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High-Throughput Short Sequence Typing Schemes for *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* Pure Culture and Environmental DNA

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Abstract: Molecular typing techniques are utilized to determine genetic similarities between bacterial isolates. However, the use of environmental DNA profiling to assess epidemiologic links between patients and their environment has not been fully explored. This work reports the development and validation of two high-throughput short sequence typing (HiSST) schemes targeting the opportunistic pathogens *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, along with a modified SM2I selective medium for the specific isolation of *S. maltophilia*. These HiSST schemes are based on four discriminative loci for each species and demonstrate high discriminating power, comparable to pairwise whole-genome comparisons. Each scheme includes species-specific PCR primers for precise differentiation from closely related taxa, without the need for upstream culture-dependent methods. For example, the primers targeting the *bvgS* locus make it possible to distinguish *P. aeruginosa* from the very closely related *Pseudomonas paraeruginosa* sp. nov. The selected loci included in the schemes are adapted to massive parallel amplicon sequencing technology. An R-based script implemented in the DADA2 pipeline was assembled to facilitate HiSST analyses for efficient and accurate genotyping of *P. aeruginosa* and *S. maltophilia*. We demonstrate the performance of both schemes through in silico validations, assessments against reference culture collections, and a case study involving environmental samples.

Keywords: molecular typing; opportunistic pathogens; neonatal intensive care units (NICUs); healthcare-associated infections (HAIs); selective medium; *Pseudomonas paraeruginosa*; eDNA; genotyping; opportunistic premise plumbing pathogens (OPPP)

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1. Introduction

Molecular typing methods are invaluable tools in infection control, helping to understand the source of specific strains and their relatedness, which is important for outbreak investigations in healthcare facilities. Primarily, molecular typing relies on multi-locus sequence typing (MLST) methods examining long DNA fragments from target genes [1]. It requires laborious procedures such as isolation of microorganisms by cultivation followed by Sanger sequencing of amplicons or analysis of whole-genome sequences (WGSs) [2–5], and many other methods involving pure cultures [6–10]. However, the application of these methods to assess the epidemiological relatedness between patients and their environment through environmental DNA profiling has been relatively understudied. This knowledge gap might lead to an underestimation of pathogenic bacteria presence, for instance, those existing in a viable but non-culturable (VBNC) state [11,12]. While molecular typing has proven effective in characterizing strains and their transmission in the

healthcare environment, its utility in linking patients to environmental sources remains an under-exploited opportunity. We have recently reported an innovative method called high-throughput short sequence typing (HiSST) to ease the monitoring of *Serratia marcescens* through PCR amplicon sequencing techniques [13]. This novel approach improves the differentiation of bacterial isolates and facilitates the exploration of diversity profiles, even among non-cultured microbial populations through direct analysis of environmental DNA.

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium belonging to the Gammaproteobacteria clade, which is reported to be ubiquitous in environments impacted by human activities [14] and to thrive in moist and wet conditions [15]. Due to its metabolic flexibility and high inherent tolerance to antimicrobial agents, this species possesses the capacity to adapt to diverse ecological niches, encompassing hospital facilities and patient devices [8,16–20]. *P. aeruginosa* is a well-known opportunistic pathogen [21,22] responsible for a wide variety of infections affecting most human organs [23]. This species has garnered significant attention due to its clinical relevance and its widespread use as a model bacterium in various biological areas [24,25]. Consequently, a considerable number of whole genome sequences are available, including numerous clonal or closely related strains (https://pseudomonas.com/, https://ipcd.ibis.ulaval.ca/, accessed on 21 August 2023). This rich dataset enables comprehensive investigations and facilitates the development of new typing schemes using next-generation sequencing such as HiSST.

Stenotrophomonas maltophilia was initially classified in the *Pseudomonas* genus in 1961, then in the *Xanthomonas* genus in 1983. Since 1993, it has been one of the more than fifteen species of the genus *Stenotrophomonas* [26]. As an ubiquitous environmental species, *S. maltophilia* can be found in aquatic and soil environments, in rhizospheres, and on plants [27]. This species is an opportunistic premise plumbing pathogens associated with nosocomial infections [26,28–30]. Multiple selective media have been developed for *S. maltophilia* [31–33], including the SM2I medium introduced by Adjidé in 2010 [34]. However, its specific composition remains to be optimized, and requires a high concentration of the expensive antibiotic imipenem. To improve *S. maltophilia* isolation, we report here a modified SM2I medium.

MLST schemes are already referenced and widely used for *P. aeruginosa* and *S. maltophilia* [35,36]. However, conventional MLST schemes are becoming outdated due to their lack of primers specifically tailored to cover the entire range of strains, especially considering more recent additions to databases. Moreover, these conventional schemes are not well-suited to large-scale epidemiological surveys due to labor-intensive upstream efforts in cultivation and isolation processes. This study was aimed at designing two HiSST schemes targeting the opportunistic pathogenic bacteria *P. aeruginosa* and *S. maltophilia*. The HiSST schemes were developed based on whole-genome sequences available in public databases, and validated with reference culture collections, environmental isolates, and environmental DNA samples from neonatal intensive care units (NICUs). HiSST offers several advantages in terms of accuracy, efficiency, and scalability in the context of large sample sizes, without requiring culture-dependent upstream procedures.

