Genomic library of *Bordetella*

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*Bordetella, Bordetella pertussis, Bordetella bronchiseptica,* phylogenetic diversity, taxonomy, virulence factors, diagnostic markers, antimicrobial resistance

**Repositories**
Sequence reads for *Bordetella bronchiseptica* isolates from France, and for *B. tumulicola, B. muralis* and *B. tumbae* type strains (newly sequenced genomes) have been deposited in the European Nucleotide Archive under BioProject number PRJEB49946.
Abstract

Background
The re-emergence of whooping cough and geographic disparities in vaccine escape or antimicrobial resistance dynamics, underline the importance of a unified definition of *Bordetella pertussis* strains. Understanding of the evolutionary adaptations of *Bordetella* pathogens to humans and animals requires comparative studies with environmental bordetellae.

Methods
We have set-up a unified library of *Bordetella* genomes by merging previously existing Oxford and Pasteur databases, importing genomes from public repositories, and developing harmonized genotyping schemes. We developed a genus-wide cgMLST genotyping scheme and incorporated a previous *B. pertussis* cgMLST scheme. Specific schemes were developed to define antigenic, virulence and macrolide resistance profiles. Genomic sequencing of 83 French *B. bronchiseptica* isolates and of *B. tumulicola, B. muralis* and *B. tumbae* type strains was performed.

Results
The public library currently includes 2,581 *Bordetella* isolates and their provenance data, and 2,084 genomes. The “classical Bordetella” (*B. bronchiseptica, B. parapertussis* and *B. pertussis*), which form a single genomic species (*B. bronchiseptica* genomic species, BbGS), were overrepresented (n=2,382). The phylogenetic analysis of *Bordetella* genomes associated the three novel species *B. tumulicola, B. muralis* and *B. tumbae* in a clade with *B. petrii* and revealed 18 yet undescribed species. A sister lineage of the classical bordetellae, provisionally named *Bbs* lineage II, was uncovered and may represent a novel species (average nucleotide identity with BbGS strains: ~95%). It comprised strain HT200 from India, two strains of ‘genogroup 6’ from the USA and six clinical isolates from France; this lineage lacked *ptxP* and its *fim2* gene was divergent. Within *B. pertussis*, vaccine antigen sequence types marked important phylogenetic subdivisions, and macrolide resistance markers (23S_rRNA allele 13 and fhaB3) confirmed the current restriction of this phenotype in China with few exceptions.

Conclusions
The genomic platform provides an expandable resource for unified genotyping of *Bordetella* strains and will facilitate collective evolutionary and epidemiological understanding of the re-emergence of whooping cough and other *Bordetella* infections.
Data summary

*Bordetella* genomes list and accession numbers: Supplementary Table S4

*Bordetella* genus phylogeny dataset (92 isolates): [https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=23&submit=1](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=23&submit=1)

*B. bronchiseptica* phylogeny dataset (213 isolates): [https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=24&submit=1](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=24&submit=1)

*B. pertussis* phylogeny (124 isolates): [https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=25&submit=1](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=25&submit=1)

iTOL interactive trees: [https://itol.embl.de/shared/117FwQAvKOoCF](https://itol.embl.de/shared/117FwQAvKOoCF)
Introduction

*Bordetellae* are beta-proteobacteria that can be found in the environment and can cause infections in animals and humans. Of the 16 currently described *Bordetella* species, the medically most important taxa *B. pertussis* (*Bp*) and *B. parapertussis* (*Bpp*), together with *B. bronchiseptica* (*Bbs*), are referred to as the ‘classical *bordetellae*’ and belong to a single genomic species [1], hereafter named the *B. bronchiseptica* genomic species (BbGS) for convenience. *B. pertussis*, and more rarely *B. parapertussis*, cause whooping cough, characterized by its typical paroxysmal cough, and kill an estimated ~140,000 children annually [2–4]. *Bp* and *Bpp* have evolved from sublineages of *Bbs* [5]. Whereas *Bp* and *Bpp* are human restricted, *B. bronchiseptica* has a broader ecological distribution and causes respiratory infections in a wide range of mammalian hosts including humans. Based on MLST [1] and genomic sequencing [4,6] two distinct *Bbs* groups were named *Bbs* complexes I and IV [1], the last one being more strongly associated to humans.

Other *Bordetella* species were described and can affect humans, although a few were so far only described as environmental. *B. holmesii* can be collected either from blood of septicemic patients or from nasopharyngeal samples of patients with pertussis-like symptoms [7–9]. *B. hinzii* is frequently carried in birds [10,11] and *B. avium* and *B. pseudohinzii* are respectively responsible of respiratory disease in poultry or birds [12] and in mice or wild rats [13]. *B. bronchialis*, *B. sputigena* and *B. flabilis* were described from respiratory samples of patients with cystic fibrosis [14,15], and *B. trematum* and *B. ansorpii* were found in infected wounds of immunocompromised patients [4,16,17]. Some *Bordetella* species have been found in environmental samples, such as *B. petrii* or the three recently described species *B. muralis*, *B. tumulicola* and *B. tumbae* [18–20]. In addition to these 16 current *Bordetella* species with standing in the prokaryotic taxonomy, additional *Bordetella* genogroups (as also named *Bordetella* genomospecies in INSDC bioproject PRJNA385118) were identified from patients with cystic fibrosis and may represent additional separate *Bordetella* species [21].

