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Reads2Resistome: An adaptable and high-throughput whole-genome sequencing pipeline for bacterial resistome characterization

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Abstract

Summary: The bacterial resistome is the collection of all the antibiotic resistance genes, virulence genes, and other resistance elements within a bacterial isolate genome including plasmids and bacteriophage regions. Accurately characterizing the resistome is crucial for prevention and mitigation of emerging antibiotic resistance threats to animal and human health. Reads2Resistome is a tool which allows researchers to assemble and annotate bacterial genomes using long or short read sequencing technologies or both in a hybrid approach. Using a massively parallel analysis pipeline, Reads2Resistome performs assembly, annotation and resistome characterization with the goal of producing an accurate and comprehensive description of a bacterial genome and resistome contents. Key features of the Reads2Resistome pipeline include quality control of input sequencing reads, genome assembly, genome annotation, resistome characterization and alignment. All prerequisite dependencies come packaged together in a single suit which can easily be downloaded and run on Linux and Mac operating systems.

Availability: Reads2Resistome is freely available as an open-source package under the MIT license, and can be downloaded via GitHub (https://github.com/BioRRW/Reads2Resistome).

1. Introduction

Antimicrobial resistance is on the rise worldwide and the CDC estimates that per year at least two million people will become infected by a drug-resistant bacteria and at least 23,000 will die as a result of such infections [1]. The primary method for determining antimicrobial resistance in clinical laboratories is culture-based antimicrobial susceptibility testing (AST). However, the declining cost of next-generation sequencing technologies has enabled increased sequencing depth and more accurate identification of antimicrobial resistance elements within bacterial genomes [2]. Additionally, PacBio and Oxford Nanopore MinION sequencing technologies provide sequencing reads longer than 10kb, improving our ability to generate complete and accurate genome assemblies. This has led to an increase in open-source bioinformatics tools which can leverage multiple types of sequencing data. When performing an analysis such as identifying antimicrobial resistance genes, a researcher might use a dozen or more individual tools with each relying on their own databases and dependencies. Here we present Reads2Resistome, a streamlined bioinformatics pipeline for quality control, assembly and resistome characterization for bacterial genomes which analyzes short reads, long reads or both in a hybrid assembly approach.

2. Methods and Implementation

Reads2Resistome is scripted using Nextflow [3], a parallel DSL workflow framework, and is integrated with Singularity [4], an open source container platform with focus towards HPC workloads. The Reads2Resistome pipeline includes three main steps: quality control, assembly, and annotation of input bacterial sequencing reads. Reads2Resistome takes long read sequences, short read sequences or both as input and performs quality control of short reads using Trimmomatic [5] and long read quality visualization using NanoPlot [6]. Both quality-controlled short reads and long reads are then assembled using Unicycler [7], which generates an assembly graph using short reads, then uses long reads to simplify the graph to generate accurate assemblies. In the event the input consists of short reads only, Unicycler employs SPAdes [8,9] for assembly and subsequently polishes the resulting graph by bridging contigs. Long read-only assembly is performed using miniasm [10] and Racon [11] employed through Unicycler. Annotation is performed with Prokka [12] using one of the provided custom databases, which are pre-built from collections of specific bacterial species and subtypes, or using the Prokka default database. Resistome characterization is performed using ABRICATE [13]. Nextflow implementation using Singularity gives version control over the various open source tools ensuring reproducible results. Reads2Resistome output contains the following for each input isolate: visualization of both raw and quality-controlled reads; assembled contigs with a corresponding assembly graph along with an assembly quality assessment; gene and resistome annotation files; genome alignment files in BAM format; and optional serovar predictions. All documentation and pipeline usage is publicly available at https://github.com/BioRRW/Reads2Resistome.

2.1 Streamline high-throughput analysis

Reads2Resistome is designed for high-throughput bacterial sequence input and performs quality control, genome assembly and subsequent genome and resistome annotation in a parallel, high-throughput manner (**Figure 1**). Reads2Resistome is able to accommodate input of different species within the same run and can perform species-specific genome assembly quality control and gene annotation. A comma-separated values (CSV) file, generated by the user, enables input of multiple different isolates regardless of the isolate identity. The user can also specify a pipeline-provided database for genome annotation or can choose to use the default database utilized by Prokka. For genome assembly quality assessment, done by QUAST, the user can optionally add a user-provided reference genome for additional reference-specific metrics. Pipeline outputs for quality control and genome quality assessment are aggregated by MultiQC into a HTML report. In addition to quality-control, assembly and annotation, genome alignments are generated for further comparison. For *Salmonella spp.* optional serovar prediction is performed using SISTR.

