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Sector: **BIOLOGICAL SCIENCES**

Speciality: **MICROBIOLOGY**

Theme :

# Comprehensive genome-based reclassification of several pathogenic species within the genera *Bordetella* and *Borrelia*.

**Presented by:**

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**Presented on** : 01/07/2025

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*By: BAGUMA AZIIZI*

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*By: NABAGALA IMMACULATE*

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*By: MUKWAYA JOSEPH*

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## Abstract

In the present study, a polyphasic taxonomic approach was employed to reassess the classification of several species within the genera *Bordetella* and *Borrelia*, using genomic, phylogenetic, and phenotypic data.

A total of 22 genome sequences of *Bordetella* were analysed to re-evaluate the taxonomic boundaries among *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*, which are traditionally regarded as distinct species despite significant genetic overlap. Phylogenetic analyses based on 16S rRNA sequences, whole-genome datasets and the core genome consistently grouped these three taxa into a single monophyletic clade, supported by strong bootstrap values (≥99%). Genomic relatedness indices, including Average Nucleotide Identity (ANI: 98–99%), Average Amino Acid Identity (AAI: 97–99%), and digital DNA–DNA hybridization (dDDH: 85–95%), exceeded recognized species delineation thresholds (ANI/AAI ≥95%, dDDH ≥70%). For the genus *Borrelia*, the analysis included two distinct groups: the first composed of *B. garinii* and *B. bavariensis*, and the second of *B. bissettiae*, *B. kurtenbachii*, and *B. carolinensis*. Phylogenetic trees constructed from both 16S rRNA and whole-genome data revealed strong clustering within each group. ANI values reached 97% between *B. garinii* and *B. bavariensis* and ranged between 96% and 98% among the second group. Corresponding dDDH values supported these results, with 77% for the first group and 63%–80% for the second. These values all exceed their corresponding thresholds for species delineation. In both genera, the additional comparative analyses, including orthologous cluster identification and functional subsystem analysis, revealed a high degree of shared gene content and functional similarity among the studied species. Collectively, all these results highlight a high genomic similarity and evolutionary relatedness among the studied taxa.

In light of these results, we propose reclassifying *B. bronchiseptica* and *B. parapertussis* as later heterotypic synonyms of *B. pertussis* within the genus *Bordetella*. Similarly, within the genus *Borrelia*, we propose that *B. bavariensis* be reclassified as a later heterotypic synonym of *B. garinii*, and that *B. kurtenbachii* and *B. carolinensis* be considered later heterotypic synonyms of *B. bissettiae*. This comprehensive reclassification clarifies the current taxonomic ambiguities within these clinically significant bacterial genera, thereby enabling more precise identification of pathogenic species which is an essential step for effective clinical diagnosis, epidemiological tracking, and public health management.



**Keywords**: *Bordetella*, *Borrelia*, polyphasic taxonomy, phylogenomics, species reclassification.

## Résumé

Dans la présente étude, une approche taxonomique polyphasique a été employée afin de réévaluer la classification de plusieurs espèces appartenant aux genres *Bordetella* et *Borrelia*, en s'appuyant sur des données génomiques, phylogénétiques et phénotypiques. Un total de 22 génomes de *Bordetella* ont été analysés pour réexaminer les frontières taxonomiques entre *Bordetella pertussis*, *Bordetella parapertussis* et *Bordetella bronchiseptica*, traditionnellement considérées comme des espèces distinctes malgré un chevauchement génétique important. Les analyses phylogénétiques basées sur les séquences de l'ARNr 16S, les jeux de données du génome complet et le génome central ont systématiquement regroupé ces trois taxons dans un clade monophylétique unique, soutenu par de fortes valeurs de bootstrap (≥99 %). Les indices de parenté génomique, incluant l'identité moyenne des nucléotides (ANI : 98–99 %), l'identité moyenne des acides aminés (AAI : 97–99 %) et l'hybridation ADN–ADN numérique (dDDH : 85–95 %), ont dépassé les seuils reconnus pour la délimitation des espèces (ANI/AAI ≥95 %, dDDH ≥70 %). Pour le genre *Borrelia*, l'analyse a porté sur deux groupes distincts : le premier composé de *B. garinii* et *B. bavariensis*, et le second de *B. bissettiae*, *B. kurtenbachii* et *B. carolinensis*. Les arbres phylogénétiques construits à partir des séquences de l'ARNr 16S et des données génomiques complètes ont révélé un regroupement marqué au sein de chaque groupe. Les valeurs ANI ont atteint 97 % entre *B. garinii* et *B. bavariensis*, et ont varié entre 96 % et 98 % pour le second groupe. Les valeurs dDDH correspondantes confirment ces résultats, avec 77 % pour le premier groupe et de 63 % à 80 % pour le second, dépassant toutes les limites établies pour la délimitation spécifique.

Dans les deux genres, les analyses comparatives supplémentaires, incluant l'identification de clusters orthologues et l'analyse des sous-systèmes fonctionnels, ont révélé un haut degré de similarité génétique et fonctionnelle entre les espèces étudiées. L'ensemble de ces résultats met en évidence une forte similitude génomique et une parenté évolutive marquée entre les taxons analysés. À la lumière de ces observations, nous proposons de reclasser *B. bronchiseptica* et *B. parapertussis* comme synonymes hétérotypiques ultérieurs de *B. pertussis* au sein du genre *Bordetella*. De même, au sein du genre *Borrelia*, nous proposons que *B. bavariensis* soit reclassée comme synonyme hétérotypique ultérieur de *B. garinii*, et que *B. kurtenbachii* et *B. carolinensis* soient considérées comme synonymes hétérotypiques ultérieurs de *B. bissettiae*. Cette reclassification exhaustive permet de clarifier les ambiguïtés taxonomiques actuelles au sein de ces genres bactériens cliniquement importants, facilitant ainsi une identification plus précise des espèces pathogènes, ce qui constitue une étape essentielle pour un diagnostic clinique efficace, le suivi épidémiologique et la gestion de la santé publique.



