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Benchmarking genome assemblers for four bacterial models based on contiguity, correctness, and completeness

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De novo **genome assembly allows for the genome reconstruction of an organism without using a reference sequence. Assembly results depend on various sequencing technologies that generate data with differing fidelity, read lengths, and coverage levels, as well as on performance of a wide variety of algorithms. These attributes generate a diversity of assemblies for each single genome, which collectively defines the pan-assembly. In this study, we aimed to benchmark pan-assemblies of the prokaryotic models *Bartonella henselae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Xylella fastidiosa*, using different attributes and their impact on metrics of the 3 C (contiguity, correctness, and completeness) criterion for selecting the best conditions for *de novo* genome assembly. Results showed that short-read assembly strategies presented higher accuracy with fewer errors (high correctness) and a high degree of completeness but lower contiguity due to fragmented assemblies. In contrast, long-read-based strategies showed high contiguity but lower completeness and accuracy. The hybrid strategy yielded the best overall results across all parameters by leveraging the strengths of both types of technology. Regarding assembly algorithms, Unicycler was the top assembler in 3 C metrics, using any of the short-read (compared to Megahit), long-read (compared to Canu), or hybrid strategies (compared to Wengan). Overall, the hybrid approach with Unicycler proved to be the best general approach for genome assembly of the four bacterial models. Finally, regarding coverage depth, increasing depth did not significantly affect assembly quality results if a minimum data level was maintained, indicating that high-quality assemblies can be achieved using moderate coverage levels. Jointly, the results of the pan-assembly provide working conditions for *de novo* genome assembly that can be applied to bacterial models of interest, guiding the selection of optimized experimental and bioinformatics conditions while reducing sequencing costs for generating high-quality sequences.**

Keywords Pan-assembly, Prokaryotes, *De Novo* genome assembly, 3 C criterion, Benchmarking

Whole genome assembly is the reconstruction of a nucleotide sequence that represents the actual genome from an organism, in which fragmented DNA pieces on average covered multiple times (or raw sequencing read data) are used[1,2]. When no prior knowledge of the source DNA sequence is assumed, a strategy called *de novo* genome assembly is considered. This approach is essential for investigating species in which a reference genome is unavailable or is not considered representative of the target genome due to a spectrum of genetic variations[3].

In *de novo* assembly, sequencing reads are assembled into consensus sequences named "contigs", that together represents most of the genome at a first level of assembly[2]. Several graph-based algorithms are available for this purpose, including Overlap-Layout-Consensus, de Bruijn and greedy approaches[4,5]. A subsequent level is the assembly of scaffolds. Scaffolding aims to bridge the gaps between the contigs using experimental data (for example mate pairs and paired end sequencing) or a reference genome[1], in which nucleotides of unsolved regions are represented with letter "N". When all gaps are solved (gap closing) by generating longer contigs[6] or Polymerase Chain Reaction[7] for example, a complete genome is assumed as a final level of assembly.

Regarding the generation of reads by sequencing technology, strategies can be separated based on the read length in short- or long-read sequencing. In short-read sequencing, such as Illumina's instruments (second-

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generation sequencing), reads can be produced with a length of up to 600 bases, accuracy > 99.9% and cost-effective[8]. In contrast, classical long-reads or third-generation strategies, such as Pacific Biosciences' (PacBio) single-molecule real-time sequencing and Oxford Nanopore Technologies' (ONT) sequencing, reads are obtained with > 10 kb and 85–90% accuracy with a higher cost[3,8]. Advances in PacBio technology have consolidated the HiFi sequencing method, which yields highly long-read sequencing with accuracy > 99.5%[9] but remains comparatively expensive. Recent advances in ONT sequencing technology have significantly enhanced both its hardware and data analysis pipelines, reducing error rates to approximately 6%[10]. Continuous methodological improvements are further increasing ONT accuracy; however, certain applications—especially those requiring single-nucleotide resolution—still benefit from the complementary use of short-read sequencing[10,11].

Notwithstanding these technologies have been widely used to determine the DNA sequence of thousands of genomes, a diversity of candidate genome sequences can be obtained depending not only on the experimental procedures but also on the bioinformatic pipelines[5,12]. These parameters include genome complexity (repeats, number of chromosomes, mobilome), sample conditions, DNA extraction protocol, reads length and sequencing technology, sequencing depth (number of times that a given nucleotide has been read in an experiment), algorithm to assemble sequence (assemblers), databases, and others[1,13]. In addition, criteria to benchmark assemblies and selecting a winner are also complicated, which depends on the aim of the study[14,15]. Due to all this, to *de novo* genome assembly is not straightforward and is still a very classical and challenging problem in bioinformatics[2,6].

To provide some insights to continue overcoming these challenges, we previously proposed the 3 C criterion to compare and assess *de novo* assemblies based on *Contiguity* (pieces of the assembled genome), *Correctness* (fidelity of the assembly) and *Completeness* (ability to assemble expected genes) using different assemblers with short- and long-reads for a bacterial model[14,16]. In line with the features of each technology and previous works[1,5,6,9,17], assemblies only using short reads (Illumina) resulted with a high correctness and completeness (not considering HiFi sequencing or improved ONT), while best values for contiguity were obtained for long-reads. Best values for all metrics were obtained when both strategies were used at the same time in hybrid assemblies.

As a proof of concept, in this current work we extend the assessment of a collection of assemblies for a specific organism, a concept that we here termed "pan-assembly", to select the best assembly based on the 3 C criterion. For this purpose, we selected four prokaryotic models in which short- and long-reads were used to assemble the genome with six assemblers (two short-reads only, two long-reads only, and two hybrid assemblers), and different levels of sequencing depth of coverage. The impact of these conditions on several 3 C parameters was quantified. Thus, the aim of the study was to benchmark pan-assemblies of the prokaryotic models *Bartonella henselae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Xylella fastidiosa*, using different attributes and their impact on metrics of the 3 C (contiguity, correctness, and completeness) criterion for selecting the best conditions for *de novo* genome assembly.

Methods

With the aim of providing conditions for generating a high-quality assembly for five bacterial models, a comparative assembly approach was implemented with three strategies (data source: short-reads, long-reads, or both), with two algorithms per strategy and different sequencing depth levels.

