with the same computational setting, completed in ~2.4 hours utilizing ~ 8 GB, while Kaiju’s database took ~ 5 hours to complete utilizing ~115 GB of memory (Table I). As for analysis time, using 12 CPU on UGA’s high memory computing node, Diamond used ~5 hour on average to classify one sample and BLASTN used ~2 hr to classify one sample. Rest of the software could finish classifying one sample within 5 minute at most (Table I).

**Differences in Microbial Profiles Classified Using Different DBs and Software**

Significant differences were identified between the microbial profiles of the rat samples when different DBs were used for classification (Table SI.1). The average number of total classified reads using the kraken2 DBs range from 10,755 (SD: 20,651) using the minikraken DB to 21,402 (SD: 27,043) using the maxikraken DB (Table SI.2). The number of reads classified under the four highest taxnomy level (Domain), Eukaryota, Bacteria, Viruses and Archaea taxa, by each DB, are presented in Figure 1a-d. The statistical significance of the differences in comparisons of the classification results for each Domain taxon classified by the four DBs were validated using the paired Wilicoxon signed rank test. The padj values for all comparisons between DBs are available in Table SI.3. For the Eukaryota taxon, all but the number of reads classified by the standard and customized DBs were found not significantly different in comparion (Figure 1a). For the Bacteria taxon, all the pairwise comparisons of the number of reads classified by the different DBs were found to be significantly different (Figure 1b). Classification results for Viruses are more similar across DBs than the results for Eukaryota and Bacteria, but still with 4 out of 6 comparisons were significantly different. In the case of Archaea, only the classification results of minikraken were found significanly different when compared with the results of other DBs, and the classification results of the other three DBs were not significantly different between each other.

To compare the profiling compositions of the different sofware, we calculated the number of total classified reads for each sample and determined these samples’ profiling compositions at multiple levels (Table SII.1). The average number of total reads classified by each software ranges from 10,955 using CLARK-s to 77,499 using Diamond (Table SII.2). The number of unique taxa classified by each software ranges from 18 by Metaphlan3 to 4816 taxa by Kaiju (Table II.S2). Furthermore, we have found that Metaphlan3 has not classified any reads in samples of Rattus R26 (R26.K, R26.L. and R26.S) and sample R22.L and R27.K, while other software have classified on average 1252 (SD: 1408), 32748 (SD: 32178), 133 (SD: 112), 111068 (SD: 113203), and 4011 (SD: 4325) reads with these five samples respectively (Table SII.2).

The number of classified reads were identified at the domain level taxa, where we closely examined the number of reads classified by each software into Eukaryota, Bacteria, Viruses, and Archaea (Figure Ie-h, Table SII.3). The number of reads classified into the Eukaryota taxon has the largest difference across the different software classification results, where only the number of reads classified by Centrifuge and Diamond were found to be not significantly different. Furthermore, due to the limitation of their DB composition, Metaphlan3, CLARK-s, and Kaiju did not report reads classified into the Eukaryota taxon (Figure 1e). Compared to reads classified into the Eukaryota taxon, the number of reads classified into the Bacteria taxon were very similar across software (Figure 1f). Only reads classified by CLARK and CLARK-s were found significantly different in Bacteria with most other software (except for when compared with Metaphlan3 and Kaiju) (Table SII.3). The classifications of Viruses by different software, on the other hand, were divided into two groups, where each group are not significantly different within each other, but different with the results classified by the software in the other group (Figure 1g, Table SII.4). The first group includes the Virsues classification results of BLASTN, CLARK, CLARK-s, Metaphlan3, and Kaiju, and the second group includes the results of Kraken2, Bracken, and Centrifuge. Diamond classification didn’t identify any reads as Viruses in the Rattus samples. Archaea’s read classification was very similar across software(Figure 1h, Table II.3), with the exception of the classification results using Centrifuge that were found to be significantly different from the other software (BLASTN, Diamond, Kraken2, CLARK, and CLARK-s). In addition, Bracken and Metaphlan3 didn’t classify any reads into the Archaea taxon (Figure 1h).