The diversity of lifestyles and medical importance of *Bordetellae* raise important questions about the origins and evolution of pathogenicity in this group [4]. Horizontal gene transfer (HGT) is likely to occur between *Bordetella* species and strains, as already observed for the O-antigen locus in *B. bronchiseptica* [22]. For example, the environmental species *B. petrii* has genomic islands with atypical G+C content [18] that were associated with the metabolic versatility of this species. The use of a unified database of genomes from all *Bordetellae* species would facilitate genomic analyses underlying the phenotypic diversity within this important bacterial genus.
Besides the large-scale evolution of *Bordetellae*, the population dynamics within *Bp* are an important topic of epidemiological surveillance, in light of vaccine-escape evolution and the possible emergence and global dissemination of antimicrobial resistance [23–25]. Major evolutionary events in vaccine antigen-related genomic features have been described, including within the promoter region of the pertussis toxin gene cluster (*ptxP*) and in the fimbriae gene *fim3* [23]. In most countries using acellular vaccines, isolates characterized by *ptxP*3 and either *fim3*-1 or *fim3*-2 alleles are currently largely predominant, whereas isolates of the ancestral *ptxP*1 genotype still predominate elsewhere, for example in China [24,26]. In addition, in countries that use acellular vaccines, an increasing proportion of *Bp* isolates are deficient for the production of the immunodominant surface protein pertactin, raising questions on future vaccine effectiveness [27]. The nomenclature of genotypic markers, sublineages and strain subtypes needs unification to facilitate collective studies of the global epidemiology and population dynamics in *Bp*.

Harmonization of the genotype nomenclature of bacterial pathogens may be achieved by the gene-by-gene approach called MLST (for multilocus sequence typing), in which allele numbers are uniquely attributed to each locus sequence variant. Bioinformatics platforms that centralize allele definitions and allow curation and open access to genotype nomenclature are critical resources for unified pathogen subtype definitions [28]. Until now, there have been two dedicated *Bordetella* genome sequence databases: Oxford University’s PubMLST (unpublished) and Institut Pasteur’s BIGSdb [29] platforms. This duality has led to nomenclatural confusion and suboptimal services to the user’s community. With the rapid developments of genomic epidemiology and biodiversity surveys, a common and optimized resource for *Bordetella* evolutionary studies and sequence variants naming is needed. Here, we aimed to set-up such a unified genomic platform and expand its features to enable addressing genus-wide evolutionary genomics questions and global tracking of important genotypes within individual species, and particularly *B. pertussis*. 
Methods

DNA preparation and genomic sequencing
Genomic sequencing was performed for 83 French *B. bronchiseptica* isolates mainly (84 out of 89) of human origin collected between 2007 and 2020, and for the type strains of *B. tumulicola, B. muralis* and *B. tumbae*.

Isolates were grown at 36°C for 72 hours on Bordet-Gengou agar (Becton Dickinson, Le Pont de Claix, France) supplemented with 15% defibrinated horse blood (BioMérieux, Marcy l’Étoile, France), and sub-cultured in the same medium for 24 hours. Bacteria were suspended in physiologic salt to reach OD\textsubscript{650} of 1, and 400 μL of the suspension were pelleted. Pellets were re-suspended in 100 μL of PBS 1X, 100 μL of lysis buffer (Roche), and 40 μL of proteinase K; heated at 65°C for 10 minutes and at 95°C for 10 minutes; and used for DNA extraction. Whole genome sequencing was performed using a NextSeq 500 system (Illumina, USA) at the Mutualized Platform for Microbiology of Institut Pasteur. For de novo assembly, paired-ends reads were clipped and trimmed with AlienTrimmer [30], corrected with Musket [31], merged (if needed) with FLASH [32], and subjected to a digital normalization procedure with khmer [33]. For each sample, remaining processed reads were assembled and scaffolded with SPAdes [34].

Development of pan-genus *Bordetella* cgMLST scheme
A genus-wide core genome MLST scheme [35], called *Bordetella* cgMLST v1.0, was established alongside a genus-wide locus annotation system (BORD loci), using the principles published for the genus *Neisseria* [36]. Six finished and annotated *Bordetella* genomes were uploaded into the BIGSdb Oxford database [37]: *Bordetella pertussis* Tohama I [38]; *Bordetella pertussis* CS [39]; *Bordetella bronchiseptica* RB50 [38]; *Bordetella parapertussis* 12822 [38]; *Bordetella petri* 12804 [18]; and, *Bordetella avium* 197N [40]. Using the GenomeComparator module of BIGSdb, the greatest number of common genes (1469) was found when *B. bronchiseptica* RB50 was used as the reference genome (even though *B. petri* 12804 had the greatest number of annotated loci, 5023). Genes described as ‘hypothetical’ or ‘putative’ were removed, resulting in 1415 loci. All loci entered into the database were given a BORD number of the format BORD000000, where the last six digits corresponded to the BB0000 numbers used in the annotation of *B. bronchiseptica* RB50 [38]. Other annotations and known aliases were included in the locus descriptions for comparison. As with other
genus-wide locus schemes, this scheme can be expanded simply by adding additional loci, to
give an expandable catalogue of the genus pangenome.