2. Materials and Methods

2.1. Development of the HiSST Scheme

Pan-genome allele databases were assembled from 45 complete genomes of *P. aeruginosa* and 23 complete genomes of *S. maltophilia* retrieved from the NCBI GenBank database with the *Build_PGAdb* module (available from the PGAdb-builder online tool) [37]. Briefly, a preliminary step involved the identification of 37 and 40 highly conserved genes with the highest number of alleles for *P. aeruginosa* and *S. maltophilia*, respectively. After aligning the alleles of each gene and removing non-overlapping ends, a sequence identity matrix was computed using BioEdit v7.2.5 [38] software. Gene fragments showing the highest polymorphism rates (i.e., highest nucleotide variations) and bound by conserved *k*-mers (allowing the design of primers) were selected. Only target amplicon sequences under 350 bp size were chosen (length compatible with Illumina PE-250 sequencing) and sub-

jected to alignment against the NCBI database using the Basic Local Alignment Search Tool (BLAST) [39]. A previously described step-by-step procedure was used to determine the specificity of the selected gene fragments [13]. Candidates for *P. aeruginosa* and *S. maltophilia* HiSST schemes included, respectively, 8 and 12 loci (i.e., nucleotide sequences of internal fragments within the previously selected genes) showing the highest polymorphism and the most specific non-overlapping ends. Finally, the selection procedure to achieve the identification of the four most discriminant loci was the same as described in our previous study [13].

2.2. Primer Design and PCR Amplification

The primers used for both *P. aeruginosa* and *S. maltophilia* HiSST schemes were designed to specifically target internal loci, as described above, composed of oligonucleotides ranging in length from 17- to 22-mers (Table 1). To evaluate the specificity and coverage of the primers, we conducted in silico tests using the software tool "Primer-BLAST" [40]. The RefSeq non-redundant proteins database was used to assess the primers specificity, while the *P. aeruginosa* or *S. maltophilia* subset RefSeq databases were used to verify the primers coverage for the corresponding species. The PCR reactions were carried out in 25 µL reaction volumes containing 0.6 U Fast-Taq DNA polymerase (Bio Basic Inc., Markham, ON, Canada), 1× Fast-Taq Buffer (Bio Basic Inc., Markham, Canada), 200 µM dNTPs, 0.4 mg/mL bovine serum albumin (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), 0.4 µM of each primer (except for *pheT* and *btuB* primers at 0.2 µM), and 2 ng/µL of extracted template DNA. A solution of 0.5x Band Sharpener (Bio Basic Inc., Markham, ON, Canada) was included in all mixtures except for the *yvoA* locus of the *S. maltophilia* HiSST scheme.

Table 1. HiSST locus-specific primer sequences and PCR cycling conditions [1].

Species	Locus		Primer Sequence (5′–3′) F: Forward; R: Reverse	PCR Amplicon Length	PCR Cycling Conditions
Pseudomonas aeruginosa	*bvgS*	F	ACGGCGACGARCTGTTGC	310	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 60 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	GGCATGGTCGGCGTAACC		
	pheT	F	GCGTGGACTTCTTCGACGC	271	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 58 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	GACAGCTCGCGGAACTTCG		
	btuB	F	GCCAAGCCGTTCTTCTCCG	330	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 58 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	CAGGTTCTGCTCGCCGTC		
	sdaA	F	ATCGTCGAGGACCGCACG	327	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 60 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	GTAGAGRTTGACCCAGTCGAGC		

Table 1. *Cont.*

Species	Locus		Primer Sequence (5′–3′) F: Forward; R: Reverse	PCR Amplicon Length	PCR Cycling Conditions
Stenotrophomonas maltophilia	*yvoA*	F	CCGAGAGCGGCATGATCGA	233	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 60 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	CAGGCARCGCATCGCCA		
	glnG	F	GTGATGTCGGCCTAYACCG	299	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 58 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	GCCACCAGYTCCTTGCC		
	tycC	F	TGTACACCGARCAGGTCGAG	249	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 58 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.
		R	TCTTGGCGTTGTGACGGATATC		
	ribA	F	CTGCCCTCGYTGGGCTA	327	Initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 60 °C for 40 s, 72 °C for 30 s and a final extension period of 5 min at 72 °C.

[1] Illumina Nextera adapter sequences were added at each 3′ end sequence of primers: 5′-TCGTCGGCAG CGTCAGATGTGTATAAGAGACAG-3′ for forward and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG-3′ for reverse primers.

2.3. Validation of the HiSST Scheme with Reference Strains

Primers were validated in vitro using reference strains obtained from diverse sources (Table S1). Selected strains for the *P. aeruginosa* HiSST scheme comprised *P. aeruginosa* (*n* = 16), *P. paraeruginosa* (*n* = 1), *P. beteli* (*n* = 1), *S. maltophilia* (*n* = 2), *Stenotrophomonas acidaminiphila* (*n* = 1), *Serratia marcescens* (*n* = 2), *Serratia liquefaciens* (*n* = 1), *Serratia rubidaea* (*n* = 1), *Klebsiella pneumoniae* (*n* = 1), *Delftia tsuruhatensis* (*n* = 1), and *Staphylococcus haemolyticus* (*n* = 1). Our tests inadvertently included an *S. haemolyticus* due to contamination of a *Pseudomonas* sp. storage culture we originally intended to examine (WGS was carried out to confirm the species). Primers from the *S. maltophilia* HiSST scheme were tested for *S. maltophilia* (*n* = 16), *S. acidaminiphila* (*n* = 1), *Stenotrophomonas nitritireducens* (*n* = 1), *Stenotrophomonas rhizophila* (*n* = 1), *P. aeruginosa* (*n* = 1), *S. marcescens* (*n* = 2), *S. liquefaciens* (*n* = 1), *S. rubidaea* (*n* = 1), *K. pneumoniae* (*n* = 1), and *D. tsuruhatensis* (*n* = 1). The strains were purified on trypticase soy broth (TSB) (Difco Laboratories, Sparks, MD, USA; Le pont de Claix, France) solidified with agar (15 g/L) (Alpha Biosciences, Inc., Baltimore, MD, USA) at 30 °C for 48 h. A single colony of each strain was inoculated in 2 mL of TSB and grown for 48 h at 30 °C for subsequent genomic DNA extraction.

