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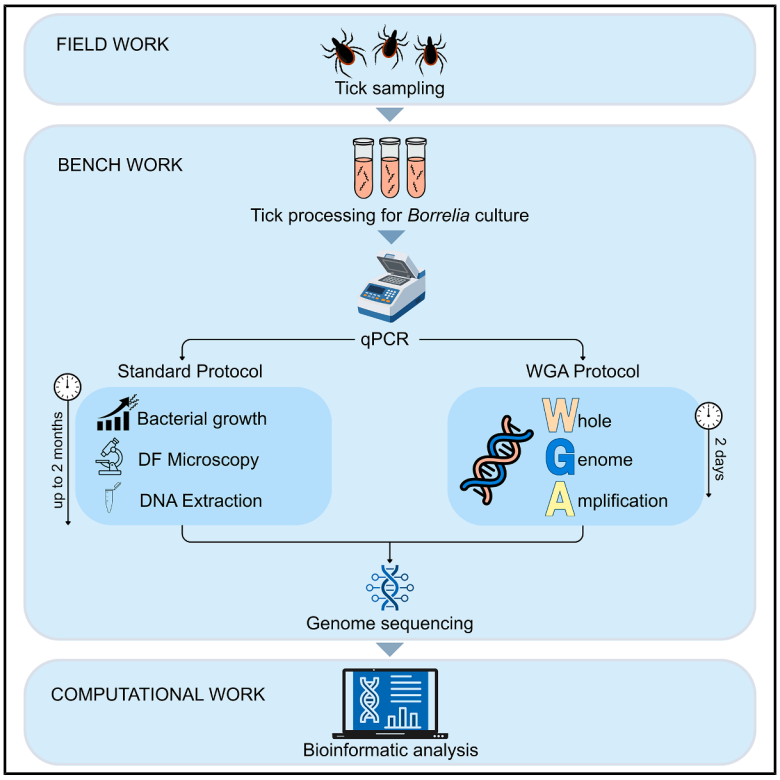
Fast and efficient *Borrelia* chromosome recovery from tick samples using whole-genome amplification

Graphical abstract



Highlights

- Whole-genome amplification enables sequencing-ready *Borrelia* DNA within 5 days
- High-quality chromosomal assemblies are generated from low-input samples
- Custom bioinformatic pipeline ensures accurate genome reconstruction
- This workflow lays a foundation for expanding *Borrelia* genomics

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In brief

Melis et al. describe a workflow that bypasses prolonged culturing of *Borrelia* by combining short-term culture with whole-genome amplification. The method delivers high-quality chromosomal genomes within 5 days, helping to expand genomic resources for understudied species and accelerating epidemiological and evolutionary research.

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Report

Fast and efficient *Borrelia* chromosome recovery from tick samples using whole-genome amplification

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MOTIVATION *Borrelia* (subgenus *Borreliella*) *burgdorferi* sensu latu (s.l.) bacteria are the causative agents of Lyme borreliosis, a multisystemic illness with an expanding epidemiology in temperate areas. Genomic studies represent a crucial tool for epidemiological investigation and for enhancing understanding of the biology and pathogenesis of the organism. However, genomic analyses on these pathogens are hindered by the difficulty of culturing procedures: standard protocols require extended incubation, substantial technical expertise, and are prone to failure, limiting timely recovery of isolates.

SUMMARY

Genomic studies of *Borrelia* are essential for understanding Lyme borreliosis epidemiology and biology but are limited by slow, labor-intensive culture methods. Here, we introduce an approach that overcomes the need for extended culture by combining a short-term culture step with whole-genome amplification (WGA) of samples derived from field-collected ticks, which can be performed in parallel with classical culturing. The protocol is paired with a tailored bioinformatic pipeline to ensure accurate assembly and robust downstream analyses. Benchmarking on multiple control isolates shows that the method produces high-quality chromosomal assemblies. We further demonstrate applicability by generating five high-quality Borrelia chromosomes from freshly collected ticks (two *B. lusitaniae*, two *B. afzelii*, and one *B. garinii*). By producing sequencing-ready DNA in 5 days rather than months, this workflow streamlines genome generation and reduces reliance on culture-based isolation, facilitating broader genomic representation of understudied *Borrelia* species and enabling future epidemiological, ecological, and evolutionary studies.

INTRODUCTION

Lyme borreliosis (LB) is the most prevalent vector-borne illness in the Northern hemisphere, with around 30,000 confirmed cases in the United States and around 85,000 reported cases in Europe each year.[1–3] A typical early sign of LB is an expanding skin lesion known as erythema migrans that, if left untreated, can progress to a disseminated infection. This may develop into severely debilitating stages characterized by arthritis, neurological disorders, carditis, and acrodermatitis chronica atrophicans.[4] LB is caused by a complex of species with a currently debated genus/sub genus name, with the latest proposal being *Borrelia* (subgenus *Borreliella*) *burgdorferi* sensu latu (s.l.) complex.[5]

The *B. burgdorferi* s.l. complex currently comprises 28 validated and *Candidatus* species.[5] These different species, and even different isolates within the same species, can cause different clinical manifestations.[3,6] For instance, *B. garinii* and *B. bavariensis* are more associated with neuroborreliosis, while *B. afzelii* mainly causes skin infections.[4,7] The genetic bases of such differences are currently unclear. In Europe, the primary vector is the hard tick *Ixodes ricinus* and the most common circulating LB-causing species

(hereafter referred to as *Borrelia*) include *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. valaisiana*, and *B. lusitaniae*.[8] While the first three species are known human pathogens, no clinical cases have been reported for *B. valaisiana*,[9] and the pathogenic potential of *B. lusitaniae* remains poorly understood.[9,10]