**Merging of the Oxford and Pasteur databases**

Two BIGSdb databases were originally designed separately for distinct purposes: while
Oxford’s PubMLST database offered MLST, *Bordetella* cgMLST v1.0 (see above) and
virulence genes schemes, the Pasteur database was designed for the sole initial purpose of
*Bordetella pertussis* genotyping, with a Bp–specific cgMLST scheme and a Bp-virulence
genes schemes [29].

To merge the data available in the two databases, we proceeded as follows. As per BIGSdb
dual design, isolates genomes and provenance data were imported into the “isolates” database,
whereas allelic definitions of MLST, cgMLST and virulence genes were imported into the
“seqdef” database.

Regarding the isolates database, we first dumped the Oxford database and uploaded it on the
Institut Pasteur server. Second, we imported Pasteur isolates into this new database. To
facilitate the understanding of historical origins of each entry, isolates identification numbers
(BIGSdb ID number) were defined as follows: isolates from the original Oxford database
were numbered from 1 to 1,914 as in the original database (*i.e.*, their identification numbers
were untouched). Second, the isolates from the original Pasteur database were numbered from
10,000 to 12,214 (original Pasteur identification number + 10000). We also completed the
comment fields for these isolates, with the old identification number being added with the
suffix word “Pasteur”. We added “putative duplicate” in this comment field if the
corresponding isolate name was present in both original databases. We also added a
“duplicate number” field in the isolates database. If two or more isolates were identified as the
same strain, they were attributed the same duplicate number, consecutively. Duplicated data
identification was based on a combined analysis of metadata and phylogeny tree
reconstruction.

At time of merging and closure, the Oxford database comprised 1,914 isolates entries, 57 of
which were private, whereas the Pasteur database comprised 2,180 entries, 2,009 of which
were private. As of January 7th, 2022, the platform resulting from the merger comprises 2,581
public isolates entries, and 4,853 isolates in total when considering private entries.
cgMLST schemes

Two core genome MLST schemes are available. The first scheme, *Bordetella* cgMLST v1.0 (hereafter referred to as cgMLST_genus), was initially defined and hosted in the Oxford platform and was designed to be applicable for the entire *Bordetella* genus. The second scheme, called cgMLST_pertussis, was originally hosted in the Pasteur database and was built for *B. pertussis* isolates genotyping only [29]. Note that only the latter scheme has attached cgST definitions, i.e., unique cgST identifiers are attached to each distinct allelic profile. Both cgMLST schemes are available in the merged library.

Genotyping schemes for vaccine antigens and virulence-associated genes

In both original databases, virulence-associated gene schemes had been defined separately. We choose to keep all loci from the virulence scheme designed in Oxford and to add some additional relevant loci. These loci were then grouped into different schemes comprising either Bp vaccine antigens (*fim2*, *fim3*, *ptxA*, *ptxB*, *ptxC*, *ptxD*, *ptxE*, *fhaB*-2400_5550), T3SS genes (*bopB*, *bopD*, *bopN*, *bsp22*, *bteA*), autotransporters (*bapC*, *brkA*, *tcfA*, *prn*, *vag8*), other toxins (*cyaA*, *dnt*) or phase biology genes (*bipA*, *bvgA*, *bvgS*). We decided to include the *prn* locus in the autotransporter scheme rather than in the *Bp* vaccine antigens scheme, because prn-negative isolates would not allow the definition of sequence types (ST) for the vaccine antigens scheme.

To make analyses of virulence-associated and vaccine antigen genes more user-friendly, common gene names used in the literature were used as locus identifier, instead of the locus tags that were initially used in the Oxford database (e.g., BORD005020 was replaced by *fim2* and BORD005021 by *fim3*). In addition, for five loci common to the original Pasteur and Oxford databases (i.e., *fim2*, *fim3*, *ptxA*, *ptxP* and *tcfA*), the allele numbering was re-defined so that the alleles of each locus would match the nomenclature found in the literature for *B. pertussis* alleles; the correspondence between the original allele numbering system and the new one is defined in Table S1. Subsequently, the numbering of alleles was simply incremented as new genomes scans led to novel allele definitions.