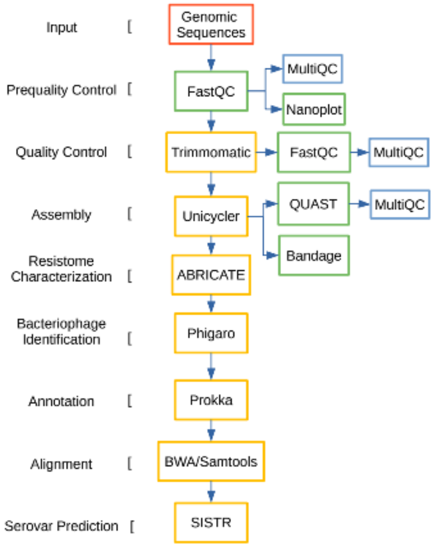


Figure 1. Key processes in Reads2Resistome pipeline.

2.2 Adaptable to cutting-edge sequencing technologies

Inclusion of long read sequences into bacteria assembly aids in resolving repeat regions of genomes and contributes to genome completeness [14]. Reads2Resistome is designed to be adaptable and flexible in that it can accommodate assembly in three different approaches: long read-only assembly, short read-only assembly, and hybrid assembly. In each assembly approach, Unicycler is used to generate genome assemblies and assembly graphs which are visualized with Bandage (**Figure 1**) [15].

2.3 Resistome characterization

Reads2Resistome characterizes resistome contents using ABRICATE and Phigaro. ABRICATE uses the assembled contigs to screen for antimicrobial and virulence genes from various databases; ARG-ANNOT antibiotic resistance gene database [16], the Comprehensive Antibiotic Resistance Database (CARD) [17], MEGARes Antimicrobial Database for High-Throughput Sequencing [18], NCBI AMRFinderPlus [19], PlasmidFinder [20], ResFinder [21] and VirulenceFinder database [22]. ABRICATE compiles results into a single report containing hits from each database and Reads2Resistome provides an output file for each isolate. Phigaro uses the assembled contigs to detect putative taxonomic annotations and the output is collected by Reads2Resistome for each isolate.

3. Case Study

Using Reads2Resistome we assembled and characterized the resistome of two bacterial isolates recovered from the ceca of 2-week old broiler chickens; SH-IC: *Salmonella enterica* serovar Heidelberg (S. Heidelberg) and EC-IC: *Escherichia coli.* (**Table 1**). Illumina, PacBio and Oxford Nanopore MinION sequences were used to test each type of assembly available under the pipeline; short read-only, long read-only and hybrid assembly.

Table 1 Summary of isolate information

Bacterial strains	Strain ID	SRA accession no.	Sequencing platform	Coverage*
Escherichia coli	EC-IC	SRR11808523	Illumina	56.95
		SRR11808522	MinION	35.35
		SRR11808521	PacBio	28.08
Salmonella Heidelberg	SH-IC	SRR11808520	Illumina	20.25
		SRR11808519	MinION	16.69
		SRR11808518	PacBio	33.14

* Coverage estimated from total quality-controlled bases divided by the genome size (*E. coli*: 4800000bp , *S. heidelberg*: 4600000bp)

Short and long read-only assemblies, regardless of the read source, resulted in the shortest run-time with an average of 6 minutes per sample. Hybrid assembly, as expected, was the most time-intensive assembly method taking on average 1 hour and 8 minutes per sample, regardless of long read source (**Table 2**).

Genome assembly and annotation metrics were compiled from QUAST and Prokka outputs. Hybrid assembly of both EC-IC and SH-IC using MinION long reads and Illumina short reads gave the fewest contigs, longest total length and highest number of annotated genes as compared to long read assembly using MinION. Hybrid assembly of both isolates using PacBio reads resulted in fewer contigs but comparable total length to that of the MinION hybrid assembly. Genome contiguity was best obtained by hybrid assembly and can be visualized with Bandage-generated graphs (**Table 4**). While hybrid and long read-only assemblies are comparable with respect to number of contigs and genome length, the long read-only assembly greatly lacked in annotated genomic features and resistome elements.

Table 2 Summary of assembly resources and run-time under various assembly conditions

Isolates included in run	Assembly Method	Elapsed time	--threads (option)	CPU-hours
EC-IC Illumina; SH-IC Illumina	Short Read	16m 13s	64	0.6

EC-IC MinION;
EC-IC PacBio;
SH-IC MinION;
SH-ICPacBio
	Hybrid	4h 31m 45s	64	5.3

EC-IC MinION;
EC-IC PacBio;
SH-IC MinION;
SH-IC PacBio
	Long Read	23m 46s	64	0.9

Annotated genes and features across all assembly methods for both isolates were considerably reduced under the long read-only assembly, while both short read and hybrid methods resulted in comparable numbers of annotated genes. We suspect this is due to relative lower quality of long reads as compared to Illumina short reads. (**Table 3**). This is mirrored in resistome characterization and bacteriophage identification. While both short read and hybrid assembly methods for both isolates resulted in comparable identified resistome elements and bacteriophages, long read-only assembly identified elements were significantly reduced (**Table 5,6**).