The read distribution at the Phylum level was also examined to increase the resolution of comparisons between the different software. At the Phylum level, the number of unique phyla taxa identified by each software ranges from 5 using Metaphlan3 to to 59 using Kaiju. We extracted the top 5 phylum taxa identified from each sample and combined reads classified to other phyla into the “p\_\_Other\_Phyla” (Figure 2). The top 5 Phyla described a large percentage of read classification for all software. However, the distribution of reads classified into different phyla taxa are different across software. For example, Virus taxon, “p\_Pisuviricota”, has contributed to over 85% (569/665) of the reads classified in sample R22.K using BLASTN (Figure 2a), while this taxon was not identified by any other software classification. Nevertheless, Metaphlan3 classified all of its reads in sample R22.K into “p\_\_Viruses\_unclassified” (Figure 2h), and CLARK and CLARK-s classified 63% (120/190) and 57% (95/166) of sample R22.K’s read into two different Virus taxa, “p\_\_Uroviricota” and “p\_\_Artverviricota” (Figure 2f-g). Kaiju also classified 21% of sample R22.K’s reads into “p\_\_Artverviricota” (34/157) (Figure 2i). Similar read distributions involving Virus taxa classification was also observed in sample R26.K, R26.S, and R27.K, where BLASTN classified 54% (657/1207), 20% (28/140), and 11% (422/3794) of reads into “p\_Pisuviricota” (Figure 2a), respectively, CLARK and CLARK-s classified a large percentage of reads into Virus taxon “p\_\_Uroviricota” (CLARK: 71% (636/900), 31/76 (41%), and 18% (201/1099); CLARK-s: 18% (50/271), 18% (7/67), 10% (83/1334), respectively) (Figure 2f-g), but other software only identified zero or a small number into a Virus taxon (Kraken2 has classified 4 reads into taxon “p\_\_Uroviricota”, Figure 2c). Except for differences in Virus taxa identification, the distribution of Bacteria reads classified by BLASTn, Kraken2, Bracken, Centrifuge, CLARK, CLARK-s, and Kaiju are relatively consistent across samples. The diversity of taxa identified by Metaphlan3 is significantly less than the one identified by other software classification. Only the most abundant taxa were capturing the majority of the classified reads with Metaphlan3 classification (Figure 2h). For example, Metaphlan3 has identified 100% of sample R7.L’s reads as “p\_\_Proteobacteria, while other software have identified 29% (SD: 12%) of R27.L’s reads as “p\_\_Proteobacteria” on average, with a unique number of Phylum taxa identified ranging from 2 by Diamond (91% of reads classified as “p\_\_Tenericutes”) to 50 by Kaiju. Diamond’s classification showed differences in read classification when compared to other software (Figure 2b). The most notiable difference is the relative abudance of taxon “p\_\_Firmicutes” across samples classified by Diamond. In the lung samples, “p\_\_Firmicutes” was classified in 17% of R22.L (SD: 9%), 20% of R26.L (SD: 9%), and 14% of R27.L (SD: 8%), but Diamond only classified 2% (133/4900) of reads as “p\_\_Firmicutes” in sample R26.L, while “p\_\_Firmicutes” taxon was not identified in R22.L and R27.L by Diamond. On the other hand, Diamond identified a relative larger proportion of reads as “p\_\_Firmicutes” in samples R27.S (24%) and R28.L (19%) compared to that of most other software (R27.S: mean: 2%, SD: 2%; R28.L: mean: 3%, SD: 3%), except for the Centrifuge classification (R27.S: 24%, R28.L: 9%).

Regarding the Species level classification, the number of reads classified under taxa (strains) with the same species was aggregated together to obtain a unique number of species classified by each software (Table SII.1). Out of all software, metaphlan3 classified the least number of species taxa, with only 18 species (Table SII.4). On the other hand, Kaiju classified the most number of distinct Species taxa, 4128 species (Table SII.4). From the species level classifications, 9 species taxon were identified by all nine software (*Leptospira interrogans*, *Leptospira borgpetersenii*, *Faecalibacterium prausnitzii*, *Bordetella pseudohinzii*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bacteroides uniformis*, *Phocaeicola vulgatus*, and *Bartonella elizabethae*) (Table SII.1). Centrifuge vs Kaiju have the largest overlapping in the number of species taxa identified (2285 taxa), followed by Kraken2 vs Centrifuge (1737 taxa) and vs. Kaiju (1723 taxa) (Table Sii.4). The species-level classification of the three software shared a total of 1379 species taxa. In addition, BLASTN shared 1253 species level taxa with Centrifuge, 1207 taxa with Kaiju, and 1126 taxa with Kraken2. CLARK and CLARK-s’s classification shared 1219 and 1059 species taxa wtith Kaiju, respectively. To assess if different software have identified the same species taxa as the most abundant taxa, species taxa with at least 10% of the reads from each sample were identified from each software’ classification. Metaphlan3 identified most of the number of unique species taxa (18 taxa), while BLASTN and Kaiju identified the least (7 taxa). CLARK vs. CLARK-s and Kraken vs. Bracken shared most of the number of taxa in this category (9 and 8 taxa, respectively). Two species taxa were identified by all software as the top ten percent most abundant species taxa, which were *L. interrogans* and *Bartonella elizabethae* (Table SII.1).