Data source and pre-processing

Sequencing data were retrieved from Sequence Read Archive database (SRA-NCBI, https://www.ncbi.nlm.nih.gov/sra) for four bacterial models: *Bartonella henselae*, *Xylella fastidiosa*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Table 1). Data were selected based on the FDA-ARGOS framework (https://www.fda.gov/medical-devices/science-and-research-medical-devices/ database-reference-grade-microbial-sequences-fda-argos) or similar projects.

Quality control was performed with FASTQC v.0.11.9 tool[18]. Trimmomatic v.0.39[19] was used for eliminating low-quality bases (Q < 30) and adapters.

Estimation of sequencing depth (also called depth or coverage, or simply coverage) was achieved by first mapping reads to the corresponding a reference sequence (from NCBI) with the BWA-MEM 0.7.5a-r405 software[20]. Then, alignment was examined with Qualimap 2.3 platform[21] to obtain several metrics, including sequencing depth.

Microorganism (model)	Short-reads		Long-reads		Reference sequence*
	SRA-ID	Depth	SRA-ID	Depth	
Bartonella henselae Houston-1	SRR2823712	450X	SRR2823712	500X	GCA_000046705.1
Escherichia coli FDAARGOS-1382	SRR15076580	621X	SRR15076579	416X	GCA_019048445.1
Pseudomonas aeruginosa AG1	SRR10387682	403X	SRR10387681	570X	GCA_009662315.1
Xylella fastidiosa GV230	SRR13994218	140X	SRR13994224	327X	GCA_014249995.1

Table 1. Sequencing data source and sequencing depth for microorganism included in this study. * Consensus sequence assembled and reported with the raw data for each model.

Pan-assembly of bacterial genomes

A standardized bioinformatics protocol was developed to assemble and annotate genomes for each bacterium. AnalysEs were run using the High-Performance Computing Cluster of the Center for Research in Materials Science and Engineering (CICIMA-HPC), University of Costa Rica. Raw sequencing data were subsampled based on sequencing depth (Table 1) using Seqtk 1.4 software (https://github.com/lh3/seqtk). Thus, trimmed reads subsets were generated with a sequencing depth of 12.5X, 25X, 50X, 100X, 200X* and 400X* (*: when possible). Depth was classified as low when < 100X, medium for 100X and high for > 100X.

Using trimmed data in each level of sequencing depth, two algorithms were implemented for each of the approaches using short-reads only, long-reads only, and hybrid (both short- and long-reads). The short-reads were processed with Unicycler v0.4.7[22] and Megahit v1.1.3[23]. Assemblers for long-reads were Unicycler v0.4.7, and Canu v1.8[24]. Finally, Wengan[25] and Unicycler v0.4.7 were implemented for hybrid assemblies. Jointly, all assemblies in the different conditions for a single genome were considered the pan-assembly.

Metrics related to assembly quality were calculated with QUAST 5.2.0 tool[26]. Gene prediction (structural genome annotation) was performed using Prokka v1.13.3[27], and results (GFF files) were used to compare assemblies based on gene content (similar to pan-genome analysis) using Roary v3.12.0[28].

Benchmarking of pan-assembly using the 3 C criterion

The 3 C criterion (Contiguity, Completeness and Correctness), defined previously in[14] was used to compare genome sequences in each pan-assembly. For this purpose, a variety of metrics were selected, as follows:

- Contiguity or number of assembled segments: the total number of fragments or contigs, N50 value (shortest contig length that needs to be included for covering 50% of the genome and other metrics were obtained using QUAST 5.2.0 tool[26].
- Completeness: the ability to assemble expected genes was assessed using the number of predicted genes by Prokka v1.13.3[27], as well as the completeness score based on analysis of orthologs with BUSCO v5.4.7[29] within the gVolante platform[30].
- Correctness: the fidelity of the assembly was estimated based on rates of mismatches and insertions/deletions of the assembly with respect to the reference sequence. Calculations were obtained for the analysis with QUAST 5.2.0 tool[26].

In addition, assessed conditions for each pan-assembly (depth level, assembly approach and algorithm/assembler) were compared and used to select the parameters for the reconstruction of each genome with the best quality possible. For this purpose, tests of statistical significance were performed with R v4.3.1 software (www.r-project.org/) using RStudio interface (www.rstudio.com). Statistical analyzes were run with parametric and non-parametric tests, as appropriate, including ANOVA tests or Kruskal–Wallis tests, T or U tests, multiple linear regression or generalized linear models (see Results). Additionally, a Hierarchical clustering and Principal Component Analysis were performed in R software to study each pan-assembly based on all metrics of the 3 C criterion.

Results

Sequencing data obtained by short- and long-reads for four bacterial models were used to generate *de novo* genome assemblies. The pan-assembly was established for each model using three strategies (based on a single technology separately or in a hybrid mode), two algorithms per strategy, and several levels of sequencing depth. The number of assemblies obtained for the organisms was 67 for *B. henselae*, 76 for *E. coli*, 69 for *P. aeruginosa*, 89 for *X. fastidiosa*.

Metrics of the 3 C criterion were calculated for each assembly, including those obtained from the comparison against the correspondent reference (consensus) sequence. Distribution of values for all assemblies evidenced a non-gaussian pattern, in which the median was used as measure of central tendency, as well as non-parametric tests (including multivariable models) for comparisons. Six metrics (two for each 3 C category) were used as key parameters for in-depth comparisons: number of contigs, N50, mismatches/100kbp, indels/100kbp, BUSCO score, and number of CDS. Using the whole set or selected metrics, pan-assembly was then described depending on sequencing strategy, assembler, and sequencing depth.

Regarding sequencing strategy, clear patterns of segregation depending on technology were found for all the models. This assessment of each pan-assembly was first done using clustering, based on the similarity among the whole set of 15 metrics of the 3 C criterion (details in Supplementary file). For *X. fastidiosa*, shown in Fig. 1-A-B, hierarchical clustering and PCA were implemented. These analyses not only assess the variation of metrics among assemblies, but also which sequences were more or less similar to each other. It is observed that clusters are defined by the sequencing strategy and then by algorithm for the assembly (assembler), rather than depth sequencing. In this line, distribution of values for the six key 3 C metrics defines a particular pattern depending on the strategy, as shown in Table 2; Fig. 2. Fragmented genome and lower N50 (lower contiguity) are reported for short-reads only, with median values of 90 and 101 890 respectively, in contrast to long-reads only (2 contigs and N50 = 2 513 184) or hybrid approaches (4 contigs and N50 = 1 441 411). In correctness, mismatches and indels are more variable with higher values for long-reads and hybrid methods. A higher completeness, based on BUSCO score and appropriate CDS number, was measured for short-reads only assemblies.