The pipeline was run using the following commands for short read-only, hybrid, and long read-only:

```
$ nextflow R2R-0.0.1.nf --assembly nonhybrid--input containers/data/input_nonhybrid.csv --output
temp/output -w temp/work --threads 64  -with-report --name R2R_Nonhybrid_Assembly
$ nextflow R2R-0.0.1.nf  --input containers/data/input_hybrid.csv --output temp/output -w temp/work --
threads 64 -with-report --name R2R_Hybrid_Assembly
$ nextflow R2R-0.0.1.nf --assembly longread --input containers/data/input_longread.csv --output temp/
output -w temp/work --threads 64 -with-report --name R2R_Long-Read_Assembly
```

Each command was executed independently on a Linux server with 128 compute cores and 504GB of memory. Resources allocated and run-time in **Table 2** were obtained from the "report.html" which is generated using the '-with-report' option.

Table 3 Summary of evaluation for assembled isolates under various assembly conditions

		Short Read	Hybrid		Long Read	
		Illumina	MinION	PacBio	MinION	PacBio
Isolate	Assembly Metrics and Annotated Features					
EC-IC	No. contigs	220	11	10	16	6
	Largest Contig (bp)	143515	3902334	3890483	4876904	3256237
	Total Length (bp)	5082305	5292549	5286973	5266949	5421221
	N50 (bp)	42032	3902334	3890483	4876904	3256237
	L50	36	1	1	1	1
	GC (%)	50.54	50.38	50.36	50.05	48.86
	tRNAs	80	90	90	32	12
	CRISPRs	1	1	1	0	0
	Predicted CDS	4765	5041	5004	4826	4837
	Annotated Genes	2277	2297	2295	430	185
SH-IC	No. contigs	57	19	8	90	5
	Largest Contig (bp)	460444	2046586	4750196	64272	2033197
	Total Length (bp)	4844513	4869998	4899506	1448734	5035134
	N50 (bp)	213070	1175028	4750196	16758	1502365
	L50	9	2	1	28	2
	GC (%)	52.1	52.08	52.1	50.44	50.52
	tRNAs	77	76	82	2	30
	CRISPRs	3	3	3	25	1
	Predicted CDS	4554	4573	4581	1387	5976
	Annotated Genes	2037	2044	2043	87	213

Table 4 Bandage graphs for isolates under various assembly conditions

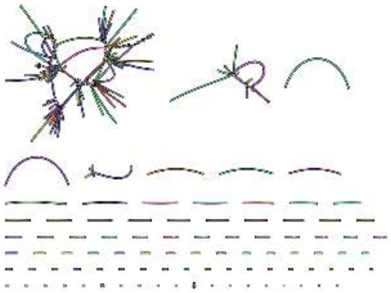
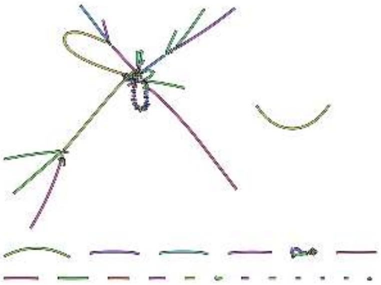
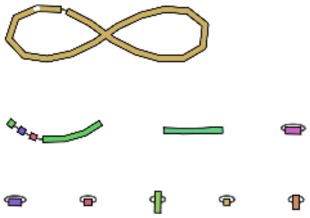
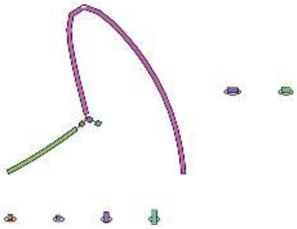
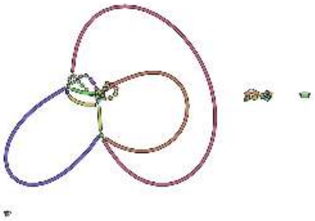
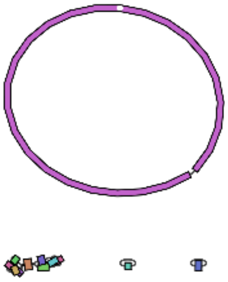
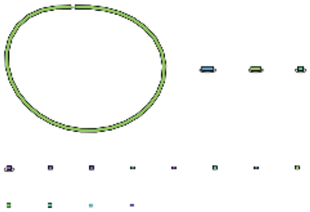
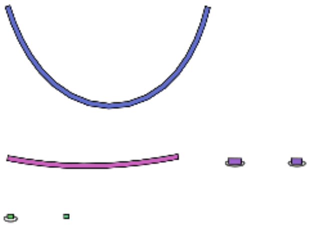
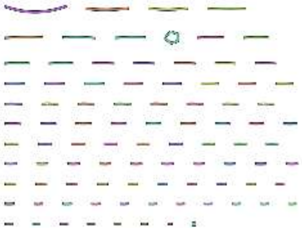
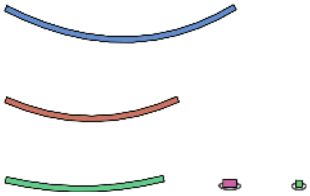
EC-IC		SH-IC	
Short read assembly			
			