**Downstream analyses for microbial community characterization**

*Within-sample diversity (α-diversity) - DBs*

To understand how differences in the classification results can directly impact the characterization of the microbial communities in each sample, we calculated two alpha indices (Shannon and Simpson) at the species level, and characterized the species richness (diversity) and evenness (abundance) of the microbial communities within each *Rattus* sample. Then, we compared these with the observed unique number of species identified by each DB and by each software (Observed). We found that although the observed unique taxa classification results across all four DBs were significanly different from each other (Figure 2a), the Shannon indexes, which emphasize the species richness within a community, obtained from minikraken DB, were found to be significantly different from the results obtained with the other DBs (Figure 2b, Table SI.4). Morever, the Simpson indexes, which weigh the microbial community’s characterization with the proportion of species of each sample, were also found similar between results of the four DBs (Figure 2c, Table SI.4). Only the Simpson’s indices obtained from the results of the standard and customized DBs comparison were found to be significantly different (Figure 2c, Table SI.4).

*Within-sample diversity (α-diversity) - Software*

The number of unique observed taxa (Table SII.5, Figure 3d) across different software were largely divergent from each other. Out of the 36 pairwise comparisons between different software, only 6 comparisons were not significantly different (Table SII.5), which were BLASTN’s observed taxa with Kraken2, CLARK, and CLARK-s, comparison between CLARK and CLARK-s, and comparison between Centrifuge and Kaiju. The Shannon indexes showed more similarity between software than the unique observed taxa, however, they still had 23 out of 36 comparisons between software significantly different (Table SII.5, Figure 3e). All the software classifications of software that were found to be similar in observed taxa, were also found to be not significantly different for their Shannon indices. BLASTN’s classification were found to be similar to the ones from Bracken and Diamond. These software similar to the Shannon indices obtained from BLASTN was also found similar with each other, ex. Bracken vs. Diamond, Bracken vs. CLARK and CLARK-s, and Diamond vs. CLARK and CLARK-s, and etc. The Simpson’s indexes were the ones that were least impacted by the differences in classification results across software. Only 7 out of 36 comparison were found to be significantly different (Table SII.5, Figure 3f). Most of these significantly different comparisons were identified to be between CLARK-s (3/7) and Centrifuge (4/7) with other software or between each other.

*Between-sample diversity (β-diversity) - DBs*

In addition to the characterization of the microbial community within each sample, relationships between the microbial communities are also very important in metagenomics studies. The pairwise relationships between every two *Rattus* samples were determined with the Bray-Curtis (BC) dissimilarity index, and clustered hierarchically. The BC indexes were found to be significantly different across all DBs, except for maxikraken and customized DBs. The hierarchical clustering analysis also shows dissimilarities across results when using different DBs (Figure 4a). Three kidney samples (R22.K, R26.K, and R27.K) were found to be clustered with one of the spleen sample (R26.S) in all four DBs’ classification. However, their relationships with another spleen sample (R27.S) changes with the type of DB used. For example, using minikraken, R27.S clustered more closely with the spleen sample R22.S before clustering with the three kidney samples, but in the clustering results of other three DBs, R27.S clustered closely with the three kidney samples and R26.S while R22.S always clustered closely with sample R28.K. Despite the changes in the lower hierarchical levels, the two major clusters describing the general relationships between samples did not change with the use of different DBs. Three Lung samples (R22.L, R26.L, and R27.L) always clustered closely together away from the other samples, while all Kidney and Spleen samples formed a separate cluster with the other Lung sample (R28.L).

*Between-sample diversity (β-diversity) - Software*

The consistency in the major clusters obtained from the between-sample relationships remained when using the classifications of different software (Figure 4b). Except for Metaphlan3, all the other software aggregated the *Rattus* samples into two large clusters: the first with three Lung samples (R22.L, R26.L and R27.L) and the second with a combination of all the Kidney and Spleen samples, and one Lung sample (R28.L). By validating all comparison with the paired Wilcoxon signed-rank test (Table SII.6), we found that BLASTN was not different from Kraken2, Bracken, and Centrifuge, while CLARK and CLARK-s are not different from most other softwares except for BLASTN, Centrifuge, and Metaphlan3, separating these software into two groups. Metaphlan3, with 5 out of 12 samples unclassified, was significantly different in the between-sample relationships with that obtained from other software (Table SII.6). The smaller clusters formed by hierarchical clustering inside the second cluster varies among software (Figure 4b). For example, BLASTN’s classification has clustered three Kidney samples (R22.K, R26.K, and R27.K) closely with each other before clustered further with other samples. This cluster was only observed in Centrifuge’s clustering. While in the clustering of other software, these three kidney samples were always clustered closer with other samples before clustered with each other. For example, in Diamond, CLARK, and CLARK-s, one of the three kidney samples has always clustered with R26.S before clustering with each other. In classification of Kraken2, Bracken, and Kaiju, both R26.S and R27.S was clustered with one of the kidney samples before the kidney samples clustered with each other. However, in BLASTN’s classification, both R26.S and R27.S was clustered with the rest of the Spleen sample first (R22.S and R28.S) before clustered together with the three Kidney samples.