Macrolide resistance

The 23S rRNA allele table was incremented with a sequence defined as allele 13, which carries the only mutation described so far as being associated with macrolide resistance [41].
To facilitate the detection of putative macrolide resistant isolates and their lineage [41,42], we built a scheme named “macrolide resistance” that includes the following loci: 23S_rRNA, fhaB (full) and four fhaB fragments. As the fhaB locus, which has a full-length size of 10,773 bp, is often fragmented in the genomic assemblies, three smaller fragments were designed to cover evenly the fhaB sequence: fhaBx-1_3193, fhaBy-3190_7183, fhaBz-7180_10773; the ranges in these locus names correspond to the begin and end positions of the three fragments. A fourth fragment was designed to cover the region comprising 2 SNPs present individually in fhaB2 and fhaB3 [24]: fhaB-2400_5550. The allele numbering for fhaB fragments was defined so that the alleles of each locus would match the nomenclature found in the literature, ie. alleles fhaB1 and fhaB2 correspond to those defined by van Loo [43], and fhaB3 to the mutation C53301T [24]. Allele fhaB3 of full size locus and restricted locus (fhaBy-3190_7183 and fhaB-2400_5550) is associated with ptxP1 isolates originating from China which exhibit a macrolide resistance phenotype [24].

**Genome scanning for defined loci**

Reference genomes were selected from either type-strain, reference genomes from literature and/or RefSeq genomes or most complete genomes available for each species (e.g., type-strain 18323 and reference strain Tomaha for B. pertussis). All these genomes are grouped into the public project “Bordetella Genus Phylogeny”. We selected two representatives for each species, when available. All these genomes are found in the “Bordetella phylogeny” public project in the BIGSdb genome library. For the cgMLST_genus scheme, missing alleles were captured by relaxing the scanning parameters to 70% identity and 70% alignment in order to capture alleles from all Bordetella species, using the reference strains. New captured alleles were defining as type alleles. Then, all future detection of new alleles were based on 90% identity, 90% alignment parameters using defined type alleles as queries. Criteria for loci associated with virulence were stricter: only new alleles sharing 90% identity, 90% alignment with a type allele and with a complete CDS were recorded (excluding the ptxP promoter locus and the fhaB fragment fhaB-2400_5500). These strict parameters were used to minimize the detection of paralogous sequences and to exclude decaying alleles.

For each species, we defined one or two reference strains taken either from literature or from the NCBI RefSeq database (Table S2). Type alleles were defined from each reference isolate in order to constrain the search space so as to ensure future consistency of allele definitions. The definition of new alleles was subject to constraining parameters. To detect alleles,
BLASTN thresholds were set to 90% of identity, 90% of alignment length coverage, and considering only the complete coding sequences (except for the ptxP locus promoter and the 23S rRNA gene, which are not protein CDSs). These strict parameters were used to minimize the detection of paralogous sequences.

**Phylogenetic analyses**

Phylogenetic analyses were performed based on concatenated alignments of individual gene loci defined in the database. We used an in-house script which combines IQtree (IQ-TREE 2.0.6, model substitution GTR+G+I) and Gubbins (2.4.1, default parameters) [44,45]. Phylogenetic trees were drawn and annotated using iTOL and FigTree [46,47].

To include the breadth of currently sampled *Bordetella* diversity, we first downloaded all genomic sequences from public databases and ran a rapid distance based phylogenetic analysis. From this, we retained all representative genomes of unique branches and down-sampled shallow branches that were represented multiple times. The resulting dataset included the putative novel species initially described as genogroups by Spilker *et al.* on the basis of *nrdA* gene sequences [21]. The *Bordetella* diversity dataset is publicly available from the genomic library as project *Bordetella* genus phylogeny ([https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=23&submit=1](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=23&submit=1)).

To analyze the phylogenetic structure within the BbGS, we selected 204 isolates: 177 *B. bronchiseptica* representative of the clonal complexes I and IV [1] and 9 others that did not belong to these complexes. This dataset was completed with strains from *B. pertussis* (n=9) and *B. parapertussis* (n=9), selected to represent the known diversity within these two taxa: for Bp, lineage I (strains B0887 and 18323) and lineage II (which includes *ptxP1* strains as including Tohama, and *ptxP3* strains) [23]; and for *B. parapertussis*, the ovine lineage (strain Bpp5) and the human lineage reference strain 12822. A public project encompassing all selected genomes is available in the BIGSdb genomic library platform (project name: *B. bronchiseptica* phylogeny; URL: [https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=24&submit=1](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=24&submit=1)).
In the same way, 124 Bp isolates selected to represent Bp genomic diversity were used to analyze the phylogenetic position of macrolide-resistant isolates. To this aim, we selected isolates representative of main ptxP branches (ptxP alleles 1, 2, 3, 19 and 21) and all genomes of isolates known to be resistant to erythromycin (n=51). The dataset is publicly available from the genomic library as project B. pertussis phylogeny (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_bordetella_isolates&page=query&project_list=25&submit=1).
Results

Genomic library contents
After merging Oxford and Pasteur databases, importing publicly available genomes, and adding three genomes of recently described species B. muralis (CIP111920T), B. tumulicola (CIP111922T) and B. tumbae (CIP111921T) that we sequenced, the genomic library comprised representatives all 16 Bordetella species currently described in the Prokaryotic taxonomy. The number of genomes varied across species, the most represented ones being the classical Bordetella species B. pertussis (n = 1,567), B. bronchiseptica (n = 618) and B. parapertussis (n = 197). Bordetella holmesii (n = 59) and the other species were represented by fewer genomes, including some by only one genome, such as B. flabilis or B. sputigena.