2.4. Validation of Molecular Typing by WGS

Three strains of *P. aeruginosa* and four strains of *S. maltophilia*, isolated from the environment, were subjected to WGS using the Illumina NextSeq 550 platform at SeqCenter (Pittsburgh, PA, USA). Trimmomatic v0.39 [41] was employed for Illumina adapter clipping and quality trimming, with a minimum average quality threshold of 30. Contig assembly from the FASTQ files of paired-end reads was carried out using the SPAdes de novo assembler v3.15.5 [42] and visualized using Bandage v0.8.1 [43]. The contigs obtained from the SPAdes output were aligned, ordered, and oriented using the most closely related reference genomes (strain PAO1 for *P. aeruginosa* genome assemblies and strain NCTC10258 for *S. maltophilia* genome assemblies) through the utilization of the ABACAS tool v1.3.1 [44] to generate a contiguous genome.

2.5. ANI Analyses

To illustrate the contrasting discriminating power of the HiSST schemes and the whole-genome sequences of *Pseudomonas* spp. or *Stenotrophomonas* spp., we used heatmap

visualization showing average nucleotide identity (ANI) values for both the selected HiSST loci and complete genomes. The estimation of percentage identities between strains was accomplished through ANIb (ANI based on BLAST+ alignment tool), using the Python package *pyani* v0.2 (15). Then, heatmaps displaying ANIb values for the HiSST schemes and whole-genome profiles were generated within the RStudio environment [45] using *circlize* [46] and *ComplexHeatmap* [47] packages (R scripts are available on GitHub https://github.com/TBourd/R_scripts_for_HiSST_scheme, accessed on 10 September 2023). The ANIb values were determined by combining the loci from both HiSST schemes. If a locus was absent in a particular strain genome, the ANIb score was set to zero. This situation arose for negative controls *Pseudomonas* spp. or *Stenotrophomonas* spp. due to the use of highly specific primers, *ribA* and *bvgS*, which exclusively target *S. maltophilia* and *P. aeruginosa* species, respectively. It appears that other species do not possess these genes or the specific *k*-mers targeted by these primers.

2.6. cgSNP Analyses

Core genome single-nucleotide polymorphisms (cgSNPs) were identified for each strain using the Snippy pipeline v4.6.0 [48]. The reference genomes used for mapping were *P. aeruginosa* PAO1 and *S. maltophilia* NCTC10258. The haplotype network tree was constructed using the SNiPlay pipeline v3 by uploading the VCF file [49] obtained through the *snippy-multi* script [50]. The tree visualization was performed in R Studio v2023.09 using the *ape* package [51].

2.7. SNP and HiSST Profile Analyses

The diversity of each sequence types (ST) included in *S. maltophilia* and *P. aeruginosa* HiSST profiles was visualized with minimum spanning trees (MST). The dataset type "Aligned Sequences (FASTA)" was employed by merging DNA sequences of the four loci (from the corresponding HiSST scheme), associated with a ST identifier, resulting in the creation of a single sequence per ST. Then, "geoBURST distance" algorithm was used to compute a full MST using the PHYLOViZ software, version 2.0a [52]. Finally, the eBURST program was employed to explore clonal complexes [53].

2.8. Validation of the HiSST Scheme with Environmental Samples

A few clinical samples were selected to demonstrate the practical application of the HiSST schemes under actual conditions. The samples were collected from two NICUs located in Montreal, QC, Canada. Sink drains and diapers worn by newborns were sampled, leading to three positive samples for *P. aeruginosa* and four positive samples for *S. maltophilia*. This selection included samples with distinct and identical HiSST profiles (=ST) originating from various sources (fecal matter from a newborn, sink drains, or faucet samples) and sample types (eDNA and isolates). Each sample was subjected to direct eDNA analysis and selective culture to assess the accuracy of the HiSST analyses. The accuracy was validated by performing WGS on environmental or clinical isolates [13].

2.9. Selective Culture Conditions for P. aeruginosa

To identify cultivable opportunistic pathogens, samples were inoculated on media selective for the species of interest. The presumptive isolates were confirmed by HiSST molecular analysis. Cetrimide medium (43.3 g/L Cetrimide Selective agar, 10 mL/L glycerol, supplemented with 15 µg/mL nalidixic acid) was used for the growth and selection of *P. aeruginosa* [54]. The inoculated agar plates were incubated at the selective temperature of 42 °C for 48 h, which promotes the production of pigmentation by *P. aeruginosa* (e.g., pyocyanin).

2.10. Adaptation of a Selective Agar for S. maltophilia

To optimize the detection of *S. maltophilia* presence in diverse HiSST PCR-positive samples, we improved the SM2I medium by adjusting its composition and replacing the

imipenem with a more cost-effective alternative, meropenem. Based on SM2I medium [34], modified SM2I (mSM2I) medium is composed of Mueller Hinton (40 g/L), maltose (40 g/L), DL-methionine (0.5 g/L), and bromothymol blue (0.06 g/L). The medium was adjusted to pH 7.1 before autoclaving, then supplemented with the antibiotics vancomycin (6 mg/L), meropenem (16 mg/L), and the fungicide amphotericin B (4 mg/L). Inoculated mSM2I plates were incubated at 30 °C for 48 h.