**DA taxa identification**

The DA taxa, which are the microbial taxa that are significantly different in abundance between two groups of samples, were identified between the samples of different tissues pairwisely. The microbial communities of the lung samples were found most distinctive from that of spleen and kidney samples despite the differences in the classification results reported by different software. Therefore, we have started with identify the DA taxa between the lung samples and the kidney samples.

Lung and kidney sample comparison

Since Metaphlan3 was not able to classify 2 lung samples and 2 kidney samples, we excluded the Metaphlan3 classification from this analysis.

*Species level*

At the species level, the number of DA taxon identified using the classification results of different software ranges from 10 (Diamond) to 596 (Centrifuge) (Table SII.7, Figure 5a), with more taxa significantly higher in abundance in the Kidney samples than in the Lung samples for all software’ classifications (Figure 5b). Five significantly abundant species (*Bordetella pseudohinzii*, *Bordetella bronchiseptica*, *Leptospira interrogans*, *Leptospira borgpeterseni*, and *Mycoplasm pulmonis*) were found by all software (Table SII.7). Kaiju, Centrifuge, and BLASTN have the highest number of DA taxa (390, 376, and 56 taxa, respectively) (Figure 5a). Although Centrifuge identified the largest number of DA species taxon, Kaiju identified the highest number of unique phylum taxa (42), which means that many of Centrifuge’s DA species have the same phylum taxonomy taxa (Figure 5a). To obtain a more generalized overview of the DA taxa identified from the classification of each software, we aggregated the species taxa into the phylum level and visualized the presence and absence of each phylum taxon as the DA taxa across the different software (Figure 5c).

*Phylum level*

At the Phylum level analysis, the taxa “p\_\_Spirochaetes”, “p\_\_Bacterodietes”,“p\_\_Protebacteria”, and “p\_\_Tenericutes” were found present for all software. Diamond missed four taxa ("p\_\_Aquificae”, “p\_\_Fusobacteria”, “p\_\_Firmicutes”, and “p\_\_Cyanobacteria”) that were identified by the rest of the software. Kaiju and Centrifuge were th only two software that reported virus taxa (“p\_\_Negarnaviricota”, and Kaiju reported “p\_\_Nucleocytoviricota” and “p\_\_Uroviricota”) as DA. Archaea taxa were only reported by Kaiju, Centrifuge, and BLASTN. All three software reported "p\_\_Euryarchaeota”, and both Kaiju and Centrifuge reported “p\_\_Candidatus Micrarchaeota” and "p\_\_Candidatus Lokiarchaeota”. Finally, Kaiju uniquely reported “p\_\_Candidatus Thermoplasmatota”.

Lung and spleen sample comparison

The DA taxa identified between lung and spleen samples were similar with those identified between lung and kidney samples (Table SII.8, Figure S2).

*Species level*

Kaiju identified the highest number of DA species (484 taxa), while Diamond identified the lowest (44 taxa) (Figure S2a). All of the DA taxa were more abundant in the lung than in the spleen samples (Figure S2b). Six species (*Mycoplasm pulmonis*, *Mycoplasma bovoculi*, *Mycoplasma neurolyticum*, *Bordetella pseudohinzii*, *Bordetella bronchiseptica*, and *Bacteroides uniformis*) were identified as the DA taxa by all software (Table SII.8), and three of these were identified as DA species by all software during Lung vs. Kidney samples comparison (Table SII.7). Kaiju has the highest number of distinct DA species taxa (335), followed by centrifuge (268), and BLASTN (46) (Figure S2a).