Phylogenetic analysis of the genus
A phylogenetic analysis of the 16 currently described Bordetella species and putative novel Bordetella species was performed, based on the alignments of the sequences of the genus-wide cgMLST scheme loci (Figure 1). As expected, the three classical Bordetella species were grouped in a common clade. A second clade comprised B. holmesii, B. avium, B. hinzii and B. trematum, consistent with Linz et al. [4], and also B. pseudohinzii and Bordetella genogroups 12, 5 and 1 [21]. A third major clade comprised B. petrii and Bordetella genogroups 7, 2 and 4. The three novel species B. muralis, B. tumbae and B. tumulicola were part of a single branch distantly associated to the B. petrii clade, consistent with 16S based phylogenetic analyses [20]. B. flabilis, B. sputigena and B. bronchialis were found in a fourth, more distant group together with genogroups 8, 9, 10 and 11. Finally, the earliest branching Bordetella species was B. ansorpii, as previously observed [4], and it was associated with genogroup 13.

The ANI metric is widely used as a guide to define novel bacterial species [49]. ANI values between representative genomes of the Bordetella genus (Table S2; Figure S1) showed that most currently described species differ by >5% nucleotide positions, with the well-known exception of BbGS members, which present ANI values between 98.3 and 99.4%. Here, we note that a large number of some previously attributed species names would need reevaluation; for example, B. petrii type strain and ‘B. petrii’ strain J51 had only 85.14% ANI, indicating that the latter should be considered as belonging to a distinct species, which remains to be defined. Likewise, ‘B. ansorpii’ strain H050680373 had an ANI of 93.48% with
B. ansorpii type strain NCTC13364<sup>T</sup> (= SMC-8986<sup>T</sup>). Some species correspond to previously defined genogroups, as observed for B. bronchialis and genogroup 3 (isolates AU3182<sup>T</sup> and AU17976), for B. sputigena and genogroup 14 (isolates LMG28641<sup>T</sup>, AU21707 and AU19157) and for B. flabilis and genogroup 15 (isolate AU10664<sup>T</sup>).

**Phylogenetic diversity within Bordetella bronchiseptica**

A phylogenetic tree of Bbs isolates, including 83 isolates from France sequenced for this study, was inferred from the cgMLST_genus gene sequences ([Figure 2 & Figure S2](#)) and revealed a primary separation of Bbs diversity into two deep lineages. We here define the minor one as lineage II of B. bronchiseptica. Lineage II comprised nine isolates, one of which is HT200 from a natural spring water in India and was previously recognized as being atypically distant from other Bbs isolates [50]. Bbs lineage II isolates from the USA (AU22978 et AU3139) were previously described as genogroup 6 and had been collected from patients with cystic fibrosis [21]. The remaining lineage II isolates were six human clinical isolates from France, collected from adults (mean age = 71.7 years) displaying pulmonary infections. These observations clearly establish the pathogenic potential of Bbs lineage II. In turn, Bbs lineage I comprised four sublineages, which we define as sublineages I-1 to I-4 in order to mirror the previous 7-gene MLST-defined clonal complexes I (here, sublineage I-1), II (Bp, sublineage I-2), III (Bpp, sublineage I-3) and IV (sublineage I-4). The ANI values between Bbs lineage II and the four sublineages of lineage I ranged from 95.09% to 95.72%, indicating that Bbs lineage II may be considered as a species distinct from lineage I, which itself equates to the BbGS or ‘classical bordetellae’.

The independent origins of Bpp<sub>hu</sub> and Bpp<sub>ov</sub> have been debated [1,6]. We note that Bpp5, the reference strain of ovine B. parapertussis, was associated with the earliest branching Bbs sublineage I-1, rendering B. parapertussis paraphyletic ([Figures 2 & S2](#)). A separate phylogenetic analysis performed using the cgMLST_pertussis scheme, instead of the genus-wide scheme, provided a nearly identical phylogenetic tree of Bbs isolates (data not shown). However, we recommend using the cgMLST_genus scheme for Bbs phylogenetic analyses, as a lower number of uncalled alleles was observed than for the cgMLST_pertussis scheme ([Figure S3](#)).
Genotyping of vaccine antigen and virulence-associated genes of *Bordetella bronchiseptica*

The allele diversity and presence/absence of vaccine antigens or virulence-associated loci were investigated within *Bbs*. For convenience, the loci were grouped into schemes labeled as Bp-vaccine antigens, Autotransporters, Phase genes, Other Toxins and T3SS.