2.11. Creating HiSST Databases

The latest HiSST databases for the two HiSST schemes were updated in April 2023 (Table S2). The initial stage of database assembly involved gathering all short sequence types (SST) relevant to the HiSST scheme for the species under study. The nucleotide Basic Local Alignment Search Tool (BLASTn [52]) parameterized with the default settings was utilized to create the *P. aeruginosa* SST databases (Table S2A) based on 513 genomes from The *Pseudomonas* Genome Database (https://www.pseudomonas.com/). *S. maltophilia* SST databases (Table S2B) were generated by a BLASTn search on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 12 July 2023) conducted against the *S. maltophilia* group (taxid:995085), using nucleotide collection (nr/nt) and the "megablast" program optimized for highly similar sequences. Any sequences with unexpected alignment lengths were eliminated and only strains comprising the four selected loci were retained in specific databases to enable ST assignment of *P. aeruginosa* and *S. maltophilia* HiSST schemes (Table 1). The specific databases were utilized to create the SST database for each locus using a script within the RStudio environment ("Step3_Create_SST.R", available in the GitHub repository: https://github.com/LaboPC/HiSST-schemes_TB, accessed on 10 September 2023). A ST identifier was generated for each HiSST profile using an R script (named "Step4_Assign_ST.R"), also accessible in the GitHub repository.

An unspecific database, comprising the closest relatives of targeted sequences, was created to include all nucleotide sequences targeted by the HiSST primers, excluding the target species. This database is designed to facilitate taxonomic assignment and identification of non-specific SSTs, involving sequences from environmental samples or isolates.

The databases were built by conducting a NCBI BLAST search on each locus. The search was performed by specifying a query subrange from 1 to the expected length of the locus, as indicated in Table 1. For *P. aeruginosa* SST databases, the first query excluded *P. aeruginosa* (taxid:287), while the second query targeted the closely-related *P. paraeruginosa* (taxid:2994495) group. In the case of *S. maltophilia* SST databases, the *S. maltophilia* group (taxid:995085) was excluded from the search set. For all SST databases, the "Somewhat similar sequences (BLASTn)" program was utilized, with a maximum of 1000 target sequences. Only aligned sequences with query coverage ranging from 99 to 100 were included in unspecific databases.

Both specific and unspecific SST databases were merged into a single fasta file per locus, with the removal of primer *k*-mers using the R script "Optional_Remove_primers.R" available in the GitHub repository (https://github.com/LaboPC/HiSST-schemes_TB, accessed on 10 September 2023). The R script "Step5_Merge_correcting_db_seq.R" was then used in three consecutive steps: (i) nucleotide sequences below the expected sequence length were eliminated, (ii) a reverse complement operation was performed to ensure uniform nucleotide sequence orientation, and (iii) sequence names were formatted and the unspecific SST databases were combined with the SST databases for *P. aeruginosa* or *S. maltophilia*. Finally, a BLASTn database was constructed for each locus using the SST databases, using the "makeblastdb" application from the BLAST+ executables [55].

2.12. Bioinformatical Pipeline for HiSST Analysis

The DADA2 pipeline [56] was adapted to the analysis of HiSST schemes, incorporating additional steps to enhance the accuracy of taxonomic assignments. The pipeline encompassed the following key procedures: (i) raw sequencing reads processing included primer sequences removal with the software Cutadapt v. 2.10 [57]; (ii) default parameters

specified in the package dada2 v1.8.0 [56] including error correction, denoising, and paired ends merging; and (iii) additional steps for HiSST analysis.

To enhance specificity, the BLASTn algorithm was utilized to filter the DNA sequences obtained from the previous step. Non-specific ASVs, corresponding to unexpected nucleotide length or unrelated to the bacterial species under study, were identified and removed. A taxon table was generated, containing only the ASVs specific to the bacterial species of interest. Additionally, two tables were created for further analysis. The first table captured the presence or absence of SST across various samples and facilitated the construction of Jaccard dendrograms to assess similarity between samples. The second table associated each sample with its corresponding ASV and assigned ST through exact matching provided valuable information for subsequent analysis and database maintenance. The entire process can be executed using the "FunHiSSTDada2.R" unified function from the dedicated GitHub repository (https://github.com/LaboPC/HiSST-schemes_TB, accessed on 10 September 2023) executed through the "Script_RUN_FunHiSSTDada2.R" R script.

2.13. HiSST Nomenclature and Assignation

The following nomenclature was used to identify HiSST loci and HiSST profiles: a "locus short sequence type" number (locus-SST) is assigned for each ASV of the individual loci; and the combination of multilocus SST of the overall HiSST profile is defined by a "sequence type" number (ST). The HiSST scheme database and R scripts are available on GitHub at URL: https://github.com/LaboPC/HiSST-schemes_TB (accessed on 10 September 2023).

2.14. Accession Number(s)

Raw sequencing reads and assembled genomes have been deposited in the Sequence Read Archive of the NCBI in the BioProject PRJNA1009139.

3. Results

3.1. Design of the HiSST Scheme for P. aeruginosa

The design of the HiSST scheme for *P. aeruginosa* involved a successive selection process. Initially, a total of 5039 alleles were identified, from which 38 alleles were carefully selected based on their profiles, with each allele having 40 to 44 unique profiles among 45 strains. The selection process resulted in 33 retained alleles (Table S3). To further refine the scheme, eight potential alleles were selected for subsequent development stages based on their high average nucleotide polymorphisms (i.e., low identity scores) at the corresponding locus and the presence of conserved *k*-mers suitable for primer design. Finally, four loci were retained for *P. aeruginosa* HiSST schemes, including gene fragments of *bvgS* (virulence sensor protein BvgS precursor (PA2583)), *pheT* (phenylalanine-tRNA ligase beta subunit (PA2739)), *btuB* (vitamin B12 transporter BtuB precursor (PA1271)), and *sdaA* (L-serine dehydratase 1 (PA2443)).