*Phylum level*

At the Phylum level, “p\_\_Bacterodietes”, “p\_\_Tenericutes”, “p\_\_Cyanobacteria” ,“p\_\_Protebacteria”, and “p\_\_Firmicutes” were as DA identified by all the software (Figure S2c). Taxa "p\_\_Aquificae”, "p\_\_Actinobacteria”,and “p\_\_Fusobacteria” were identified by all software except for Diamond. Archaea phylum, "p\_\_Euryarchaeota”, was still the Archaea taxon identified by BLASTN, Centrifuge, and Kaiju, however, the rest of the Archaea taxa were either only identified by Kaiju and Centrifuge, or Kaiju alone. Virus taxon, “p\_\_Negarnaviricota”, was only identified by Centrifuge as differentially abundant, while Kaiju only identified the virus taxa “p\_\_Nucleocytoviricota” and “p\_\_Uroviricota”. Morever, CLARK also reported the virus taxon, “p\_\_Uroviricota”, as significantly abundant.

Kidney and Spleen sample comparison

Finally, we futher identified the DA taxa between Kidney and Spleen samples (Table SII.9, Figure S3).

*Species level*

The number of species identified ranges from 6 by Diamond and 57 by BLASTN (Figure S3a). More taxa were identified significantly abundant in the Kidney samples than in the Spleen samples, especially at the genus level (Figure S3b). Kaiju, the software that identified the second highest number of distinct DA taxa at the species level, has five out of ten distinct taxa reported as viruses (Figure S3a). In general, only 1 species (*Leptospira interrogans*) and 4 phylum taxa (“p\_\_Spirochaetes”, “p\_\_Bacteroidetes", "p\_\_Cyanobacteria”, and “p\_\_Proteobacteria”) were classified by all software (Table SII.9, Figure S3c).

*Phylum level*

The Phylum taxon “p\_\_Firmicutes” was identified as the DA taxon by all software except for Diamond. Kaiju identified as a DA taxon the virus taxon, “p\_\_Negarnaviricota”.

***Leptospira* detection.**

*Different software*

*Leptospira* was identified in the three tissues of all four subjects by the nine software, however, each software reported *Leptospira* in different samples (Table II). Centrifuge is the only software that reported *Leptospira* in all of the 12 Rattus samples, where 8 unique *Leptospira* species were identified (8 from the pathogenic group, 1 from the saprophytic group) (Table SIII.1). Kaiju also identified *Leptospira* from 9 out of 12 samples with 8 unique species (7 from the pathogenic group, 1 from the saprophytic group) (Table SIII.1). Kraken2, following Centrifuge and Kaiju, classified 6 *Leptospira* in 6 samples with 3 unique species all from the pathogenic group (Table SIII.1). Except for Metaphlan3, all software identified *Leptospira* from two of the kidney samples (R22.K and R28.K), which have on average 31 (SD: 3) and 84,344 (SD: 2.2) reads classified under *Leptospira* (Table SIII.2), respectively. BLASTN and CLARK identified *Leptospira* from a lung sample (R22.L), which was also identified by Centrifuge, Kaiju, and Kraken2. Metaphlan3 only identified *Leptospira* in one of the kidney samples (R28.K). All samples identified by at least three software have at least a total of 30 reads classified under *Leptospira* (Table SIII.2). Samples that were only identified by Kaiju or Centrifuge have on average only 2 (R27.K, SD: 1) to 15 (R26.L, SD: 2) reads classified under *Leptospira* (Table SIII.2).

*Different Kraken2 databases*

In addition to the differences in *Leptospira* detection by the different software, the diagnosis of *Leptospira* was dissimilar when different databases were used for Kraken2’s classification (Table SIV). Kraken2’s analyses with the maxikraken DB identified *Leptospira* in all samples, while standard and customized DB identified *Leptospira* in two Lung samples (R22.L and R27.L). Standard DB also identified *Leptospira* in the three Spleen samples (R22.S, R27.S and R28.S). In addition to the metagenomics approaches, the diagnosis of *Leptospira* in the kidney samples was validated using three traditional methods (PCR/DFA/Culture), *Leptopsira* was identified in two kidney samples (R22.K and R28K) by all three methods, but only identified by PCR in the third kidney sample (R26.K) (Table II).

**Discussion**

Profiling the microbial taxonomies from biological specimens allows a better understanding of the microbial communities of samples collected for different fields of studies (Coyte, Schluter and Foster, 2015; Gilbert and Lynch, 2019). The field of metagenomics, developed with the advancement of NGS technologies, allows scientists to build a complete and discriminatory microbial profile with virus, archaea, and bacteria taxa for samples collected from their environments of interest (Jovel *et al.*, 2016). These metagenomic profiles can be used to detect relevant pathogens in clinical and epidemiological investigations (Qin *et al.*, 2012; Knights, Lassen and Xavier, 2013) and to observe the interactions between micro-ecosystems and their changing environments (Handley, 2019).