T3SS genes are known to be present within the BbGS [6]. However, isolates of the classical *Bordetella* species differ in the levels of expression of their T3SS *in vitro* [51]: although *Bbs* and ovine *Bpp* are able to produce T3SS effectors such as BteA, in human adapted *Bp* and *Bpp*, T3SS expression is blocked at post-transcriptional level [3,51]. The insertion of an alanine in position 503 of BteA from *B. pertussis* leads to the attenuation of *B. pertussis* cytotoxicity [52]. This insertion was found in 8 of 11 strains of *Bbs* sublineage II-2 (*Bp*), corresponding to alleles 1 and 6. We devised a nomenclature of sequence types based on the combination of alleles at the loci *bteA, bopB, bopD, bopN* and *bsp22*; these are referred to as T3STs. For T3SS genes *bteA, bopB, bopD, bopN* and *bsp22*, most alleles were specific of (sub)lineages with some minor exceptions. T3STs were largely specific for *Bbs* sublineages (Figure 2 & S5). Sublineage I-1 comprised 30 T3STs, the main ones being type 16 and type 14, and sublineage I-4 comprised 33 T3ST types. Hypervirulent *Bbs* strains have been defined previously based on an enhanced activity of the T3SS, for example strain 1289 (BIGSdb ID: 1039; T3ST: 19) in sublineage I-1 or strain Bbr77 (BIGSdb ID: 1040; T3ST: 20) in sublineage I-4 [53,54]; however, they do not share the same T3ST. Interestingly, sublineage I-2 (*Bp*) isolates all belonged to T3ST-3, whereas isolates of sublineage I-3 (*Bpp*) all had T3ST-2, except for strain Bpp5, the ovine representative, with T3ST-18. T3ST were defined for 8 of 9 *Bbs* isolates of lineage II, all being distinct. The T3ST nomenclature therefore provides a complementary classification system of *B. bronchiseptica*, useful for distinction of subtypes within the two major sublineages of lineage I, and within lineage II.

For most other loci, alleles were specific for either *Bbs* lineage I or II, or for unique sublineages within lineage I (see details in the Supplementary text and in Figure S5). Consistent with its large (~5%) genomic divergence from the BbGS, alleles observed within *B. bronchiseptica* lineage II were unique for this lineage. In addition, even though only few isolates have been sampled so far for this lineage, we noted a high diversity of alleles indicating that it forms a genetically diverse group, suggesting phenotypic variability. Some
loci including fim2, ptxP, dnt, tcfA, vag8, prn or bipA had no allele tagged according to our stringent criteria. When relaxing these criteria, we noted that ptxP, prn and dnt were absent in Bbs lineage II, whereas fim2, bipA and tcfA presented partial matches (< 90%) with existing alleles in almost all isolates.

**Allele diversity and macrolide resistance in B. pertussis**

A phylogeny of Bp isolates, which were selected with a focus on macrolide resistance, was built using the 2,038 loci of cgMLST_pertussis scheme (Figure 3). Three isolates of the early ptxP2 branch correspond to Bp lineage II-a as defined by Bart et al. [23]. The remaining isolates fell in the ptxP1 and ptxP3 branches, which represent the main subdivisions of Bp lineage IIb.

Bp isolates were screened with the *macrolide resistance* scheme to detect alleles associated with this phenotype. Allele 13 of the 23S_rRNA locus is specific for erythromycin resistant B. pertussis isolates, whereas alleles 1, 2 and 20 correspond to susceptible ones. Allele 13 was found in 46 of 51 isolates from China [24] and in two isolates previously described as erythromycin resistant: one from USA (ATCC:BAA-1335D-5, [42]) and one from France (FR4991, [41]). When checking our entire library, which contained 1,567 Bp isolates, no additional resistant isolate was found. In a previous study, Xu et al. [24] showed that allele fhaB3 characterized by the mutation C5330T was found in a unique branch of the phylogenetic tree of Bp, and that this branch corresponds to macrolide-resistant isolates from China. Here, macrolide-resistant isolates from China shared the same allele for fhaB (full): fhaB-39. Only one exception was apparent, for isolate L14404 which had a 15-nucleotide insertion at position 6959, defined as allele fhaB-40. In our library, the alleles observed in isolates of the fhaB3 branch were defined as fhaBy-3190_7183 allele 3, fhaB-2400_5550 allele 3 and 23S rRNA allele 13 (following the nomenclature from literature [24]). Whereas all isolates from China with 23S rRNA allele 13 have the fhaB3 allele, the opposite was not true, as some isolates (PT2019, ERR030030, ERR361878, SRR1610553) have the fhaB-2400_5550 allele 3, but 23S rRNA allele 1. Hence, the fhaB3 allele marks a broader lineage, of which a subset of isolates possesses the 23S rRNA allele 13. As observed in the phylogenetic tree, macrolide-resistant Bp isolates from China all fell in a single branch (characterized by both fhaB2400_5500 allele 3 and 23S rRNA allele 13). The two other resistant isolates, collected in the USA and France, belonged to the ptxP3/fim3-2 branch and were not grouped together, indicating three independent evolutionary origins of macrolide-
resistance in these isolates and the Chinese ones. The macrolide resistant isolates from the USA and France both displayed allele 1 for fhaB2400_5500 locus and allele 13 for 23s_rRNA.