For *B. henselae*, *E. coli*, and *P. aeruginosa*, similar results were obtained during the clustering analysis (Supplementary Figs. 1–3) and median comparison among strategies, shown in Table 2 for each pan-assembly. Interestingly, for all the four models, the number of CDS were higher and BUSCO score were lower under long-

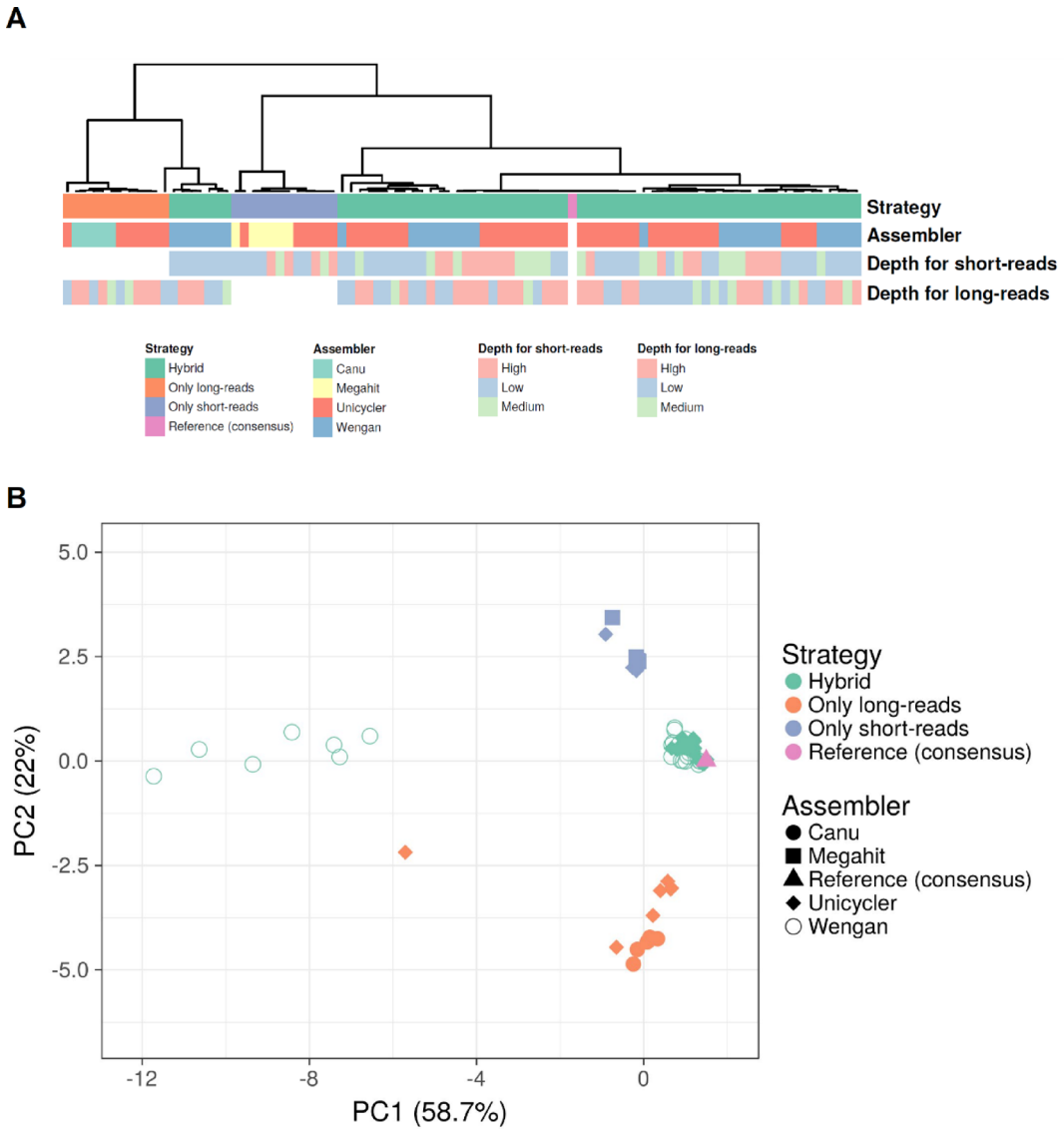

Fig. 1. **Assessment of the genome pan-assembly of *X. fastidiosa* using clustering algorithms based on metrics of the 3 C criterion.** Different conditions regarding strategy, assembler and sequencing depth were used to assemble the genome, resulting indifferent sequences that can be compared using a profile based on contiguity, correctness, and completeness (3C). In the panassembly,(A) Hierarchical clustering and (B) Principal Component Analysis (PCA) show that sequencing strategy and algorithm,unlike sequencing depth, impact the assembly when compared to the expected (reference) sequence.

reads only approaches, unlike other methods which remained homogeneous and closer to the values of the reference sequence.

In the comparison of assemblers, differences were revealed even using the same sequencing strategy. For *X. fastidiosa*, results for Megahit and Unicycler (short-reads) were similar among clustering analysis (Fig. 1A, B) and key 3 C metrics (Fig. 3). However, differences in values and dispersion were evidenced using long-reads (Canu vrs Unicycler) or Hybrid (Wengan vrs Unicycler), indicating that the algorithm influences the final assembly. This appreciation was also verified for the other prokaryotic models, as shown in Supplementary Figs. 1–3.

Besides, conditions of those assemblers belonging to the vicinity of the reference sequence were considered as the relevant parameters to obtain the expected sequence. In *X. fastidiosa*, these conditions were the use of Unicycler as assembler under a hybrid strategy (Fig. 1A,B). This is supported by the high value for the explained variance of 80.7% (PC1 + PC2) in the PCA analysis. The profile of long-reads only approaches (with Canu or Unicycler), as well as a few hybrid cases with Wengan, resulted with the more discordant profiles. Again, proximity to the reference sequence is not associated with the sequencing depth for short- or long-reads used for the assembly (Fig. 1A).