Bacterial genomics now is a key tool in microbiological research and allows to refine phylogenetic reconstructions, to improve epidemiological characterizations, and to discover genomic determinants of pathogenicity, virulence, and resistance to antimicrobial agents. Unfortunately, despite several notable efforts,[11–14] *Borrelia* genomics have been lagging behind compared to other pathogens, with relatively few high-quality genomes publicly available for species outside *B. burgdorferi* s.s.[15]

The unusual genome architecture of *Borrelia*, which combines a linear chromosome with up to 21 linear and circular plasmids (ranging from 5 to 84 kbp in size) represents a challenge for genomic studies.[11,16] Additionally, an even greater challenge lies in the difficulty of obtaining pure cultures. Isolation from patients typically relies on tissue biopsies or aspirates, an invasive procedure that is rarely recommended and not always feasible. Culturing of *Borrelia* from ticks or patient material is inefficient: it requires 1–2 months of incubation depending on the species, and in our experience yields successful isolates from only about 25% of PCR-positive samples (Fingerle et al., unpublished). Moreover, culture-based approaches are not only slow but also labor-intensive and resource-demanding, limiting their scalability for large epidemiological or comparative genomics studies. As a result, many *Borrelia* isolates risk never being sequenced, leaving important gaps in our understanding of the diversity and pathogenic potential of this genus. A potential alternative could be whole-genome capture,[17] considered a valid tool for DNA isolation of *B. burgdorferi* s.s. from *Ixodes* samples. The method is, however, based on specific probes, and its performance on other members of the group is uncertain, especially for understudied and/or divergent species as *B. lusitaniae*.[18]

These drawbacks highlight the need for alternative strategies to obtain *Borrelia* genomes without relying solely on culture.

Here, we describe a workflow that, after a first step of pre-enrichment in *Borrelia* medium, combines whole-genome amplification (WGA) with dedicated bioinformatic analyses, enabling the recovery of sequencing ready *Borrelia* samples from single ticks within a total of 5 days. WGA allows amplification of genomic DNA from very small starting quantities, bypassing the need for long culture periods and enabling genome sequencing from early-stage cultures or low-input samples. In fact, as little as picograms of starting DNA is sufficient for WGA, compared to the nanograms required for genome sequencing even with the more sensitive Illumina library preparation protocols. Our approach shows substantially higher success rates compared to conventional methods, while remaining compatible with parallel PCR-based diagnostics and classical culturing procedures.

RESULTS

We designed a lab protocol based on WGA to be performed in parallel to the standard protocol for *Borrelia* isolation. This enables the generation of *Borrelia* genome sequences faster and with a higher success rate. The new protocol is compatible with the standard culture protocol which can be performed in parallel with the remaining material. To validate the approach, we first evaluated the quality of WGA genomes from pure cultures. Then, we applied the protocol and the associated bioinformatic pipeline to fresh tick samples.

Overview of the experimental design

Two approaches were used for the generation of *Borrelia* genomic DNA: a standard culturing protocol (hereafter: standard protocol)[11] and a protocol that leverages WGA (hereafter: WGA protocol) (Figure 1). Three pure cultures of *Borrelia* isolates (IT-B1, IT-G1, and IT-G2), previously obtained from ticks and stored in our collection, were processed with both protocols. These samples are hereafter referred to as "positive pure culture controls" and were used to compare the quality of the genomes generated with both protocols. We then tested WGA protocol on freshly collected ticks (see Table S1 for sample details).

WGA enables accurate *Borrelia* genome assemblies

To assess the performance and reliability of the WGA approach, we first evaluated its impact on genome quality, testing it on three *Borrelia* positive pure culture controls, specifically one *B. burgdorferi* s.s. and two *B. garinii* samples. DNA was extracted from these cultures and subjected both to WGA and to standard DNA extraction workflow and then sequenced using Illumina and nanopore platforms.

We initially assessed the uniformity of coverage by comparing the two approaches across the three samples. As expected, coverage plots from the WGA protocol exhibited a more uneven distribution across the chromosome compared to the smooth, uniform coverage obtained with the standard protocol (Figure S1). This pattern reflects the amplification biases inherent to WGA methods. Importantly, however, no chromosomal regions were left uncovered in WGA assemblies. This indicates that, despite minor biases, the WGA approach provides comprehensive chromosomal representation suitable for downstream analyses.

Using the same hybrid assembly method for both standard and WGA-derived datasets, we generated complete genome assemblies for the three *Borrelia* samples and subsequently compared their quality and completeness (Table 1). In both *B. garinii* isolates, IT-G1 and IT-G2, the chromosome was assembled into a single contig both from pure culture and from WGA. For IT-B1, a complete chromosome assembly was obtained only with the WGA protocol, while the standard approach produced a more fragmented result. Despite the lower nanopore sequencing yield observed for IT-B1 using the standard protocol (Table S2), coverage across the genome was generally consistent, with only a single coverage drop detected (Figure S1). Lower yield reduced the availability of long reads spanning challenging genomic regions, together with assembler-specific limitations, may have impaired contig assembly. The WGA-based approach, by contrast, provided sufficient read support to overcome this limitation and produce a complete chromosome assembly.