Hybrid assembly			
MinION	PacBio	MinION	PacBio
			
Long read assembly			
MinION	PacBio	MinION	PacBio
			

Table 5 Summary of resistome characterization for assembled isolates under various assembly conditions

		Short Read	Hybrid		Long Read	
		Illumina	MinION	PacBio	MinION	PacBio
Isolate	Database	Number of identified elements				
EC-IC	ARG-ANNOT	8	8	8	7	8
	CARD	48	48	48	44	43
	MEGARes	58	58	58	53	52
	NCBI	4	4	4	3	4
	PlasmidFinder	7	7	7	5	5
	ResFinder	4	4	4	3	4
	VirulenceFinder	70	72	72	55	55
SH-IC	ARG-ANNOT	8	8	8	6	5
	CARD	29	30	30	6	9
	MEGARes	36	37	37	6	16
	NCBI	4	4	4	4	3
	PlasmidFinder	3	3	3	2	2
	ResFinder	5	5	5	5	4
	VirulenceFinder	105	105	106	44	99

Table 6 Summary of identified bacteriophages under various assembly conditions

	Short Read	Hybrid		Long Read	
	Illumina	MinION	PacBio	MinION	PacBio
Isolate	Bacteriophage family regions identified				
EC-IC	*Siphoviridae* *Myoviridae* *Myoviridae*	*Siphoviridae* *Siphoviridae* *Siphoviridae* *Myoviridae* *Myoviridae* Unknown	*Siphoviridae* *Siphoviridae* *Siphoviridae* *Myoviridae* *Myoviridae* Unknown	*Siphoviridae*	None
SH-IC	*Siphoviridae* *Podoviridae* *Myoviridae* Unknown	*Siphoviridae* *Podoviridae* *Myoviridae* Unknown	*Siphoviridae* *Podoviridae* *Myoviridae* Unknown	None	None

4. Limitations

Reads2Resistome is designed to be deployed on Linux servers with at least 16GB of RAM and at least 16 compute cores. Running Reads2Resistome on a personal machine will allow full completion of the pipeline but time requirements will be daunting using less than 16 compute cores. Prokka gene annotation custom databases consist of *Escherichia coli, Campylobacter, Salmonella, Enterococcus and Staphylococcus*; all other input samples will use the default Prokka database. Currently serovar prediction is only provided for *Salmonella spp*. using SISTR. Additionally, obtaining high-quality genome assemblies along with accurate resistome characterization is dependent on the quality and depth of sequencing obtained for the input isolates.

Conclusion

Reads2Resistome provides a streamline high-throughput analysis pipeline for the assembly, genome annotation and resistome characterization for bacterial sequenced reads. The pipeline can perform three methods of assembly; short read-only, long read-only or a hybrid method utilizing both short and long reads. The user can generate an input CSV file containing multiple isolate samples from different species, all of which can be fed into the Reads2Resistome pipeline under a user-specified assembly method. Pipeline output is generated for quality control, assembly and annotation for each isolate. The pipeline is executable on both Mac and Linux operating systems and is well-suited for institutions and organizations which maintain, or have access to, a high-performance cluster for the analysis of "big data."

Results from our case study indicated that obtaining a highly contiguous genome assembly with robust gene annotation, bacteriophage identification, and resistome characterization is best obtained under a hybrid assembly approach. While hybrid assembly is the most time-intensive assembly method, it produces the most complete annotated genome in our case study [23,24]. Long read-only assembly is able to produce a respectable genome length with high contiguity but falls short when annotating genomic features.

The ability to input multiple samples via the input CSV allows users to analyze hundreds of samples regardless of their identity. This prevents tedious bash scripting and collecting of various output files and ensures consistent and correct naming of all output files. Pipelines such as bacass from nf-core [25] provide genome assembly under short, long and hybrid approaches but do not offer resistome annotation. PRAP [26] and sraX [27] offer robust resistome analysis but do not provide genome assembly within the pipeline. Therefore, Reads2Resistome is the unique in providing a robust pipeline for genome assembly, genome annotation and resistome identification.

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Conflict of Interest: none declared.

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