The HiSST scheme for *P. aeruginosa* has a minimum similarity threshold of 96% among its 196 STs (Figure S1). The pairwise ANIb genomic similarity score was greater than 98% to distinguish the different *P. aeruginosa* strains (Figure 1B). In comparison, using the four concatenated loci of the *P. aeruginosa* HiSST scheme yielded a 94% ANIb similarity score between *P. aeruginosa* strains. In contrast, the sequences of other *Pseudomonas* spp. retrieved from NCBI genomic database exhibited less than 84% nucleotide identity with the STs of the *P. aeruginosa* HiSST scheme (Figure 1A,B). The discriminating performance of the HiSST scheme was also demonstrated during primer–BLAST tests. The specificity of the primers was supported by all hits affiliated to *P. aeruginosa* but one sequence from the strain NCTC10783 identified as a *P. fluorescens* isolate. Actually, the genome sequence of strain NCTC10783 corresponded to *P. aeruginosa* [58] in the genome comparison analysis (Figure 1A,B), further supporting the specificity of the HiSST scheme.

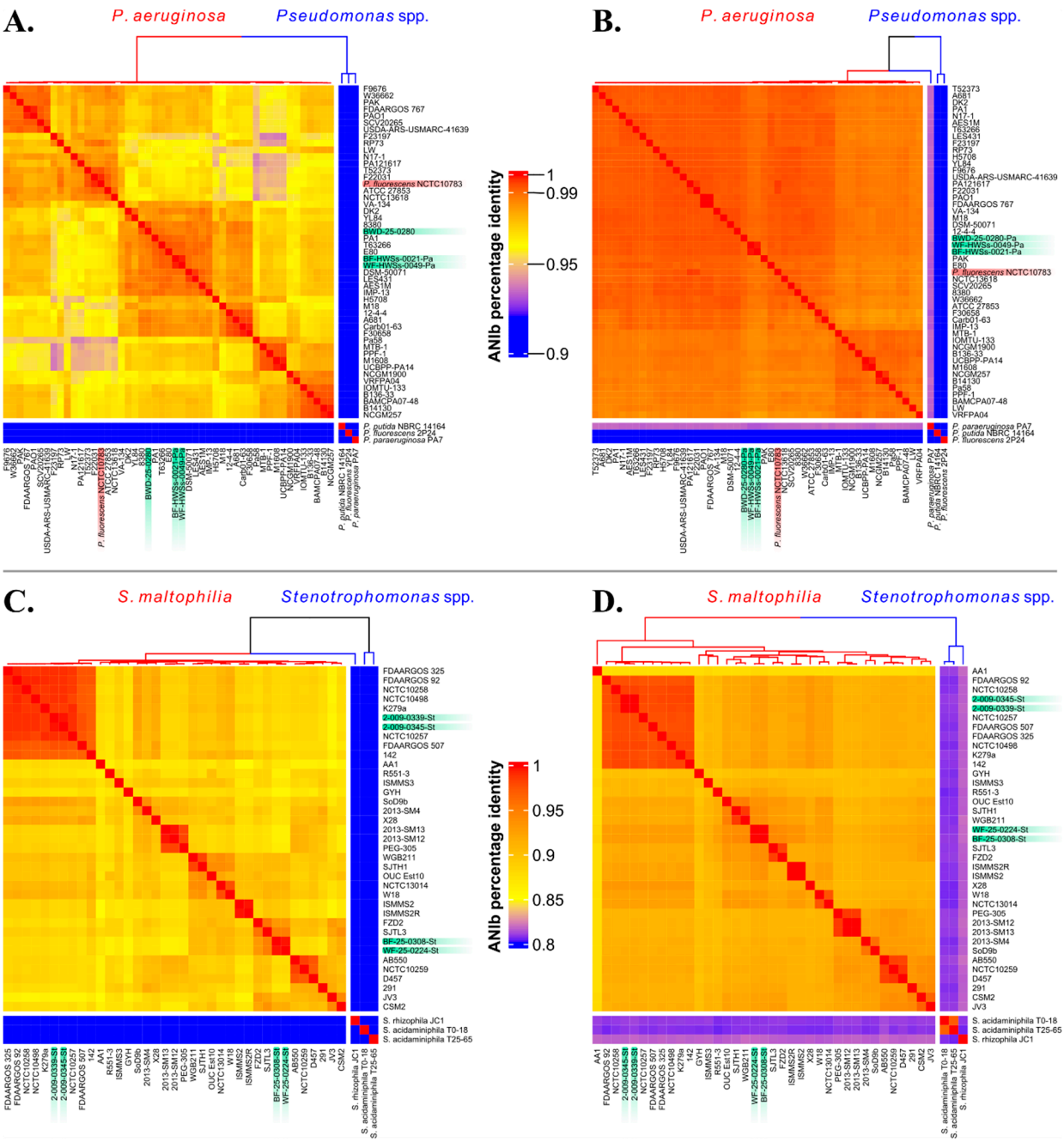

Figure 1. Discrimination of *Stenotrophomonas* spp. and *Pseudomonas* spp. based on the HiSST scheme and whole-genome sequences. The heat map reports the ANIb score of (**A**,**C**) the four concatenated loci of the HiSST scheme and (**B**,**D**) genome similarity. The more cells turn from blue to red, the higher the ANIb score, and the more the strains are genotypically similar. Strains highlighted in green were derived from eDNA (BWD-25-0280) or DNA (samples ending with "Pa" or "St") isolated from the NICU surveyed in this study. The strain highlighted in red is a misassignment of a *P. aeruginosa* strain as *P. fluorescens* (NCTC10783).