Overall, chromosomal sequences were nearly identical across methods, with only minimal SNP differences ($\leq$4 per genome)

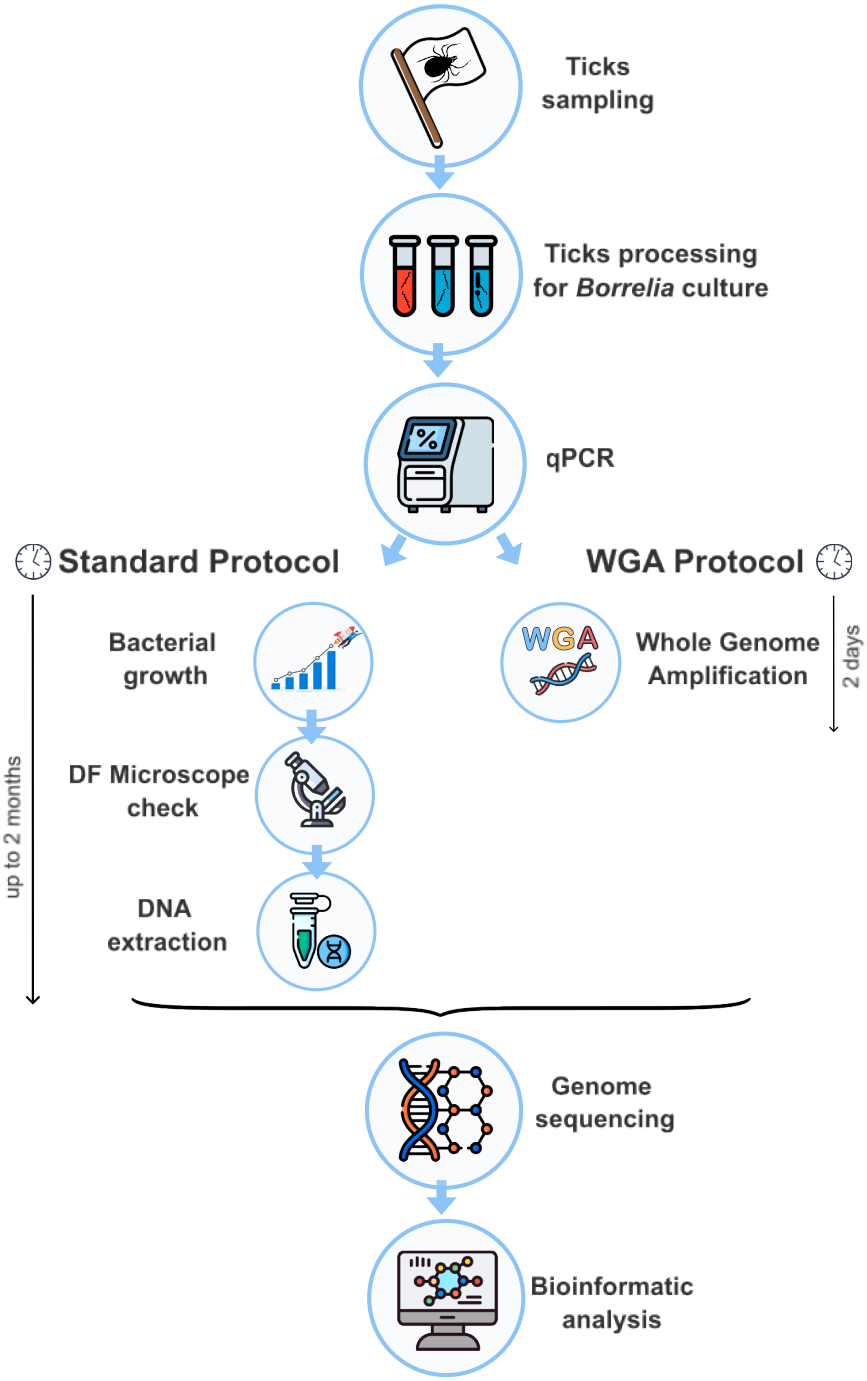

Figure 1. Workflow diagram
Comparison between standard protocol and the newly optimized WGA protocol.

and comparable completeness scores (BUSCO: 98%–100%; CheckM2: 92.85%–96.44%). Assemblies from WGA samples tended to be slightly smaller, with repeated telomeric regions not fully reconstructed—as observed in IT-B1—and fewer plasmids recovered (3–5 vs. 6–9), occasionally showing structural rearrangements such as plasmid-chromosome fusion (e.g., lp36 in IT-G1).

Plasmid assembly proved particularly challenging across all samples and methods. In the three culture controls, plasmids such as lp54, cp26, lp17, lp36, lp28-2, lp28-7, and multiple cp32 variants were detected. WGA assemblies recovered only a subset of plasmids, with some appearing incomplete or absent. Notably, lp54 is considered constitutive[19,20] but was missing in IT-B1 for both protocols. However, a targeted PCR assay[19] detected lp54 in this isolate, indicating that the failure to recover lp54 was not due to true plasmid loss. The fragmentation of the IT-B1 chromosome observed with the standard protocol, along with the absence or partial recovery of several plasmids, likely results from a combination of factors. These could include biological and culture-related issues such as long-term storage, multiple passages (13), and potential DNA degradation together with technical limitations in the assembly process, including difficulties in resolving repetitive or low-coverage regions with the assembler, and the naturally low copy number of *Borrelia* plasmids.[21]

Borrelia genomes from fresh tick samples

We then applied our protocol to newly collected ticks (Table S3). Twenty-six specimens of *I. ricinus* were collected, sterilized and put in MKP medium for *Borrelia* isolation. After 3 days, all samples were tested for *Borrelia* using qPCR, and positives (8 samples out of 26, 30%) were centrifuged, a process that efficiently enriched *Borrelia* cells in the pellet allowing us to retrieve a sufficient amount of DNA for genome amplification (Table S4—sheets 1 and 2). These samples were thus subjected to WGA and sequencing with Illumina and nanopore technologies.