For the other bacterial models (Supplementary Figs. 1–3), hybrid approaches with Unicycler also resulted closer to the reference than other assemblies. Moreover, unlike *X. fastidiosa*, short-reads approaches tended to present a more different profile of 3 C metrics from reference when compared to the long-reads approaches. In

3 C component		Contiguity		Correctness		Completeness	
Model and strategy		Contigs	N50	Mismatches	Indels	CDS	BUSCO score
Bartonella henselae	Short-reads only	83	76,191	21.0	2.6	1539	100.0
	Long-reads only	4	1,572,283	32.8	65.6	2039	79.8
	Hybrid	3	1,879,773	36.0	12.3	1661	100.0
	Reference	1	1,931,047	0.0	0.0	1631	100.0
Escherichia coli	Short-reads only	51	260,187	6.3	0.2	4590	100.0
	Long-reads only	3.5	3,227,861	4.3	69.8	6058	73.8
	Hybrid	3	4,884,996	3.3	3.3	4651	100.0
	Reference	1	4,973,085	0.0	0.0	4553	100.0
Pseudonomas aeruginosa	Short-reads only	214	62,878	4.4	0.3	6520	98.4
	Long-reads only	2	7,185,317	39.6	341.6	11,428	38.3
	Hybrid	8	2,215,256	15.9	21.3	6655	98.4
	Reference	1	7,190,208	0.0	0.0	6620	100.0
Xylella fastidiosa	Short-reads only	90	101,890	9.0	1.8	2218	100.0
	Long-reads only	2	2,513,184	6.3	164.0	3630	50.0
	Hybrid	4	1,441,411	13.8	9.0	2381	99.0
	Reference	1	2,514,993	0.0	0.0	2369	100.0

Table 2. Comparison of median values for key metrics of the 3 C criterion in the assessment of genome pan-assembly of four bacterial models.

all cases, these patterns were provided with high support for the explained variance of the PCA, with 72.9%, 83.5% and 78% for *B. heneselae*, *E. coli*, and *P. aeruginosa*, respectively.

On the other hand, a comparative genomics approach was implemented to describe the pan-assembly based on gene content. As found in Figs. 4 and 5 for the four models (blue lines: presence of the gene in each assembly), the well-defined clusters suggest that the gene content profile is established -again- by sequencing strategy and assembler, but independent on sequencing depth (after a minimal value). For *X. fastidiosa* (Fig. 4), gene fragmentations are identified for long-reads only and some hybrids with Wengan (when sequencing depth for short-reads is 12.5X). Gene fragmentations for long-reads, as well as some specific hybrid cases with Wengan, are also present in the other models (Fig. 5). In *P. aeruginosa*, loss of genes is evidenced when using Wengan even for > 100X depth for short-reads, besides no assemblies were established for lower levels with the same assembler. Unlike this case, using Unicycler in a hybrid mode, sequences were built using at least 25X depth for short-reads.

A final assessment of the parameters studied here (predictors: strategy, assembler, and sequencing depth) and their effect on the key 3 C metrics (response variable) was done using generalized linear models (GLM) for the pan-assembly of each organism. As summarized in Table 3 for all the four bacteria, significant values (<0.01) were obtained for the very most cases of the 3 C metrics using strategy and assembler as predictors. Only scarce significant values were evidenced for sequencing depth for short- or long-reads among response conditions.

Finally, the subsequent analysis was conducted to determine the minimal sequencing depth to achieve an assembly with a similar quality to assemblies obtained with depth ≥ 100X data under the same strategy and assembler (Table 4). Using short-reads only approaches, requirements of depth for Unicycler and Megahit were always the same for all the bacterial models (12.5X for *E. coli* and *X. fastidiosa*, and 25X for others). A similar situation was observed for long-reads only approaches, in which 25X is enough to assemble the sequence for all bacteria but *X. fastidiosa* (with 12.5X). In the case of hybrid approaches, Wengan was demonstrated to always need more sequencing depth in comparison to Unicycler, including requirements of > 100X depth for short-reads (*B. henselae* and *P. aeruginosa*). In *B. henselae*, Wengan also demanded 100X depth for long-reads. When sequencing depth ≥ 25X (in some cases even with lower values), hybrid Unicycler was always able to assemble the sequence.

Jointly, whole results of pan-assembly analyses indicate that the assemble sequence and the 3 C metrics are significantly impacted by sequencing strategy and assembler, but not by sequencing depth after a minimal level is considered for the four bacterial models.

Discussion

De novo genome assembly allows for the genome reconstruction of an organism without using a reference sequence[31]. However, the assembly results depend on various sequencing technologies that generate data with differing fidelity, read lengths, and coverage levels, as well as on performance of a wide variety of assemblers (algorithms)[13,32]. In this study, we compared pan-assemblies under these conditions and their impact on *de novo* genome assembly for four bacterial models, using the 3 C criteria -contiguity, correctness, and completeness- for evaluation.

Regarding contiguity, our results indicate that short reads present significant variability in the number of contigs, suggesting high fragmentation in the resulting assemblies and reduced N50 values (low contiguity), in contrast to when long reads are used either alone or in hybrid formats. Thus, the use of long-read technology