Each locus contained 17 to 36 informative sites (Table S4). The ST profiles remained highly heterogeneous and distinguishable (Figure 2A). Among the 196 STs identified, the eBURST program classified eight potential clonal complexes (Figure S1). Each group consists of only two STs, including two closely related groups (groups 6 and 7 at eBURST level 2). The diversity observed among *P. aeruginosa* strains was found to be representative of the whole-genome profiles (Figure S2). Notably, clonal complexes were preserved

regardless of whether the analysis was based on whole-genome profiles or the HiSST scheme. Furthermore, strains sharing identical STs were found to be likely of clonal origin.

The number of selected loci (n = 4) for the HiSST scheme was deemed optimal for discriminating *P. aeruginosa* strains (Figure 2C). In fact, the maximum cumulative frequency of STs was achieved using only three loci: *pheT*, *btuB*, and *sdaA*. Among these loci, *pheT* demonstrated the highest discriminating power, distinguishing most *P. aeruginosa* strains, with 79 unique SSTs identified among 513 *P. aeruginosa* isolates (Table S2A). This was followed by *btuB* (64 SSTs), *sdaA* (54 SSTs), and *bvgS* (35 SSTs). Although a notable decrease in discriminating power was observed with the fourth locus, *bvgS*, it remains highly relevant for distinguishing *P. aeruginosa* from other *Pseudomonas* spp. Recently, Rudra et al. proposed a revised classification for the outlier clade of *P. aeruginosa* PA7 (=NCTC 13628T = ATCC 9027) as a novel species, *Pseudomonas paraeruginosa* sp. nov. [59]. The HiSST scheme developed in this study is exclusively specific to *P. aeruginosa* due to the inclusion of locus *bvgS*, which is absent from *P. paraeruginosa*, as supported by BLASTn results and in vitro tests conducted on *P. (para)aeruginosa* PA7 (=NCTC 13628T = ATCC 9027).

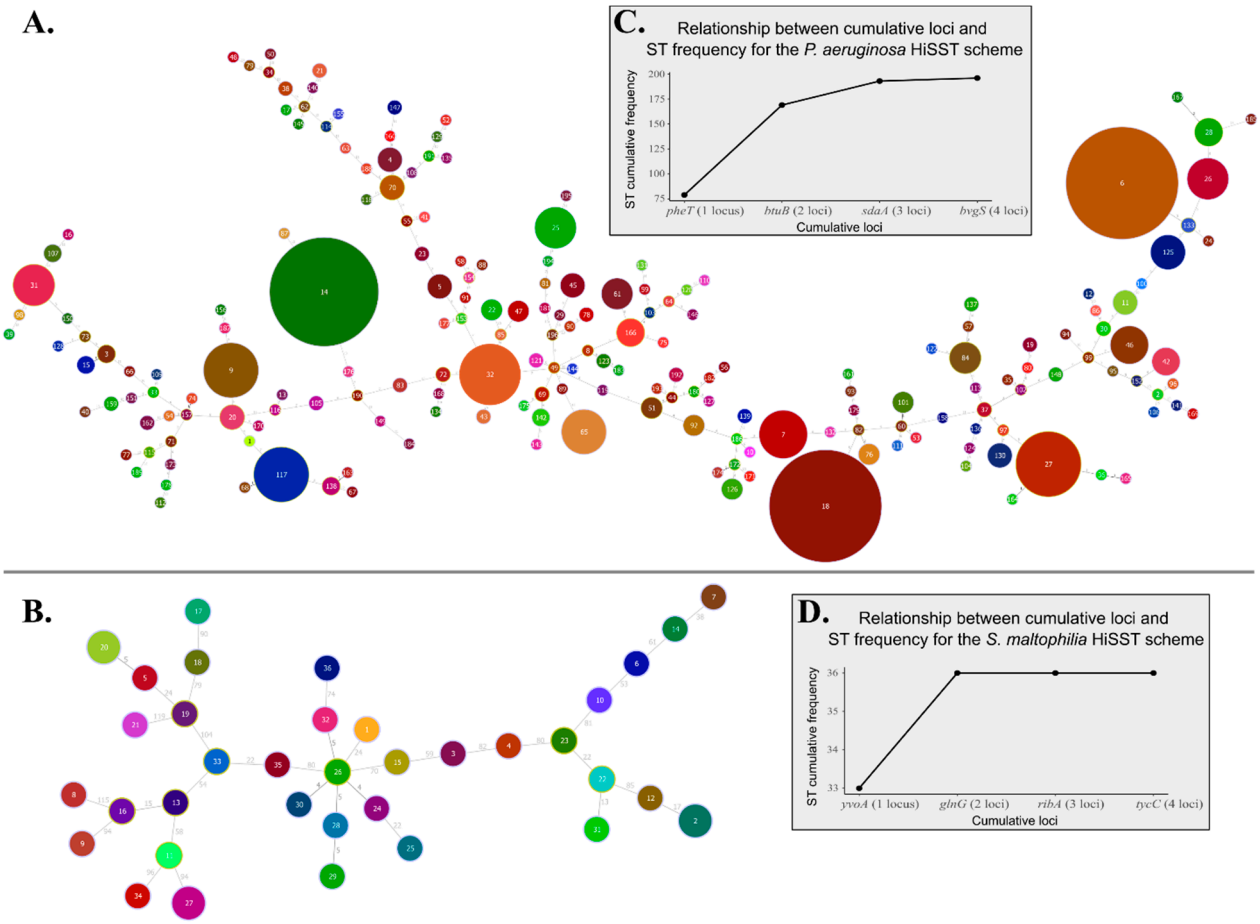

Figure 2. Minimum spanning trees for HiSST schemes of (**A**) *Pseudomonas aeruginosa* and (**B**) *Stenotrophomonas maltophilia* based on SNP analysis. The distance labels represent the number of discriminating SNPs between neighbouring genotypes. Each pie chart label refers to sequence type (ST) identifier of the corresponding HiSST scheme. The ST diversity is displayed by the grey boxes, illustrating the relationship between ST frequency and the number of loci included in the (**C**) *P. aeruginosa* or (**D**) *S. maltophilia* HiSST scheme.