We also defined a Bp vaccine antigens scheme, which groups the loci ptxP, ptxABCDE, fim2, fim3 and fhaB2400_5550. Bp vaccine antigens sequence types (BPagST) were defined for this scheme, corresponding to the unique combinations of alleles at the individual loci. These STs were distributed along the Bp tree (Figure 3). For example, BPagST34 marked the sublineage with allele fim3-4, whereas BPagST9 corresponded to most isolates of the fim3-2 branch. BPagST37 (allele 3 for fhaB2400_5550 locus) corresponded to macrolide resistant fhaB3 lineage of Xu et al. [24]. The macrolide-resistant isolate from France was characterized by BPagST68, whereas no BPagST could be determined for the macrolide resistant isolate from the USA, because its allele at the fim3 locus was not defined due to a frameshift. We suggest that the BPagST sequence types will be useful for future tracking of specific sublineages of Bp.

Loci variation within other schemes (Other toxins, Autotransporters, T3SS and Phase biology schemes) are detailed in the Supplementary Text.
Discussion

We have set-up a unified genomic platform, available at https://bigsdb.pasteur.fr/bordetella/, by merging the data from the two previously existing PubMLST and Pasteur BIGSdb-Bordetella databases. This merger has enabled allelic nomenclature unification and has led to reassemble on the same platform, genotyping schemes that will facilitate the study of Bordetella diversity at different phylogenetic breadths. We have also expanded the genomic library with public sequences and sequenced genomes from the French NRC. The resulting unified platform for Bordetella genotyping will ease the future curation and expansion of Bordetella genomic data by a community of users and curators.

Two complementary cgMLST schemes are available on the genomic platform. While the cgMLST_genus scheme should be preferred for genus-wide phylogenetic analyses and within individual species including B. bronchiseptica, the cgMLST_pertussis scheme comprises more loci that are conserved within Bp, and its added discriminatory power will be useful for strain identification and comparison within Bp. Recently, a whole-genome MLST (wgMLST) scheme comprising even more loci (n =3,506; 1,822 of which are common with our cgMLST_pertussis scheme) was proposed for Bp subtyping, and concordant results were obtained for classification of outbreak and sporadic isolates from the USA using the cgMLST and wgMLST schemes [55]. As the later provides slightly higher discrimination among Bp isolates than the cgMLST_pertussis scheme, it might provide valuable additional genotyping information for local transmission investigations, and may be incorporated into the unified platform in the future.

We provided examples of applications of the genomic library and its associated genotyping schemes, from the analysis of the genus-level diversity of species, to strain genotyping within Bp. Regarding the phylogenetic structure of Bordetella, we provide a complete picture of the relationships among the 16 Bordetella species that have current standing in the Prokaryotic taxonomy, and confirm the existence of approximately the same number of additional putative species ([21]; PRJNA385118). This work shows that the taxonomy of the Bordetella genus will need future updates, and the unified Bordetella genomic library should facilitate the recognition of strains belonging to the same putative novel Bordetella taxa, thereby enhancing knowledge of their microbiological, ecological or pathogenic properties.
An important finding of this work is the identification of a novel *B. bronchiseptica* lineage, which we provisionally named *Bbs* lineage II. This lineage is clearly distinct from the classical *Bordetella*, which we therefore propose to define strictly as the BbGS, *i.e.*, encompassing the previously described *Bbs* lineages I-1 and I-4 together with *Bp* and *Bpp*. The average ANI between *Bbs* lineage II and the BbGS members is borderline regarding the species definition threshold (95-96%), and the relevance of elevating *Bbs* lineage II as a taxonomic species or subspecies separate from *B. bronchiseptica* remains to be defined. All but one isolate from this lineage were collected from humans, demonstrating the pathogenic potential of *Bbs* lineage II, as for lineages I-1 and I-4. Virulence factors content and vaccine antigens sequence variants are unique to lineage II. Whether lineage II has distinctive epidemiology or pathogenic features should be the subject of future studies.

A rarely isolated subgroup of *Bpp* causes pneumonia in sheep and has been referred to as *Bpp*<sub>ov</sub> in order to mark its distinctness from *Bpp*<sub>hu</sub>, the human *Bpp* [56]. *Bpp*<sub>ov</sub> remains an under-studied lineage within the BbGS. So far, very few isolates of ovine *Bpp* have been reported, mainly from New Zealand and Scotland, and all were collected before 2006 [56–58], suggesting that this lineage is very rare, if at all still circulating. Bpp5 is the only *Bpp*<sub>ov</sub> representative for which a genome sequence is currently available. More genomes of *Bpp*<sub>ov</sub> would be needed to define the phylogenetic position and unique genomic features of this group within the BbGS.