While long-term pure cultures provided high-quality *Borrelia* DNA with minimal contamination, samples obtained from early cultures derived directly from tick material and processed using WGA inevitably contained co-amplified non-*Borrelia* DNA. Such contaminations can complicate downstream genomic analyses, leading to biased assemblies or erroneous conclusions if not properly accounted for. To address this issue, we developed an ad hoc bioinformatic pipeline. We first used Kraken2[22] to perform taxonomic classification. In the three long-term cultures

Table 1. WGA and standard protocol results comparison

	Standard protocol	WGA protocol	Standard protocol	WGA protocol	Standard protocol	WGA protocol
	IT-B1		IT-G1		IT-G2	
Species	*B. burgdorferi* s.s.		*B. garinii*		*B. garinii*	
Chromosomal contigs	8	1	1	1	1	1
No. of plasmids[a]	9	3	6	5	7	4
Plasmids	lp28-2, lp36, cp26, cp32-1, cp32-3+7, cp32-5, cp32-9, cp32-11, cp32-12	cp32-3 (partial), cp32-7 (partial), cp32-11 (partial)	lp54, lp28-7, lp36, cp26, cp32-4, cp32-9	lp54, lp36£, cp26, cp32-4, cp32-9	lp54, lp36, lp17, cp32-1, cp32-5, cp32 -6, cp26	cp32-1, cp32-5, cp32-6, cp26
Chromosome size (bp)	951,498	908,642	905,758	905,754	906,465	906,465
N50	213,238	908,642	905,758	905,754	906,465	906,465
Completeness % (BUSCO)	100	98	98	98	99.7	99.7
Completeness % (CheckM2)	96.44	93.9	93.26	92.85	94.99	94.11
Contamination % (CheckM2)	0.27	0.27	0.48	0.48	0.44	0.43
ST	20	20	187	187	177	177
cgST	440	440	444	444	438	438

Comparison of WGA and standard protocol for three pure culture samples.
[a]Independent linear or circular sequences. £ This plasmid was fused to the chromosomal telomere by the assembly and after inspection was manually separated.

used as controls, Kraken2[22] classified over 97% of reads as *Borrelia*, confirming the high purity of these samples. However, fresh tick samples amplified using WGA exhibited variable levels of contamination of reads assigned to non-*Borrelia* taxa (Table S5). Within these, the proportion of tick-derived reads was consistently low, never exceeding 2.5% of total reads assigned to the Ixodidae family, confirming that centrifugation allows efficient separation of bacterial cells from tick DNA. Most of the contaminants are represented by other bacteria, known symbiont and tick-borne pathogens, including *Spiroplasma*,[23] *Midichloria*,[24] and *Rickettsia*.[25] Furthermore, Kraken2 classification provided species-level confirmation of *Borrelia* isolates across all samples, validating the identity of both standard and WGA-derived datasets.

Three out of eight samples, which showed a high contamination level (reads assigned to the *Borrelia* genus <30 Mb; <1% of total), were excluded from downstream analyses. We observed that samples with lower numbers of reads assigned to *Borrelia* generally showed qPCR cycle threshold (ct) values above 20 after WGA. However, this ct value cannot be considered a strict threshold for sequencing performance, as sequencing output may be influenced by multiple factors, including variability in WGA efficiency and the presence of non-target DNA, such as DNA from other bacteria or the tick host, which can be co-amplified during WGA.

Remarkably, sample IT-L3, showing only 4.73% of reads assigned to *Borrelia*, still achieved 130X coverage, and was thus maintained. The full Kraken classification results for all samples can be found in Table S5.

All non-*Borrelia* short reads identified by Kraken2 were removed prior to downstream bioinformatics steps. After filtering, the retained *Borrelia* reads were successfully assembled to nearly complete (BUSCO scores spanning from 93% to 100%) and contiguous genomes with contigs number ranging from 4 to 52 and N50 values spanning from 129,633 to 905,836 bp, reflecting good contiguity (Table 2). Notably, four out of five genomes (IT-A1, IT-A3, IT-L3, and IT-G3) reached chromosome-level resolution, with a completely reconstructed linear chromosome and several plasmid sequences recovered.

Plasmids were typed based on the presence of PFam32, PFam49, PFam50, and PFam57/62 following established literature. Assignment to a known plasmid type was performed only when a PFam match was detected. In total, 28 contigs potentially carrying partial plasmid sequences remained untyped. Some of these untyped contigs could represent previously uncharacterized plasmids, warranting future dedicated investigation, while others may simply be incomplete due to missing PFam domains, reflecting the known limitations of our assembly and typing approach for *Borrelia* plasmids. Completeness of plasmid assemblies varied due to several factors, including sequence similarity among cp32 variants, structural complexity, and differences in copy number, which can hinder full reconstruction even from high-quality assemblies. Plasmid recovery varied between isolates, but lp54 was recovered as a complete plasmid in all five genomes, consistently with reports on its stability.[19,20] Similarly, cp26, another constitutive plasmid, was detected in three out of five isolates. The absence of cp26 in WGA-amplified DNA in samples IT-A3 and IT-L3 has been validated through standard PCR. As cp26 harbors genes essential for *Borrelia* growth,[16] we assume its presence in all spirochetes.