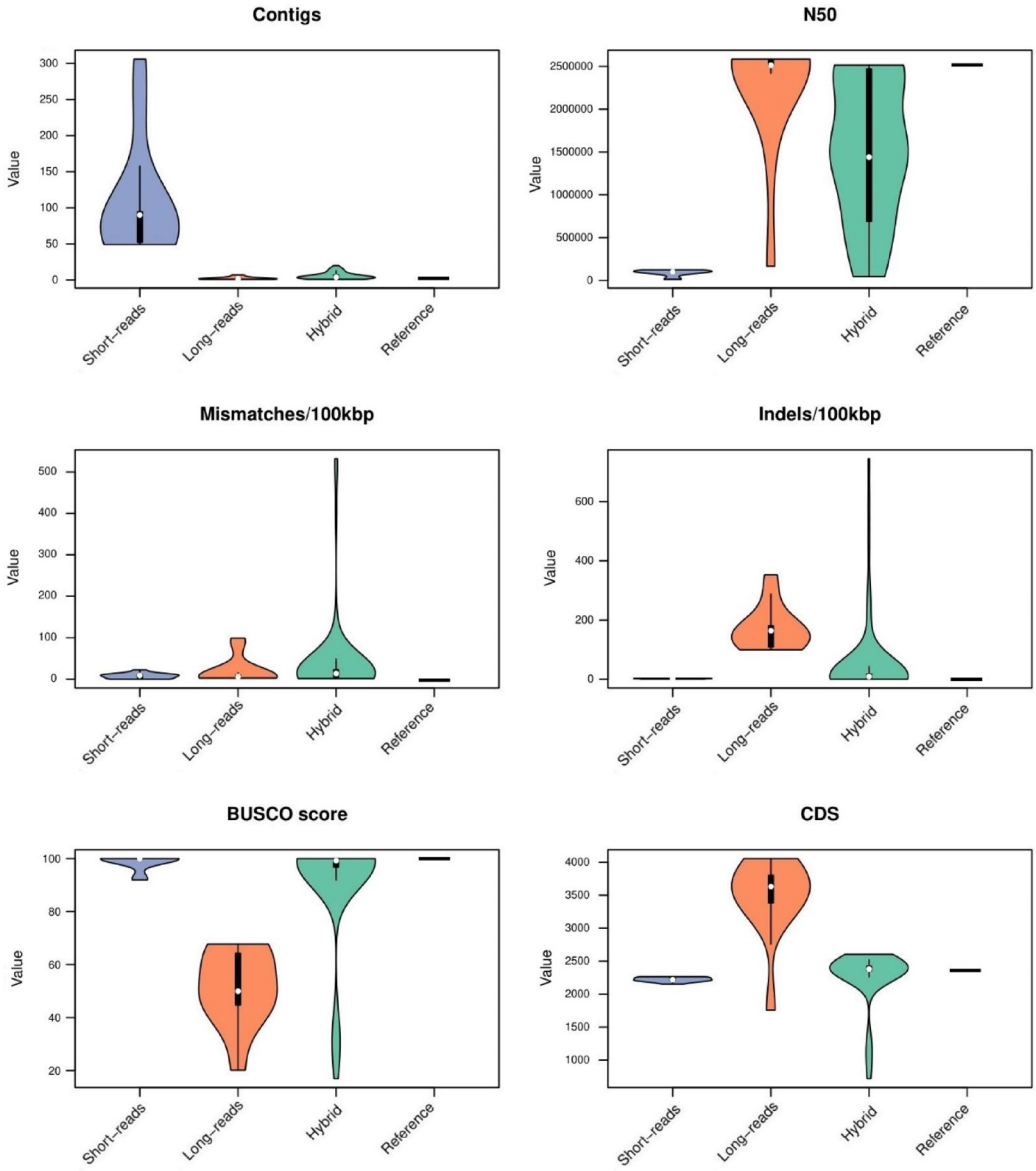

Fig. 2. Comparison of key metrics of the 3 C criterion among sequencing strategies for the genome pan-assembly of *X. fastidiosa*. Violin plots were used to assess distribution of each parameter according to the use of short-reads only, long-reads onlyor hybrid approaches (which included all six algorithms and several levels of sequencing depth). In comparison to the referencesequence, the contiguity (contigs and N50) resulted with best values for long-reads and hybrid strategies, while a more confidentcorrectness (mismatches and indels) was found for short-reads only. Completeness (BUSCO score and CDS) was found to be closeto the reference sequence for short-reads only approaches.

improves contiguity and potentially leads to better genome reconstruction under this criterion, even allowing for the potential circularization of bacterial genomes, as previously demonstrated[22,33]. In another benchmarking strategy, the performance of long reads was also outstanding, with a low number of contigs and high N50 values[34]. However, a detailed inspection of the fragments revealed assembly errors that were not apparent when only contiguity was assessed. These findings have also been highlighted in other comparative studies[35,36]. This underscores the need for diverse metrics to evaluate assemblies, as we propose with the 3 C criteria[14,16].

In the context of correctness or fidelity evaluation, significant differences were reported in the error rates for the four bacterial models. Short reads demonstrated a lower incidence of errors compared to long reads. In other studies, using Illumina and Oxford Nanopore data for various bacterial models, the results support our findings, with a low error rate detected when working with short-read data, including regions containing repeats[14,35,36]. Thus, in applications where fidelity is a priority, short reads may be preferable to minimize specific errors such as those caused by indels and technical sequencing errors[33,37].

Regarding completeness, the best performance was identified in assemblies with short reads and hybrid strategies. This effect was also evident in the pan-assembly analyses based on CDS gene content, with significant