3.2. Design of the HiSST Scheme for S. maltophilia

For *S. maltophilia*, a total of 2077 alleles were initially identified, and from these, 40 alleles were selected based on their profiles, with each allele exhibiting 22 unique profiles

across 23 strains. This selection process resulted in 39 retained alleles (Table S3). Among these, eight potential alleles were kept for further development of the HiSST scheme. Ultimately, four gene fragments were retained, encompassing *yvoA* (HTH-type transcriptional repressor YvoA), *glnG* (nitrogen assimilation regulatory protein), *tycC* (tyrocidine synthase 3), and *ribA* (GTP cyclohydrolase-2).

The minimal similarity threshold among *S. maltophilia* strains was determined to be 86% for the four loci (Figure S3). The ANIb similarity score for the HiSST scheme was determined to be 85%, effectively distinguishing between *S. maltophilia* strains. In comparison, the ANIb score obtained through whole-genome analysis was over 87% (Figure 1C,D). Furthermore, genomes that belong to the same ST are clearly clones, as also demonstrated by the cgSNP analysis (Figure S4). These results suggest a comparable discriminating power between a whole-genome analysis and the HiSST scheme.

In contrast with *P. aeruginosa*, the loci targeted by the *S. maltophilia* HiSST scheme exhibited a high diversity rate, with a range of around 10% estimated diversity for the four loci, encompassing 62 to 81 informative sites (Table S4). This contributes to the high heterogeneity of STs even though the database for *S. maltophilia* is not as extensive as that of *P. aeruginosa*, with only 39 complete genomes available compared to 513 strains used for the *P. aeruginosa* HiSST scheme. No clonal complex was identified using the eBURST program.

Among the four loci, *yvoA* and *glnG* demonstrated the highest discriminatory power, with 33 unique SSTs identified, followed by *ribA* (31 SSTs) and *tycC* (30 SSTs). The maximum cumulative frequency of STs was achieved using only *yvoA* and *glnG* (Figure 2D), but *ribA* demonstrated the highest species specificity to *S. maltophilia*.

3.3. mSM2I Agar Selective for S. maltophilia

mSM2I was found to be highly specific for the selection of *S. maltophilia*, effectively improving its detection in various environmental samples (Bourdin et al., in preparation). All the reference strains tested for the *S. maltophilia* HiSST scheme (Table S1) were also grown on mSM2I. The medium successfully inhibited the growth of other species, including strains from three other *Stenotrophomonas* species, while allowing *S. maltophilia* to thrive after 48 h of incubation at 30 °C. The mSM2I medium appears green at neutral pH, but turns blue in the presence of *S. maltophilia* as the pH rises into the alkaline range. *S. maltophilia* colonies are easily recognized by their olive to dark green hue.

3.4. Validation and Application of the HiSST Schemes

The primers, originally designed in 2019, have maintained their specificity up to the time of writing this report (May 2023), as confirmed by a recent in silico analysis, and subsequent laboratory validation through PCR testing using reference strains (Figures S5 and S6 represent tests conducted in 2023). All *P. aeruginosa* and *S. maltophilia* strains exhibited optimal PCR amplicon of the correct size when subjected to their respective HiSST primers. As anticipated, amplification was observed for *P. paraeruginosa* with primers *pheT*, *btuB*, and *sdaA*, while no amplification was obtained with primers targeting the *bvgS* locus, thereby indicating a higher specificity for *P. aeruginosa*. None of the four primer pairs yielded amplification for other *Pseudomonas* spp. or other bacterial genera tested.

The *ribA* primer pair displayed the utmost specificity of the *S. maltophilia* HiSST scheme, resulting in amplification exclusively for the targeted species. However, amplifications were observed for *S. acidaminiphila* and *S. nitritireducens* with the *tycC*, *glnG*, and *yvoA* primers. Additionally, the *glnG* primer demonstrated the ability to amplify each tested *Stenotrophomonas* spp. Although certain primers lack species specificity, these limitations are mitigated by the inclusion of highly specific primer pairs (*bvgS* for *P. aeruginosa* and *ribA* for *S. maltophilia*), the genus-level specificity of all primers, and the integration of a BLAST analysis into the DADA2 pipeline, thereby enhancing the analysis of HiSST schemes and facilitating the removal of unspecific ASVs, if necessary.

The primers were used in an extensive study aimed at characterizing the ecology of three opportunistic pathogens, including *P. aeruginosa* and *S. maltophilia*, in the sink

environment of two NICUs, resulting in HiSST analyses of thousands of samples (Bourdin et al., in preparation). The results supported the specificity of each primer sets, even when applied to complex samples containing a high concentration and diversity of bacteria, such as biofilms or water from sinks. There was a concordance between the HiSST schemes and ANIb analysis of whole genomes (Figure 1), as well as cgSNPs (Figure S4). Regarding *P. aeruginosa*, two faucet samples (aerator and tap water) collected from the same sink over a one-month time interval exhibited the same ST (samples BF-HWSs-0021-Pa and WF-HWSs-0049-Pa). Strains were successfully isolated from both samples, genotyped by HiSST, and analyzed by WGS, confirming them as likely clonal strains (Figure 1B and in green on Figure S2). Another eDNA sample, BWD-25-0280, obtained from a sink, also tested positive for *P. aeruginosa*. The strain was successfully isolated and exhibited the same HiSST profile as the eDNA sample, which was further confirmed by WGS.