Table 2. Newly processed *Borrelia*

	IT-A1	IT-A3	IT-L1	IT-L3	IT-G3
Species	*B. afzelii*	*B. afzelii*	*B. lusitaniae*	*B. lusitaniae*	*B. garinii*
Total n. of contigs	13	6	52	4	11
No. of chromosomal contigs	1	1	32	1	1
No. of non-chromosomal contigs	12	5	20	3	10
Typed plasmid sequences	lp38+lp28-7+lp28-2+lp28-9, lp32-10+lp17, lp54, cp32-9+7, cp26, lp28-4, cp32-6, cp32-5, cp32-1	lp54, cp32-3	lp54, cp26, cp32-9, lp28-4	lp54	lp54, cp32-6+9, lp32-10, lp28-4, cp32-3, cp26
N50	900,623	905,836	129,633	897,080	903,272
Genome size (bp)	1,268,012	1,023,634	1,122,796	958,798	1,162,899
Completeness% (BUSCO)	99.7	99.4	93.4	99.1	100
Completeness% (CheckM2)	97.1	98.54	89.46	95.13	95.81
Contamination% (CheckM2)	0.78	0.18	2.23	0.52	0.38
ST	347	1,203	1,201	1,204	743
cgST	439	441	ND	442	436

Assembly statistics, quality metrics, and typing results for the five samples processed exclusively with the WGA protocol. ND stands for not determined.

Therefore, its apparent absence is consistent with potential limitations in plasmid recovery associated with WGA protocol, rather than a real biological absence or shortcomings in the bioinformatic pipeline.

WGA genomes allow in-depth downstream analyses

To assess the genotypic diversity of the sequenced isolates, we first performed multilocus sequence typing (MLST) using the standard 8-locus *Borrelia* scheme.[26,27] All samples, comprising both positive controls and fresh samples were successfully typed, resulting in the assignment of three novel sequence types (STs) for three fresh samples IT-A3, IT-L1, and IT-L3 (Table 2).

To further contextualize our isolates within the global diversity of *B. burgdorferi* s.l. complex, we built a minimum spanning tree (MST) based on all the complete MLST profiles associated with a sample deposited in *Borrelia* PubMLST. The MST (Figure 2) shows the overall population structure of *B. burgdorferi* s.l., with clear species-level clustering. The two *B. afzelii* isolates (IT-A1 and IT-A3) were embedded within the main *B. afzelii* cluster (colored in light orange in Figure 2), in line with the species assignment. Isolate IT-B1 also grouped within the central cluster corresponding to its species, *B. burgdorferi* s.s. (colored dark blue in Figure 2). The *B. lusitaniae* isolates (IT-L1 and IT-L3, colored dark green in Figure 2) formed a divergent point and a peripheral branch, reflecting their distinct genetic background. The *B. garinii* isolates, IT-G1, IT-G2, and IT-G3, clustered within the species *B. garinii*.

We subsequently performed core genome multilocus sequence typing (cgMLST) analyses.[28] In contrast to MLST, which relies on a few housekeeping loci (eight MLST loci for *Borrelia*), cgMLST exploits several hundred conserved loci across the genome, enabling much finer resolution (the *Borrelia* cgMLST scheme contains 639-loci). All samples were assigned to novel cgMLST types, with the exception of one *B. lusitaniae* isolate (IT-L1) where cgST assignment failed. Initially, cgMLST assignment failed for both *B. lusitaniae* isolates, IT-L1 and IT-L3, with only 590 and 629 of 639 loci successfully assigned, respectively. In this case, the loci were present but were too divergent to meet the established thresholds (99% query coverage and 97% identity) for allele assignment in cgMLST. This outcome reflects the fact that *B. lusitaniae* was not included in the development genome set of the cgMLST scheme[28] as high quality genomes were not available for this species by this time. After seeding the *Borrelia* PubMLST database by some manual curation including assigning exemplary alleles that were slightly different in lengths to those of the previously included alleles, the cgMLST scheme performs well for the species *B. lusitaniae*, and one out of the two samples, IT-L3, was successfully assigned to a cg-ST. IT-L1 was not assigned to a cg-ST due to the presence of only 590 loci out of 639, likely reflecting an incomplete genome, as also suggested by its relatively low BUSCO score (93.4%).

The previous absence of high-quality *B. lusitaniae* genomes in the *Borrelia* PubMLST and other databases underlines the understudied status of this species and the need for broader genomic representation of neglected species.

DISCUSSION

In this study, we developed and validated an optimized workflow integrating WGA in the standard *Borrelia* culturing procedures, to enable faster, easier, and more successful genome sequencing. Overall, our WGA-based protocol allows

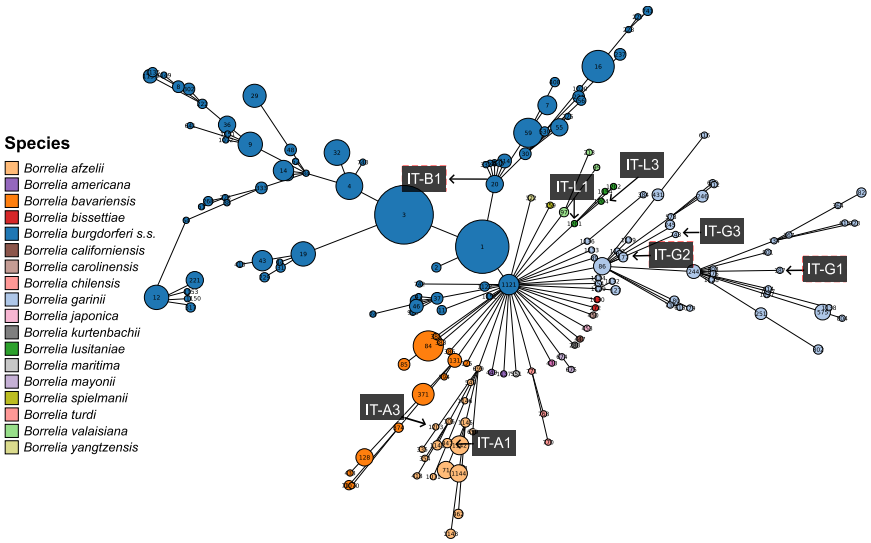

Figure 2. MST of *Borrelia burgdorferi* s.l. complex

MST of *Borrelia burgdorferi* s.l. complex based on 8-locus MLST profiles. The tree was constructed with the built-in BIGSdb GrapeTree tool using all available STs associated with a genome and a complete MLST profile (n = 521), together with the eight isolates sequenced in this study. Each node represents a ST, with its size proportional to the number of isolates corresponding to the ST. Colors correspond to species as indicated in the legend. Edges connect STs according to allelic differences. Isolates from this study are highlighted with black boxes and labels, with an additional red dashed border for positive controls (IT-B1, IT-G1, and IT-G2).