*Bp* is the medically most important *Bordetella* species, being the most frequent cause of important infections in humans. The unified *Bordetella* genomic platform provides specific tools for characterizing genes linked to *Bp* vaccine antigens and virulence. These were organized into several genotyping schemes that might be used separately for specific biological or epidemiological questions. Where available, we defined allele identifiers in accordance with their literature numbering. This will disambiguate, in particular, the genotyping of vaccine antigens within *Bp*. The population of *Bp* has been noted to evolve in response to the selective pressure exerted by vaccination, in particular in countries using the acellular vaccines [23,59]. The proposed vaccine schemes will help keeping track of the circulating strains and defining the prevalence of particular sublineages, including those that are deficient for pertactin production [27,60,61].
We also created a genotyping scheme dedicated to macrolide resistance, the possible dissemination of which is an important point of public health concern. To enable easy identification of the macrolide-resistant sublineage that is currently highly prevalent in China, we defined this scheme as combining the causative allele 23S rRNA with the associated marker fhaB3. This scheme thus provides a convenient way to distinguish ‘out of China’ dissemination of the fhaB3 sublineage, from parallel evolution of macrolide resistance in other locations.

An important current limitation of the genomic library is its contents imbalance towards genomic sequences from acellular vaccine-using high-income countries, as observed for other pathogens [62]: our database contained only few genomes from Africa (0.2 %), Asia (1.9 %) or Oceania (2.1 %) as compared to those from North America (13.6 %) or Europe (58.7 %). In the future, efforts should be made to generate and incorporate more genomic sequences from world regions that are currently underrepresented.

In conclusion, the unified Bordetella genomic library was built with the intention to facilitate and harmonize the genotyping of strains of this important bacterial genus, and should represent a useful tool even for genomic epidemiology consortia unfamiliar with genomic analyses. It provides a uniformization of genetic variants designations, which will clarify the communication on genotypes and will enable a collective understanding on the biodiversity and epidemiology of Bordetella. We provide a timely solution for genomic studies of Bordetella pathogens, and in particular Bp, the reemergence of which is partly caused by evolutionary and epidemiological dynamics of public health concern, including vaccine escape and antibiotic resistance emergence.

**Author contributions**

**Conceptualisation, Supervision**: SBrisse designed and coordinated the study. **Methodology, software, validation and data curation**: SBridel performed the merging the two databases into BIGSdb-Pasteur and the phylogenetic analyses, as well as extensive databases curation (metadata and duplicated data cleaning). VB performed in-depth analyses of virulence and antigen loci sequences and established the literature-based allelic nomenclature. SH, KAJ and MCJM created the Bordetella cgMLST v1.0 scheme. KAJ & MCJM provided the data from BIGSdb Oxford and developments of the BIGSdb platform. NA, AL, SG, JT and EM analyzed Bordetella strains at the French NRC and performed genomic sequencing. BB
provided support for the BIGSdb-Pasteur platform deployment and maintenance. Writing-Original draft Preparation, Review and Editing: SBridel, VB and SBrisse wrote the original draft. All authors contributed to and approved the final version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1: Phylogenetic analysis of the *Bordetella* genus.
The phylogenetic tree was obtained based on the concatenated multiple sequence alignments of the 1,415 core gene sequences from the *cgMLST_genus* scheme; recombination was accounted for using Gubbins. The tree was rooted on the branch leading to strain FB-8, which was the earliest branching genome in a phylogenetic analysis that included the external group *Ralstonia solanacearum* strain IBSBF1503 (Figure S4). Leaves are labelled with the identifier of the strain in the BIGSdb database, followed by the strain name.

Figure 2: Cladogram of *Bordetella bronchiseptica*.
The analysis was performed with 186 *B. bronchiseptica* genomes, and representatives of the phylogenetic diversity of *B. pertussis* (9 genomes) and *B. parapertussis* (9 genomes). The recombination-purged concatenated multiple sequence alignment of 1,415 core gene loci (*cgMLST_genus* scheme) was used. The tree is rooted on lineage II, which is the most divergent clade. Branch lengths were not used in order to ease readability of groups and isolates; see Figure S5 for the corresponding phylogram. For each isolate, the host is represented using a leaf symbol, where available (see key in Figure S5). The numbers corresponding to T3SS sequence types are indicated along the external circle around the tree; only the identifiers of main T3STs are indicated.

Figure 3: Phylogenetic analysis of *Bordetella pertussis*.
The phylogenetic tree was obtained based on the recombination-purged concatenated multiple sequence alignments of the 2,038 core genome loci of the *cgMLST_pertussis* scheme. The distribution of macrolide resistance is indicated. The three outer circles represent (from innermost): 23S_rRNA alleles, fhaB alleles and the vaccine antigen sequence types (AgST).
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