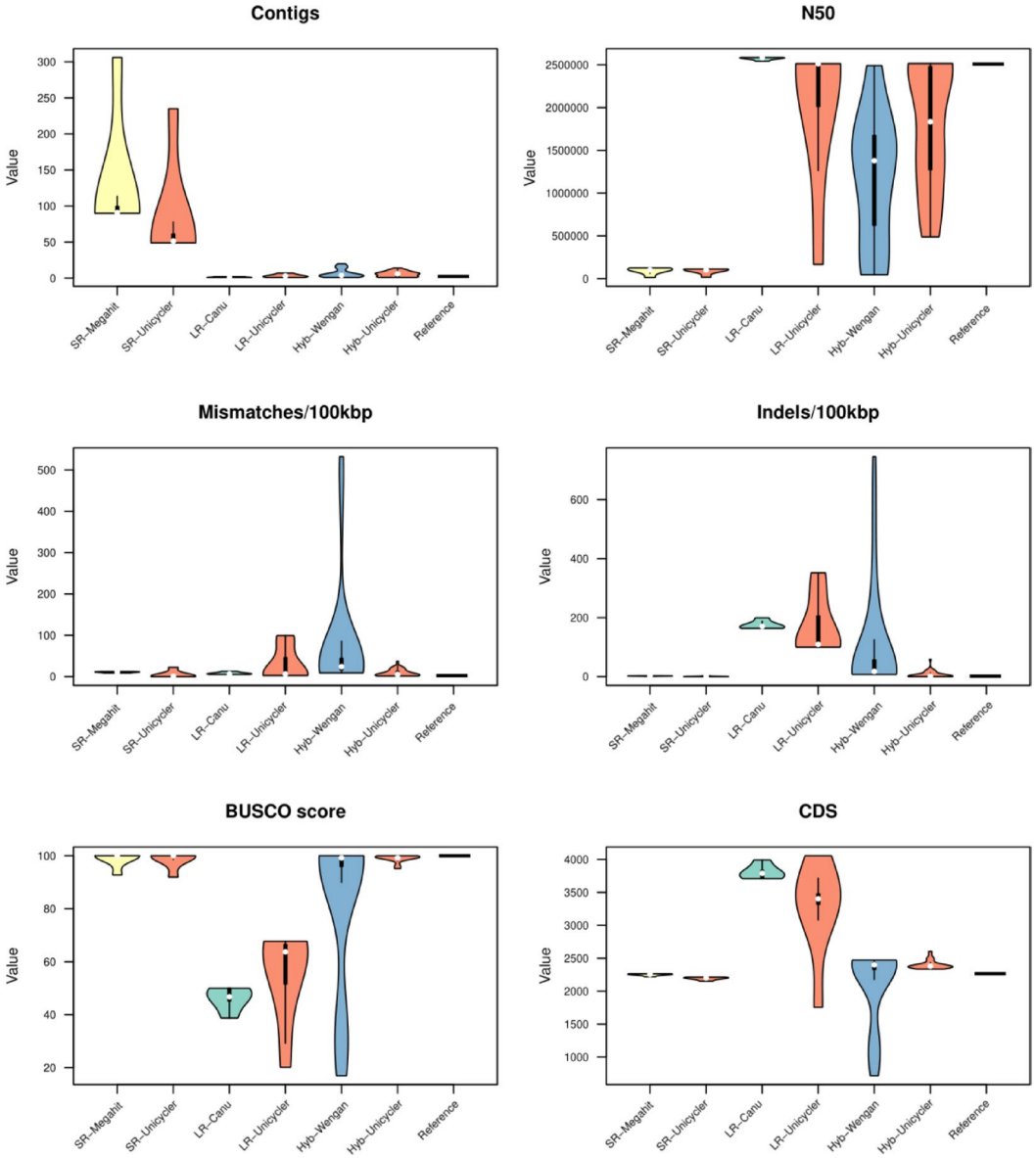

Fig. 3. **Comparison of key metrics of the 3 C criterion among assembler for the genome pan-assembly of *X. fastidiosa*.** Violin plots were used to assess distribution of each parameter according to the use of different assemblers (which included severallevels of sequencing depth).

reductions mainly in hybrids assemblies using Wengan and low-coverage conditions. Although long reads can span more complex and broader genomic regions, gene prediction is incomplete due to assembly errors, a characteristic of these strategies with lower fidelity[38]. These findings reflect the capability of short reads to generate assemblies with high completeness, despite inherent limitations such as raw data length and low contiguity[33].

For hybrid strategies, the high completeness is associated with the resolution of ambiguities in genomic regions characterized by repetitive or highly complex sequences provided by long reads[39,40]. In other works, the best results are justified by the resolution achieved with long assembled fragments[37,41], although this contrasts with other cases where long reads are associated with lower completeness and gene identification due to frameshifts from sequencing errors and their impact on gene prediction[14]. This latter effect is also evidenced by the high number of CDS (compared to the reference sequence) found for long reads but not for other strategies.

Due to the lower fidelity of long-read strategies, the need to continue optimizing long-read sequencing techniques and minimizing errors without compromising assembly integrity is emphasized[42]. Notable advances in this area include the implementation of polishing strategies for assemblies generated from long-read data, which have significantly improved assembly completeness[33,43]. In parallel, the development of high-fidelity long-read sequencing technologies—such as PacBio HiFi[9,38,44] —and optimized ONT platforms incorporating new flow cells and deep learning–based data processing have further enhanced sequencing accuracy and reliability[10,11]. These two scenarios were not included in this study.

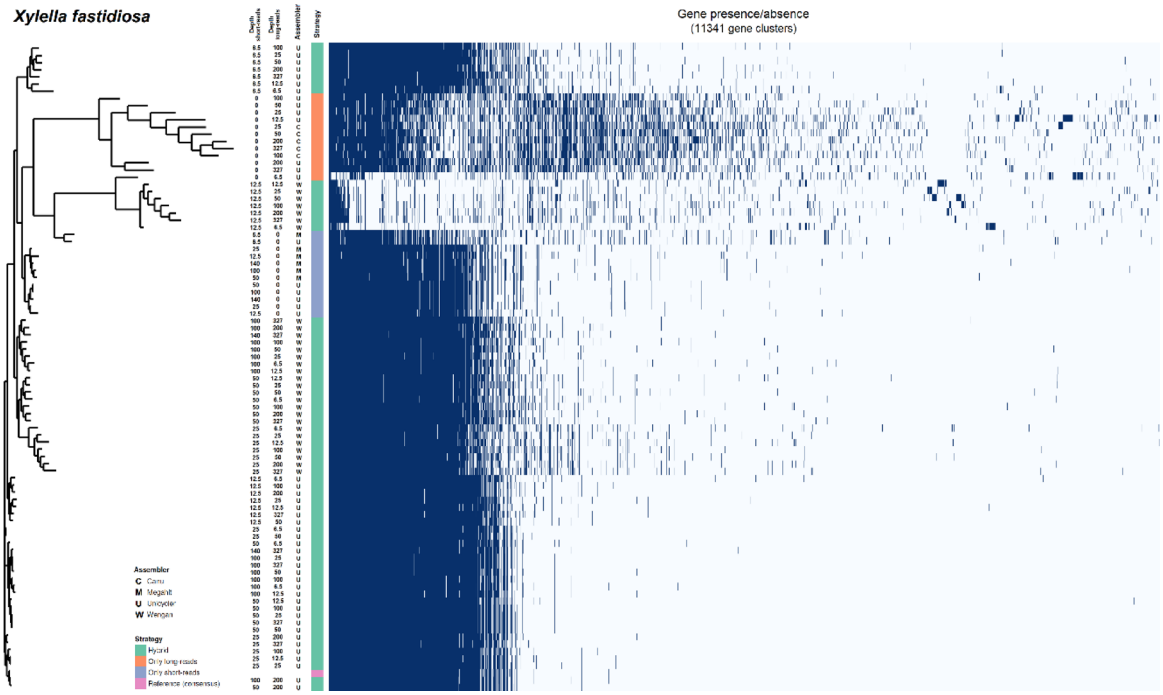

Fig. 4. Comparison of gene content predicted for each sequence of the genome pan-assembly of *X. fastidiosa*. Based ongene content (presence: blue), a pan-genomic approach was used to assess all assemblies. Expected genes (based on thereference sequence) were found for most cases using short-reads, hybrid approaches with Unicycler and some hybrids with Wengan.Other Wengan-based hybrid assemblies, as well as long-read only cases, resulted in an unexpected profile with a different number ofgenes and apparently several "exclusive" genes. Despite some exceptions, no association between sequencing depth and the genecontent outcome is evidenced.