a substantial improvement in the success rate and reduces the sample-to-sequencing timeline. Whereas standard protocols require 30–60 days of culture and frequently fail to yield pure isolates, the WGA-based workflow can be completed in just 5 days, producing sequencing-ready samples.

Our results demonstrate that WGA enables the recovery of high-quality chromosome assemblies even from low-input samples or early-stage cultures, overcoming a major bottleneck in generating genomic data for this difficult to grow bacterium. In this study, we incorporated long-read nanopore sequencing alongside Illumina reads to improve assembly contiguity and to support the reconstruction of more complete genomes, particularly for building reference-quality assemblies. The combined use of WGA with both short- and long-read sequencing is not intended to be applied routinely to large numbers of samples, as this approach may be too costly for pathogen surveillance programs. Rather, the primary aim of this protocol is to generate high-quality, reference-grade genomes from otherwise inaccessible or low-yield samples, thereby expanding the available genomic resources for poorly represented *Borrelia* species and lineages. Once such reference genomes are established, they can serve as a base for downstream analyses based on more scalable and cost-efficient short-read sequencing strategies.

While WGA-derived assemblies yield highly accurate and complete chromosomal reconstructions, plasmid recovery is often incomplete due to intrinsic sequence complexity, high similarity among cp32 variants, and amplification biases associated with WGA.

However, the chromosomal information generated is sufficient to support a broad range of genomic epidemiology applications, including sequence typing (MLST and cgMLST), comparative genomics, and studies of strain diversity, population structure, and geographic distribution. Crucially, this strategy makes it possible to recover genomic data from isolates with limited success of culturing, and it has already enabled the identification of novel STs, expanding the representation of under-sampled species such as *B. lusitaniae* in public databases.

Limitations of the study

This study aimed to propose an alternative experimental approach for retrieving *Borrelia* genomes. While the protocol was effective in recovering complete chromosomal sequences, the WGA-based approach failed to reconstruct the full plasmid repertoire. Consequently, this protocol is not suitable for comprehensive plasmid-level analyses.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Greta Bellinzona (greta.bellinzona@unipv.it).

Materials availability

This study did not generate new, unique material.

Data and code availability

- All sequencing reads and genome assemblies generated in this study have been deposited in NCBI under BioProject SRA: PRJNA1330164 and submitted to PubMLST. Sample-specific accession numbers, PubMLST identifiers, and relevant metadata are provided in Table S3.
- All scripts generated in this study are publicly available on GitHub at https://github.com/MIDIfactory/Borrelia-WGA-Project and are also available at https://doi.org/10.5281/zenodo.18934974.
- Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.S.; methodology, D.S., S.M., and G.B.; formal analysis, C.C.B., L.M., and G.B.; investigation, S.M., I.M., A.V., C.C.B., G.B., and L.M.; data curation, L.M., G.B., S.M., M.V., and S.H.; supervision, D.S., G.B., and G.M.; resources, D.S., P.P., C.B., V.S., S.G., F.B., G.M., V.F., and