In this study, we classified the microbial profiles of twelve samples collected from four wild rat subjects. These rats were captured in the Caribbean island of St.Kitts, and are the major reservoir species for *Leptospira*, contributing to the transmission and maintenance of this pathogenic bacteria in the island (Boey *et al.*, 2019). Rats harbor *Leptospira* in their kidneys and are a significant source of environmental contamination (Saito *et al.*, 2013; Rawlins *et al.*, 2014; Costa *et al.*, 2015; Boey, Shiokawa and Rajeev, 2019; Rajeev *et al.*, 2020). In addition to kidney samples, we also classified the microbial profiles of samples from lungs and spleen. We identified the microbial profiles of these samples, which might contain many potential rodent pathogens, using nine different shotgun metagenomics sequencing taxonomic classification software. The differences in the classification outputs were compared and analyzed to address how the use of different taxonomical profiling software on the same dataset could affect the results of microbiome characterization, which could lead to different biological conclusions in the downstream analyses, as well as to divergent diagnosis of the pathogen *Leptospira*.

Previous benchmarking studies (Escobar-Zepeda *et al.*, 2018; Ye *et al.*, 2019) have performed comprehensive analyses on these software’ speed and performance (sensitivity, specificity, precision, and accuracy). However, these benchmarks have been generally based on *in silico* datasets or with the support of laboratory synthetic samples. Differences among the microbial taxonomical profiles classified with different tools can be the result of reporting false positive or false negative taxonomies using different algorithms, DBs, or software. These differences sometimes seem negligible in the benchmarking studies for tools with similar algorithms but can lead to diverging biological conclusions in the downstream analyses depending on the questions being asked. These biases originated from these differences have been understudied; therefore, it is crucial to demonstrate the effect of these biases with real biological data, to raise awareness for their existence and identify the potential factors that lead to incorrect biological conclusions in a metagenomics study.

**Biases introduced by DB selection**.

Incorrect taxonomical profiling of the collected samples’ microbial community can start from choosing an incorrect DB. All current taxonomical profiling software require a large number of computational resources for DB building and storage. Some software, such as Kraken2, provide an alternative pre-built DB for users with inefficient computing resources, which minimize the size of the DB to be loaded into a machine with RAM as low as 8 GB. This DB is built with all libraries included within the standard Kraken2 DBs but it down samples the size of the sequences included using a hash function. There were also multiple versions of Kraken2’s DBs provided by the science community that can be easily downloaded and updated frequently. For example, the Langmead lab builds the most recent version of Kraken2’s standard database based on NCBI’s RefSeq library routinely. In addition, the Loman lab has built a Kraken2 DB with the inclusion of draft genomes that were not included in the Refseq library. Both of these two Kraken2 DBs are freely available to use online, replacing the workload of building a database from scratch. However, all three databases mentioned above included the human genome as the only Eukaryotic genome in the database, which is not the host of our dataset. The biases introduced from host genomes included in the DB for metagenomics analysis have been address previously (Pereira-Marques *et al.*, 2019). Therefore, we built a separate DB (customized database for our dataset) with the inclusion of the two Rattus hosts genomes on top of the standard DB. We found that although the number of reads classified using different DBs differ significantly from each other, the characterization of each sample’s microbial communities will not be largely biased by the use of different DBs. In our analyses, only the richnesses of the samples (Shannon indexes), which accounts for the rare species within the community, obtained from the miniKraken DB were significantly different when compared to other software. On the other hand, the eveness of each sample’s microbial community measured with Simpson’s index was mostly consistent across the classifications with different DBs. For microbial communities between samples, we found that only the higher level clusters describing the most distinctive relationships between samples were consistent across the the classifications of all DBs. Sophisticated relationships between samples were altered by the biases introduced from DB selection.

**Resources required to use different software**

Metagenomics software can be classified into two different categories, alignment-based and alignment-free. The alignment based software, which suffers greatly from slow speed and the need of large resources, were generally thought to have high sensitivity. On the other hand, the alignment-free software utilize relatively small computational resources and significant improvement in speed of the analysis.In our study, BLASTN and Diamond, were the two most time intensive software. They took two and five hours, respectively, on average to complete the analysis for one sample, while other software took at most three minutes for the same task. The time and resources required to build the DBs for the alignment-free software became the trade-off for the speed of the analysis itself. For example, the building of CLARK’s DB took almost two days with 400 GBs of memory used. Fortunately, most of the software included in our study have pre-built DBs distributed with the release of the software (except for CLARK, CLARK-s, Diamond, and Kaiju). However, if the analysis requires the identification of taxa that are not included in these pre-builit DBs, the time and resources added to the metagenomics profiling analysis will increase significantly.