Regarding algorithms, this study observed statistically significant differences in the performance of assemblers for genome reconstruction. Their effect was evident in the size of the contigs produced (N50 and number of contigs), the number of expected genes reconstructed or completeness (BUSCO Score and CDS), fidelity or correctness (indels and mismatches), and the resolution of pan-assemblies based on gene content. Overall, the choice of algorithm has direct implications for the quality and utility of the assembled genome[5,45,46]. Additionally, as evidenced here, using exactly the same input data within the same assembly strategy, fidelity results increase for certain algorithms[47].

Considering all parameters, assemblies using Unicycler performed best in each strategy for the bacterial models studied. As supported by the literature in various benchmarking studies, Unicycler is currently one of the algorithms with the best reported performance for bacterial genome assembly, whether using long reads, short reads, or a hybrid mode[14,35,43,48]. In studies with only long reads, Unicycler also stood out as the top-performing algorithm[35], though in other studies, Canu has been reported as performing better[46–48]. In our study, Canu showed good values in contiguity and moderate accuracy and completeness, but its performance did not surpass that of Unicycler. However, it is worth noting that Canu is optimized for maximizing performance in cluster computing environments[40]. Regarding the use of Megahit for assemblies with only short reads, it has been reported as a high-performing assembler compared to other strategies[48–50], even comparable to Unicycler, mainly in terms of completeness and accuracy. A strength of Megahit is its execution time, which is much faster compared to Unicycler. For hybrid assemblies, the other algorithm used was Wengan. In this study, it performed well but depended on high coverage to produce high-quality assemblies, unlike Unicycler in hybrid mode. This observation contrasts with a previous report where Wengan was highlighted for generating quality assemblies even in low-coverage situations[25]. In other metrics, Wengan has been reported with superior performance compared to other algorithms[25,46]. However, Wengan showed suboptimal performance in contiguity, with low N50 values in another study[38]. In summary, Unicycler showed the highest concordance with reference genome data compared to other algorithms, regardless of whether long reads, short reads, or hybrid modes were used. The hybrid mode demonstrated the best overall performance.

Lastly, the final evaluation of the pan-assembly focused on the possible correlation between quality of the assembly and coverage depth. The results suggest that there is no statistical correlation between coverage depth (after a minimum level) and 3 C criteria metrics, with a few exceptions. These results are consistent with several reports in the literature regarding this parameter in genome assembly analyses, showing no association between increased depth (in both long and short reads) and improved assembly quality[43,51,52]. One study highlighted that values of 40X do not change the results in cases of lower coverage, and ultra-deep sequencing can lead to algorithm saturation and a significant increase in computational resource usage[43]. It has also been reported that

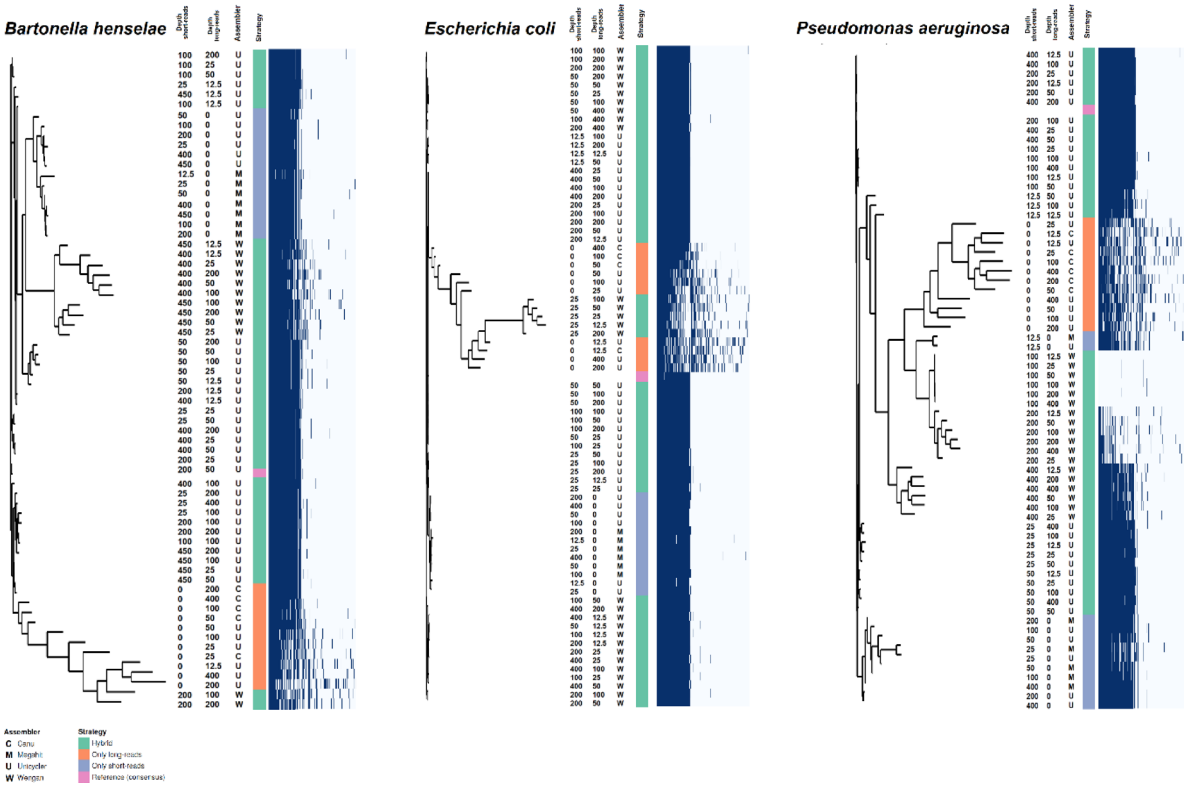

Fig. 5. **Comparison of gene content predicted for each sequence of the genome pan-assembly of *B. henselae*, *E. coli*, and *P. aeruginosa*.** Based on gene content (presence: blue), a pan-genomic approach was used to assess all assemblies. Expectedgenes (based on the reference sequence) were found for most cases using short-reads, hybrid approaches with Unicycler and somehybrids with Wengan. Other Wengan-based hybrid assemblies, as well as long-read only cases, resulted in an unexpected profilewith a different number of genes and apparently several "exclusive" genes. Despite some exceptions, no association betweensequencing depth and the gene content outcome is evidenced.

very low coverage levels of 16x are sufficient to assemble a complete genome, but accuracy improves with values around 30X[46]. In other studies, the minimum coverage level has even been reported as low as 10X[51,53]. These minimum values are very similar to our results for the four organisms studied. It should be noted that in certain applications, such as genetic variant imputations, there are recommendations for a minimum coverage level for decision-making[53,54], but these are not focused on *de novo* genome assembly. From a practical perspective, this aspect of coverage depth is highly relevant because it means that high-quality assemblies can be generated using moderate coverage levels, without the need for ultra-deep sequencing (> 100X), translating into significantly lower sequencing costs.