A.S.; writing – original draft, S.M., G.B., and D.S.; and writing – review and editing, all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Media and culturing condition
- METHOD DETAILS
  - Samples collection and processing
  - Molecular processing—qPCR and WGA
  - Whole-genome sequencing (WGS)
  - Read quality control
  - Genome assembly and quality assessment
  - Plasmids identification and typing
  - Typing
  - Read mapping and coverage analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
IT-B1 - *B. burgdorferi* s.s.	National Reference Center for Borrelia - Oberschleissheim, Germany	N/A
IT-G1 – *B. garinii*	National Reference Center for Borrelia - Oberschleissheim, Germany	N/A
IT-G2 – *B. garinii*	National Reference Center for Borrelia - Oberschleissheim, Germany	N/A
Chemicals, peptides, and recombinant proteins		
CMRL Medium (10X)	ThermoFisher	Cat# 21540026
Bacto™ Neopeptone	ThermoFisher	Cat# 211681
HEPES	SigmaAldrich	Cat #H3375
Sodium Citrate	SigmaAldrich	Cat# S4641
Glucose	SigmaAldrich	Cat# G8270
Sodium Pyruvate	SigmaAldrich	Cat# P2256
N-acetyl-D-Glucosamine	SigmaAldrich	Cat# A3286
Gelatin	SigmaAldrich	Cat# 48722
Albumine Bovine Solution 35x in DPBS	SigmaAldrich	Cat# A7979
Rabbit serum	ThermoFisher	Cat# 16120099
Critical commercial assays		
REPLI-g Single Cell Kit	Qiagen	Cat# 150343
Illumina DNA Prep	Illumina	Cat# 20060060
Qubit dsDNA BR Assay Kit	ThermoFisher	Cat# Q32850
NucleoSpin Tissue Kit	Macherey-Nagel	Cat# 740952
SsoAdvanced Universal SYBR Green Supermix	BioRad	Cat# 1725270
Deposited data		
Raw and analyzed data	This paper	NCBI, BioProject PRJNA1330164
Plasmid Reference dataset	Hepner et al.[11]	https://doi.org/10.1186/s12864-023-09500-4
B. garinii reference genome (CP075232.1)	–	NCBI accession number: CP075232.1
B. burgdorferi s.s. reference genome (CP161191.1)	–	NCBI accession number: CP161191.1
New *Borrelia* ST	This paper	PubMSLT IDs listed in Table S3
Oligonucleotides		
Borrelia spp. F-GAGTCTTAA AAGGGCGATTTAGT *Borrelia* spp. R-CTTCAGCCT GGCCATAAATAG	Michelet et al.[29]	N/A
Software and algorithms		
FastQC v.0.12.1	–	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
MultiQC v.1.23	Ewels et al.[30]	https://github.com/MultiQC/MultiQC/releases
fastp v.1.0.1	Chen et al.[31]	https://github.com/OpenGene/fastp
Kraken2 v.2.1.16	Wood et al.[22]	https://github.com/DerrickWood/kraken2
Unicycler v.0.5.1	Wick et al.[32]	https://github.com/rrwick/Unicycler
BUSCO v.6.0	Tegenfeldt et al.[33]	https://busco.ezlab.org/busco_userguide.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CheckM2 v.1.0.2	Chklovski et al.[34]	https://github.com/chklovski/CheckM2/releases
Bandage v.0.8.1	Wick et al.[35]	https://www.qt.io/development/download-open-source
GrapeTree v.1.5.0	Zhou[36]	https://github.com/achtman-lab/GrapeTree/releases
Bowtie2 v.2.5.4	Langmead et al.[37]	https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.5.4/
Samtools v1.21	Danecek et al.[38]	https://github.com/samtools/samtools/releases/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Media and culturing condition

Borrelia causing-LD was grown in MKP medium, at a temperature of 33°C and with CO_2 concentration around 5%. Specifically, one liter of MKP medium was prepared as follows: 675 mL water, 75 mL CMRL-1066 (10x), 2.3 g Neopepeton, 4.5 g HEPES, 0.5 g Sodium citrate, 2.3 g Glucose, 0.6 g Sodium pyruvate, 0.3 g N-acetyl glucosamine, 1.5 g Sodium bicarbonate. pH was adjusted with NaOH to reach 7.6 pH. After filtering with 0.22 μm syringe filter, the medium was additioned with 150 mL of gelatin 7% (pre-autoclaved at 120°C for 15 min), 50 mL of bovine serum albumin 35% in DPBS and 50 mL of rabbit serum, previously inactivated at 55°C for 30 min.

METHOD DETAILS

Samples collection and processing

We used three pure culture controls of *Borrelia* spp. previously isolated from *I. ricinus* ticks between 1989 and 1996 in northern Italy which had undergone approximately 13 culture passages. The cultures, stored at −80°C in the laboratory of the National Reference Center for *Borrelia* (Oberschleissheim, Germany), were revived using the Modified Kelly-Pettenkofer (MKP) medium, supplemented with 6% of rabbit serum at 33°C, in 8 mL glass tubes.[28]

In parallel, a total of 26 ticks (nymphs and adults) were collected via dragging in multiple locations in northern Italy and identified as *I. ricinus* using morphological keys[39] (see Table S1 for collection details). Single individuals were then processed by surface sterilization, cut with a sterile scalpel and cultured in 1.5 mL plastic tubes with 350 μL of MKP medium, supplemented with antibiotics (Sulfamethoxazole 45 μg/mL, Fosfomicin 170 μg/mL and Amikacin 25 μg/mL).[40]

Molecular processing—qPCR and WGA

Positive culture controls were cultured for 7–14 days and examined daily under dark field microscope (Nikon - ECLIPSE) to check the concentration and viability of spirochetes, until they reach a concentration of 10^7 per mL. Volumes of 20 mL of positive cultures were centrifuged at 3500 rcf for 20 min. The pellet was recovered and DNA extraction was performed using the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. In parallel, a 150 μL volume of each culture was centrifuged at 7100 rcf for 10 min and the pellet resuspended in 4 μL of PBS. WGA-protocol was then performed according to the manufacturer instructions (REPLI-g Single Cell Kit, Qiagen).

Newly collected ticks were subjected to culture protocol as described above. Three days after tick processing, a volume of 2 μL of each new culture was collected and subjected directly to qPCR, to assess the presence of *Borrelia*. Amplification of a fragment of 23S rRNA gene, was performed using real-time PCR. SsoAdvanced Universal SYBR Green Supermix (BioRad) was used with primers at a final concentration of 400 nM and the thermal protocol was as follows: 95°C for 5 min, 40 cycles at 95°C 15s and 60°C 30s, and melt curve from 55°C to 95°C with increasing increments of 0.5°C per cycle.[29] PCR-positive cultures were then checked under dark-field microscopy. Cultures were determined as PCR-positive if specific amplification was observed with a threshold cycle <32.

A second aliquot of 150 μL of medium of qPCR-positive samples was collected and subjected to the WGA-procotol. First, the aliquot was centrifuged at 7100 rcf for 10 min. Then, to evaluate the separation of *Borrelia* from tick DNA in the 150 μL aliquot from the culture, after centrifugation, both pellet and supernatant were subjected to qPCR specific for *Borrelia* (23S rRNA gene) and a tick housekeeping gene (*calreticulin*).[29,41] WGA was then performed as above. A control qPCR for *Borrelia* (same condition as before) was performed after WGA protocol, and all the samples were further processed for sequencing. We empirically determined that the samples with a threshold cycle of >20 once sequenced produced a very low number of reads assigned to *Borrelia*, making them unsuitable for downstream analyses. Quantification of *Borrelia* DNA before and after WGA is reported in Table S4.