To compare the metagenomics profiles classified by each software, we chose the standard DBs provided by the developers of these software. If the standard DBs were not indicated, we built the DBs with the genomes of Bacteria, Archaea, Viruses, and Human available in NCBI’s RefSeq library, which are the compositions for most of the pre-built DBs. CLARK-s’ DB was required to be built on top of a CLARK DB of the same composition, but when the DB was built with the genomes of Bacteria, Archaea, Viruses, and Human, the building was suspended by the software with the error message “the number of targets exceeds the limit (16383)”. This limitation was reported to the developer of CLARK-s, but it has not been resolved by the time this manuscript was drafted. We bypassed the limitation by building the DB with Bacteria, Archaea, and Viruses genomes separately, and combining the classifications using each DB at end of the analysis. In addition, Metaphlan3, which identifies the microbial taxon with marker genes, does not have an option to build a customized DB, only the marker DB distributed by the developer could be used for profiling.

**Biases in microbial profiles introduced from software selection**

At the Domain level, Eukaryota taxon has contributed the most to the dissmimilarities between the different software classifications. Almost all pairwise comparisons between the Eukaryota profiles classified by each software were found significantly different between each other. Compared to the number of reads classified under Eukaryota, the number of reads classified under Bacteria, Viruses and Archaea taxa by different software were much more similar between. The classifications of Centrifuge, CLARK, and CLARK-s were frequently identified significantly different from the ones of other software regarding the number of reads mapped to Bacteria and Archaea. The classifications of Viruses, on the other hand, was found separated into two groups where the classifications within a group were similar (group1: BLASTN, CLARK, CLARK-s, Metaphlan3 and Kaiju; group2: Kraken2, Bracken and Centrifuge). Diamond did not identify any reads as viruses. This division in virus classifications was further validated by the virus classifications at the lower taxonomy levels. The samples with large percentage of reads classified under virus taxa by group1 software were not profiled by software in group2. Although software in group1 were more sensitive in virus identification than of group2 software, the exact virus taxa and the correspondent number of reads using different group1 software were not consistent. The virus taxon identified by BLASTN in high abundance was not identified by any other software included in the analysis. Except for the samples with virus classifications, the profiling of Bacteria taxa was found mostly consistent across the software at both Phylum and Genus level. Only the classifications of Metaphlan3, which could only identify a few taxa from each sample with high abundance, and Diamond, which reported conflicting profiles in Firmicutes identification at the Phylum level (Bacillus at Genus level) with the classification of all the other software, were different from other software in Bacteria classification.

Compared to Phylum and Genus levels, the classifications at the Species level were more divergent across software. Although most software have reported more than 1,000 unique Species taxa from the Rattus profiles (except for Bracken and Metaphlan3), only nine species were identified by all software included in this analysis, and only 2 species were found overlapping in taxa with at least 10% in relative abundance.

**Microbial community characterization**

In addition to the differences in microbial profiles classified by different software, the differences across the richness of each samples’ microbial community were significant in the majority of the comparisons across software. Most of these were found between the classifications of Kraken2, Metaphlan3, Centrifuge, and Kaiju with other software. However, species abundance measurements (Simpson’s index) were mostly not affected by use of different software. The characterizations of the relationships between-samples were divergent across software. However, the most discriminatory relationships within the rat samples (between lung and other samples) were captured by most of the software (except for Metaphlan3), but the descriptions of the more subtle relationships between samples were not reported consistently across software.

**Differences in differential abundant taxa**

In order to address potential biases introduced from software selection with biological significance, we identified the DA taxa between samples of different tissues in a pairwise fashion. From the between-sample relationship analyses, all software reported that the microbial communities of lung samples were distinct from the kidney and spleen ones. Following this observation, we performed analyses to answer the following questions: What are the taxa that contributed the most to the differences in the microbial profiles between different rat tissues? Were DA taxa identified from lung vs. kidney and lung vs. spleen samples similar to each other? Were the number of DA taxa reported from kidney vs. lung samples comparison less than those reported when compared to the lung samples? Metaphlan3 was excluded from this analysis due to the fact of not classifiying 5 out of 12 samples in the dataset. The classifications of all DA taxa reported at the species level were largely different across software. The largest range in the number of differentially abundant taxa reported by different software were found in the analysis between lung and kidney samples, where the software with the least DA taxa identified, Diamond, reported 10, and the software identified most DA taxa, Centrifuge, reported 596. Despite the large differences in the number of taxa identified, there were still a small number of overlapping species identified across the results of all software. We also found similarities in the software-overlapped DA taxa between lung vs kidney and lung vs. spleen analyses, where two *Bordetella* species and a *Mycoplasm* species were reported by all software in both analyses. More DA identified were overlapped across software at the Phylum level. In addition to the overlapped DA taxa, Kaiju and Centrifuge were more likely to report more taxa as differentially abundant than the other software. These two software were also the only two that reported both viruses and archaea taxa as DA taxa (BLASTN only reported Archaea and CLARK only reported viruses). Diamond was found least sensitive in differentially abundant analyses for all three comparisons between tissue samples, where phylum taxa identified by all the other software were frequently not identified by Diamond.