In the investigation of *S. maltophilia*, two faucet samples (aerators and tap water) were found to be colonized by the same ST, as indicated by the HiSST analysis of the eDNA samples. The HiSST analysis of *S. maltophilia* isolates confirmed their identical genotypes, which were further validated by WGS, confirming their clonality. A similar case was observed in fecal samples collected from the same newborn on different dates (one week apart), where the same ST was identified in both samples. The isolation of strains and subsequent WGS analysis confirmed the consistency of these findings.

4. Discussion

Identifying discriminant alleles for *P. aeruginosa* poses a significant challenge. Strain differentiation primarily arises from the presence or absence of genes rather than nucleotide polymorphisms, indicating extensive genomic plasticity largely attributed to frequent horizontal gene transfers [25,60,61]. HiSST is a robust genotyping tool that provides highly reliable predictions for assessing clonality between strains [13,62]. However, in cases where two strains exhibit identical HiSST profiles, it is highly recommended to use WGS, especially for epidemiological investigations involving *P. aeruginosa* [63]. WGS analyses can confirm if two isolates are clonal, despite the very low probability of non-clonal strains having the same HiSST profile within a single sample. Like for any typing methodologies, it is important to note that the presented HiSST scheme, while effective for the current strain repertoire, may eventually need to be updated considering evolving databases and the inclusion of new strains that might not be targeted by the existing HiSST primers. This will ensure its continued applicability and accuracy in capturing the full diversity of *P. aeruginosa* strains. An intriguing finding of this study is the exclusive presence of the *bvgS* allele in *P. aeruginosa* and its absence in all other *Pseudomonas* species. This distinctive genetic marker further strengthens the ability to differentiate *P. aeruginosa* from related *Pseudomonas* species, which include the closely related *P. paraeruginosa* sp. nov, underlining the specificity and utility of the HiSST scheme.

The loci in the *S. maltophilia* HiSST scheme demonstrated a higher level of polymorphism than those of *P. aeruginosa*, contributing to the heterogeneity of STs. While the database for *S. maltophilia* is limited compared to *P. aeruginosa*, the scheme showed promising specificity, with the *ribA* locus being highly specific to *S. maltophilia*. Indeed, the *ribA* locus is seldom found in other *Stenotrophomonas* spp. Furthermore, the primers designed for *ribA* are exclusively specific to *S. maltophilia* (Figure S6 and primer–BLASTn query). Including the fourth locus *tycC* takes a more conservative approach to ensure maximal genotyping distinction. This consideration considers the limited size of the *S. maltophilia* database and the eventual occurrence of unknown genotype variants where certain loci may fail to amplify.

Both HiSST schemes demonstrate superior or similar differentiating power compared to the ANI score, based on whole-genome comparisons, and the cgSNPs analysis. The HiSST schemes effectively reflect the clustering of strains according to their whole genomes, enabling the discrimination of strains and identification of potential clonal strains.

Furthermore, the analysis of the HiSST schemes was enhanced by incorporating additional steps and improvements into the DADA2 pipeline. This led to more accurate taxonomic assignment and the generation of informative tables, facilitating in-depth investigations and analysis.

5. Conclusions

We developed HiSST schemes for *P. aeruginosa* and *S. maltophilia* based on four loci of conserved, yet polymorphic, genes. As a demonstration, we utilized the HiSST schemes on samples collected from a hospital environment to successfully achieve the characterization of the genotype diversity of *P. aeruginosa* and *S. maltophilia*. The results demonstrate the accuracy and reliability of the HiSST genotyping method, both in comparison to whole-genome ANIb and cgSNPs analysis, and through the confirmation of clonal relationships using isolates and WGS. The genotyping schemes developed here will represent powerful tools for both ecological and epidemiological investigations. They enable the detection and identification of bacterial genotypes from environmental or isolated DNA in various samples, addressing limitations of culture-dependent methods that might underestimate bacteria, including pathogens present in a viable but non-culturable (VBNC) state within biofilms [11,12].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12010048/s1, Figure S1: UPGMA tree of 196 HiSST sequence types (STs); Figure S2: Haplotype tree illustrating the genetic relationships among 63 *P. aeruginosa* strains; Figure S3: UPGMA tree of 36 HiSST sequence types (STs); Figure S4: Haplotype tree illustrating the genetic relationships among 43 *S. maltophilia* strains; Figure S5: In vitro tests and validation of primers designed for HiSST schemes of *P. aeruginosa*; Figure S6: In vitro tests and validation of primers designed for HiSST schemes of *S. maltophilia*; Table S1: Reference strains used for HiSST scheme validation; Table S2A: HiSST database for *P. aeruginosa* scheme; Table S2B: HiSST database for *S. maltophilia* scheme; Table S3: Highly specific and distinguishing loci retained during the selection process for HiSST scheme development; Table S4: Comprehensive list of single-nucleotide polymorphisms (SNPs) obtained through sequence alignment for all short sequence types.

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Data Availability Statement: The HiSST scheme database and R scripts are available on GitHub at URL: https://github.com/LaboPC/HiSST-schemes_TB (accessed on 10 September 2023). Raw sequencing reads and assembled genomes have been deposited in the Sequence Read Archive of the NCBI in the BioProject PRJNA1009139.

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