In the newly sequenced isolates, the presence of some constitutive plasmids (i.e., cp26 and lp54) was confirmed with standard PCR, following the protocol described by Bunikis et al.[42] As positive controls, we included DNA from isolates of different *Borrelia* species (i.e., *B. afzelii*, *B. garinii, B. lusitaniae*).

Whole-genome sequencing (WGS)

All DNA samples (DNA from positive controls from the standard protocol, DNA from WGA of positive controls and fresh tick samples) were subjected to DNA quantification using Qubit (dsDNA Quantification Assay, Broad-range) and followed by nanopore and Illumina sequencing. Standard Library Preparation was performed for Illumina sequencing using Illumina DNA Prep following manufacturer instructions (final concentration of each sample was 0.2 ng/μL). Libraries were then sequenced on a MiSeq machine and v2 (500 cycles) reagents multiplexing in order to obtain 1Gb per sample. In parallel, aliquots of the same DNA samples were subjected to nanopore sequencing (outsourced to the company Eurofins, using Oxford Nanopore GridION, flow cell R10.4.1, using v14 library prep chemistry, declaring a minimum of 210 Mb of sequencing data per sample, Dorado basecaller version: dna_r10.4.1_e8.2_400bps_sup@v4.3.0).

Read quality control

Illumina and nanopore reads were quality-checked using FastQC v.0.12.1[43] and summarized using MultiQC v.1.23.[30] Adapters and low-quality bases (Q < 20) were trimmed in Illumina reads using fastp v.1.0.1.[31]

All quality-filtered reads were then classified using Kraken2 v.2.1.16[22] with the 'core_nt' database. Default settings were used except for –confidence 0.1, which sets the minimum confidence score for taxonomic assignments, and –minimum-hit-groups 3, which requires at least three distinct k-mer hit groups to support a classification. This step was used both to identify and remove potential contaminant reads (i.e., non-*Borrelia* taxa), and to confirm the taxonomic identity of the *Borrelia* species present in each sample. KrakenTools[44] '*extract_kraken_reads.py*' script was employed to retrieve reads assigned to the *Borreliella* genus (taxid: 64895) and its subordinate taxa, ensuring that only genus-specific sequences were retained for downstream analyses.

Nanopore reads were not subjected to taxonomic filtering or read-level classification. These reads were used exclusively within the hybrid assembly framework (more details provided in "Genome Assembly and Quality Assessment") to scaffold Illumina-based contigs, and any long reads not supporting Illumina contigs are not used and therefore do not contribute to the final assemblies.

Genome assembly and quality assessment

Hybrid genome assemblies were generated using Unicycler v.0.5.1,[32] which integrates short Illumina reads and long nanopore reads for improved assembly. Unicycler was run with default parameters in bold mode. Assemblies were polished within Unicycler using both read types and manually curated. Assembly quality was then assessed using BUSCO v.6.0,[33] using the Spirochaetia lineage dataset, to evaluate completeness. Contamination was assessed using CheckM2 v.1.0.2.[34] Assemblies generated from the same sample using different protocols (standard vs. WGA) were compared using blastn within Bandage v.0.8.1.[35]

Plasmids identification and typing

Plasmids were first identified in the assembled genomes through manual inspection using Bandage v.0.8.1.[35] Plasmid nomenclature was assigned according to the paralogous gene families (PFam) they contained, specifically PFam32, PFam49, PFam50, and PFam57/62.[16,19,20,45,46] The identification process followed the general approach outlined by Hepner et al. (2023), using the same reference PFam set of sequences. To detect PFam32 plasmid partitioning gene loci within the assembled contigs, we employed BLASTN v.2.16.0[47] using default parameters. Loci showing at least 86.5% nucleotide identity and a minimum of 90% alignment coverage were considered valid matches and used to assign plasmid designations accordingly.

Typing

Multi-locus sequence typing (MLST) and core genome MLST (cgMLST) typing were performed using the assembled genomes of each isolate as input, following the published schemes.[26–35,39–48] Assemblies were uploaded to the PubMLST database for *Borrelia* spp. (https://pubmlst.org/),[49] where MLST and cgMLST alleles, sequence types (STs) and core genome sequence types (cgSTs) were automatically assigned. A minimum spanning tree (MST) was generated using GrapeTree v.1.5.0,[36] as implemented in the PubMLST platform[49] using the MLST profiles. MLST profiles were retrieved for all available *Borrelia* genomes (n = 519, 8-locus scheme) and combined with the eight novel isolates sequenced in this study. The MST was constructed using the "MSTree V2" algorithm with default parameters.

Read mapping and coverage analysis

To assess whether the amplification and sequencing process introduced any systematic coverage biases across the chromosome, both raw Illumina reads and nanopore reads were mapped for each sample to the reference genome of the *Borrelia* species identified by Kraken2 in each sample, using Bowtie2 v.2.5.4.[37] The reference genomes used were: *B. garinii* (CP075232.1) and *B. burgdorferi* s.s. (CP161191.1). Mapping quality and coverage metrics were assessed using Samtools v1.21.[38] Coverage uniformity plots were created using a custom Python script (Figure S1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Genome assembly quality was assessed using standard quantitative metrics. Assembly contiguity was evaluated using the N50 statistic, while genome completeness and contamination levels were estimated using CheckM based on lineage-specific marker genes as described in the sections above. The resulting metrics are summarized in Tables 1 and 2.