*Leptospira* diagnostic sensitivity comparison

To assess the sensitivity of shotgun metagenomics as a tool for pathogen diagnosis, we identified the presence of the zoonotic pathogen *Leptospira* in all of our tissue samples. Centrifuge and Kaiju were found most sensitive in diagnosing *Leptospira*, where Centrifuge reported the presence of *Leptospria* in all the samples. Except for Centrifuge and Kaiju, the rest of the software were consistent in *Leptospira* identification in only two of the kidney samples (R22.K and R28.K) and in one Lung sample (R22.L), where the reads classified under *Leptospira* were relatively more abundant. Since *Leptospira* were mainly depositted in the kidney of rats before infecting or contaminating other mammals or environment through urination (Adler and de la Peña Moctezuma, 2015), we diagnosed the presence of *Leptospira* using three traditional methodologies (PCR/DFA/Culture) in the kidney samples alone i. We found that most software included in our analysis has similar sensitivity in *Leptospira* identification with traditional methods, except for PCR. In addition, Centrifuge reported the presence of *Leptospira* in samples that were not reported by any other software or a traditional method. This identification could be due to Centrifuge’s better performance in sensitivity, or as a result of false positive reporting. Furthermore, we found that *Leptospira* was in the same kidney sample if maxikraken DB was used for Kraken2’s analyses. Kraken2 with maxikraken DB also reported *Leptospira*’s presence in all samples. We hypothesize that the sensitivity of *Leptospira*’s diagnosis may improve with more draft genomes of *Leptospira* included in the database, because most of Leptospira species’s genomes were only available in the draft format.

The inconsistencies found between the results of different metagenomic software showed that significant biological conclusions from metagenomic profiling analyses have the potential to be only the artifacts of the software’ algorithms. Shotgun metagenomics sequences might be too short for current taxonomical profiling software to differentiate microbial taxonomies between similar genomes (Tran and Phan, 2020). The use of real-world datasets has the advantage of addressing this challenge, reminding the investigators to stay skeptical with the classification results obtained from the profiling software. On the other hand, benchmarking the software’ performances with the real-world dataset, in contrast to using *in silic*o datasets, has the limitation of lacking knowledge about the true microbial compositions within each sample, which means we could not evaluate the performance of software based on their degrees of accuracy and sensitivity, nor giving direct suggestions on software’ selection. In addition, metagenomics profiling has been broadly utilized in many fields of studies, including clinical, pharmaceutical, as well as ecological. Each field utilizes microbial profiles differently based on the biological question proposed. Our choice of the real-world dataset could only address a limited number of software selection biases. We suggest researchers from different study fields to be aware of the possible error-prone conclusions made from metagenomics profiling analysis, and evaluate it objectively comparing it to other traditional methods (e.g. PCR, culture, or serotyping).

Advancement in sequencing as well as computational technologies allows modern-day biological research to move to a brand-new era. However, while benefiting from the powerfulness and convenience of technologies, we should always critically analyze and validate software outputs based on our prior knowledge.

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**Conflicts of interest**

No conflict of interest declared.

**Repositories**

The raw sequence files (FASTQ) were submitted to the NCBI Sequence Read Archive under the Bioproject accession number: PRJNA717669. The individual isolates can be accessed under the following Biosample accession numbers: SAMN18507082 - SAMN18507091. All scripts for this publication are freely available on the following Github link: <https://github.com/salvadorlab/MetagenomicsToolsEvaluation>.

**Data summary**

The raw sequence files (FASTQ) were submitted to the NCBI Sequence Read Archive under the Bioproject accession number: PRJNA717669. The individual isolates can be accessed under the following Biosample accession numbers: SAMN18507082 - SAMN18507091. The short-read archive accession numbers are listed in Table S1.

**Ethical Approval**

Rats were captured following protocols approved by the Ross University School of Veterinary Medicine (RUSVM) IACUC (approval # 17-01-04).

**Supporting Information**

**References**