In summary, once a minimum data level is reached, coverage depth does not significantly affect assembly quality results across the various characteristics evaluated by the 3 C criteria, for which no statistical correlation was observed. This contrasts with the influence of strategy (technology) type and assembly algorithms on the quality of the resulting assembly. These results are based on genome sequencing, and our previous works on other molecular strategies[55,56], will be considered to continue working on these bacterial models at the local biological context.

It should be mentioned that this study has some limitations. This study focused on four bacterial models, each with their own genomic complexity, but it could be extended to other models, including eukaryotic organisms in further analyses. Other methodological strategies, such as genome polishing or high-fidelity long-reads data obtained using HiFi sequencing ot enhanced ONT platforms, were not assessed in this comparison. Additionally, other 3 C criteria metrics, execution time and computational resource usage could be valuable for comparing assembly conditions in other studies.

Conclusions

In this study, the pan-assembly of four bacterial models (*B. henselae*, *E. coli*, *P. aeruginosa*, and *X. fastidiosa*) was assessed using contiguity, accuracy, and completeness metrics (the 3 C criteria). The benchmarking strategy showed that short-read assemblies presented higher accuracy with fewer errors (high correctness) and a high degree of completeness but lower contiguity due to fragmented assemblies. In contrast, long-read-based strategies showed high contiguity but lower completeness and accuracy. The hybrid strategy yielded the best overall results across all parameters by leveraging the strengths of both types of technology. Regarding assembly algorithms,

Bacterial model	3 C metric (response variable, Y)	Predictor (factors, X_i)			
		Strategy	Assembler	Depth for short-reads	Depth for long-reads
B. henselae	Contigs	***5.72E-07***	***0.0007***	0.3921	***0.0090***
	N50	***3.27E-05***	***0.0023***	0.3786	0.0124
	Mismatchs	***1.09E-05***	0.0144	0.1267	0.3386
	Indels	***2.95E-06***	***1.09E-05***	0.1986	0.2241
	CDS	***2.79E-06***	0.0591	0.8925	0.1900
	BUSCO score	***0.0011***	***2.12E-05***	0.1749	0.3063
E. coli	Contigs	***5.55E-06***	***0.0001***	0.0729	***0.0006***
	N50	***4.59E-05***	***0.0008***	***0.0059***	***0.0003***
	Mismatchs	0.3936	***0.0043***	***2.43E-06***	0.1331
	Indels	***1.44E-07***	***4.14E-06***	0.1184	0.0432
	CDS	***1.48E-09***	***0.0010***	0.2439	0.0651
	BUSCO score	***3.00E-08***	***0.0081***	***0.0001***	0.9368
P. aeruginosa	Contigs	***1.92E-07***	***4.58E-05***	0.0224	0.5472
	N50	***7.19E-08***	***0.0009***	0.1138	0.4283
	Mismatchs	***2.36E-08***	***3.74E-06***	0.3527	0.4355
	Indels	***1.33E-10***	***6.06E-07***	0.2463	0.4424
	CDS	***2.13E-07***	***9.47E-05***	0.2224	0.9103
	BUSCO score	***0.0004***	***3.79E-07***	0.2384	0.2384
X. fastidiosa	Contigs	***1.52E-08***	***4.15E-05***	0.0318	0.0472
	N50	***2.14E-08***	***3.49E-06***	0.0230	***0.0069***
	Mismatchs	0.0424	***7.59E-08***	***0.0002***	0.1449
	Indels	***5.14E-07***	***1.31E-06***	***0.0004***	0.3025
	CDS	***6.61E-08***	***0.0005***	0.1496	0.4162
	BUSCO score	***5.09E-06***	***0.0026***	***5.26E-10***	0.6539

Table 3. Statistical significance (p-values*) for generalized linear models in the association of assembly parameters (strategy, assembler, and sequencing depth) and a key metric of the 3 C criterion in the assessment of genome pan-assembly of four bacterial models. * Values with $p < 0.01$ (99% confidence) are presented with italics and bold font.

Model	Assembly parameters	Short-reads only		Long-reads only		Hybrid	
		Megahit	Unicycler	Canu	Unicycler	Wengan	Unicycler
B. henselae	Depth for short-reads	25X	25X	-	–	200X*	25X
	Depth for long-reads	–	–	25X	25X	100X*	25X
E. coli	Depth for short-reads	12.5X	12.5X	-	–	50X	12.5X
	Depth for long-reads	–	–	25X	25X	25X	12.5X
P. aeruginosa	Depth for short-reads	25X	25X	-	–	400X**	25X
	Depth for long-reads	–		25X	25X	25X	12.5X
X. fastidiosa	Depth for short-reads	12.5X	12.5X	–	–	50X	12.5X
	Depth for long-reads	–	–	12.5X	12.5X	12.5X	6.5X

Table 4. Minimal sequencing depth to achieve an assembly with a similar quality to assemblies obtained with depth ≥ 100X data under the same strategy and assembler. * Genome assembly was only achieved using this minimal value (i.e. no genome sequence was obtained using lower depths). ** Genome assembly was achieved with lower depths but with a reduced quality.

Unicycler was the top assembler in 3 C metrics, using any of the short-read (compared to Megahit), long-read (compared to Canu), or hybrid strategies (compared to Wengan). Overall, the hybrid approach with Unicycler proved to be the best general approach for genome assembly of the four bacterial models. Finally, regarding coverage depth, increasing depth did not significantly affect assembly quality results if a minimum data level was maintained, indicating that high-quality assemblies can be achieved using moderate coverage levels. In summary, these results of the pan-assembly provide working conditions for *de novo* genome assembly that can be applied to bacterial models of interest, guiding the selection of optimized experimental and bioinformatics conditions while reducing sequencing costs for generating high-quality sequences.

Data availability

The raw sequencing data (short and long reads), as well as the assembled genomes used in this study, are available in GenBank and SRA databases (NCBI), with the accession number detailed in Table 1.

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Author contributions

J.A.M.M. participated in the conception and design of the study. H.R.M., V.M.L. and J.A.M.M. performed experimental assays and data analysis. J.A.M.M. drafted the manuscript. All authors were involved in its revision and the final approbation of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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