**Mots-clés** : *Bordetella*, *Borrelia*, taxonomie polyphasique, phylogénomique, reclassification des espèces.

## المُلخّص

في هذه الدراسة، تم اعتماد منهج تصنيفي متعدد الأساليب لإعادة تقييم تصنيف عدد من الأنواع التابعة لجنسي *Bordetella* و*Borrelia*، وذلك بالاستناد إلى بيانات جينومية وفيزيولوجية ونشوء وتطور (فيليلوجينية).

تم تحليل 22 تسلسلاً جينوميًا من *Bordetella* بهدف إعادة تحديد الحدود التصنيفية بين *Bordetella pertussis* و *Bordetella parapertussis* و*Bordetella bronchiseptica*، والتي تُصنّف تقليديًا كأنواع مستقلة رغم التشابه الجيني الكبير فيما بينها. أظهرت التحاليل الوراثية المعتمدة على تسلسلات جين S rRNA16 وبيانات الجينوم الكامل والجينوم الأساسي أن هذه الأنواع الثلاثة تشكل تفرعًا أحادي الأصل (monophyletic clade) مدعومًا بقيم دعم إحصائي مرتفعة (bootstrap ≥ 99%).كما تجاوزت مؤشرات التقارب الجينومي، بما في ذلك متوسط تشابه النيوكليوتيدات (ANI: 98–99%)، ومتوسط تشابه الأحماض الأمينية ( AAI: 97–99%)، والتهجين الرقمي للحمض النووي (dDDH: 85–95%)، العتبات المعتمدة لتعريف الأنواع (ANI/AAI ≥ 95%، و dDDH ≥ 70%). أما بالنسبة لجنس *Borrelia*، فقد شمل التحليل مجموعتين متميزتين: الأولى تضم *B. garinii* و *B. bavariensis*، والثانية تضم *B. bissettiae* و*B. kurtenbachii* و*B. carolinensis*. وقد أظهرت الأشجار التطورية المبنية على تسلسلات جين S rRNA16 والجينوم الكامل تجمعًا واضحًا داخل كل مجموعة. بلغت قيم ANI نسبة 97% بين *B. garinii* و *B. bavariensis*، وتراوحت بين 96% و98% داخل المجموعة الثانية، بينما دعمت نتائج dDDH هذه النتائج بنسبة 77% للمجموعة الأولى و63–80% للمجموعة الثانية، وهي جميعها تفوق العتبات المقررة لتعريف الأنواع. كشفت التحليلات المقارنة الإضافية، مثل تحديد العناقيد الجينية المتجانسة (orthologous clusters) وتحليل النُظُم الوظيفية، عن درجة عالية من التشابه الجيني والوظيفي بين الأنواع المدروسة في كلا الجنسين. وتشير هذه النتائج مجتمعة إلى وجود تقارب جينومي وتطور مشترك واضح بين هذه الأنواع.

واستنادًا إلى هذه النتائج، نقترح إعادة تصنيف كل من *B. bronchiseptica* و*B. parapertussis* كمرادفات غير متماثلة لاحقة (later heterotypic synonyms) لـ*B. pertussis* ضمن جنس *Bordetella*. وبالمثل، نقترح إعادة تصنيف *B. bavariensis* كمرادف لاحق غير متماثل لـ*B. garinii*، و*B. kurtenbachii* و*B. carolinensis* كمرادفين لاحقين غير متماثلين لـ*B. bissettiae* ضمن جنس *Borrelia*. تُسهم هذه المراجعة التصنيفية الشاملة في توضيح الغموض القائم في التصنيف الحالي لهذه الأجناس البكتيرية ذات الأهمية السريرية، مما يُمكّن من التعرف بدقة على الأنواع الممرضة، وهو ما يُعد خطوة أساسية في التشخيص السريري الفعال، ورصد الحالات الوبائية، وتعزيز إدارة الصحة العامة.



**الكلمات المفتاحية**: *Bordetella*، *Borrelia*، التصنيف المتعدد الأساليب، التصنيف الجينومي التطوري، إعادة تصنيف الأنواع.

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# List of abbreviations

**ANI** – *Average Nucleotide Identity*

**AAI** – *Average Amino Acid Identity*

**dDDH** – *Digital DNA-DNA Hybridization*

**OGRIs** – *Overall Genome Relatedness Indices*

**WGS** – *Whole-Genome Sequencing*

**NGS** – *Next-Generation Sequencing*

**GC-MS** – *Gas Chromatography-Mass Spectrometry*

**HPLC** – *High-Performance Liquid Chromatography*

**MS** – *Mass Spectrometry*

**MLSA** – *Multilocus Sequence Analysis*

**MUMi** – *Maximal Unique Matches Index*

**HGT** – *Horizontal Gene Transfer*

**SPR** – *Subtree-Pruning-Regrafting*

**UPGMA** – *Unweighted Pair Group Method with Arithmetic Mean*

**SP** – *Sum-of-Pairs*

**ICNP** – *International Code of Nomenclature of Prokaryotes*

**LPSN** – *List of Prokaryotic Names with Standing in Nomenclature*

**GBDP** – *Genome Blast Distance Phylogeny*

**RF** – *Relapsing Fever*

**LBRF** – *Louse-Borne Relapsing Fever*

**TBRF** – *Tick-Borne Relapsing Fever*

**LB** – *Lyme Borreliosis (Lyme Disease)*

**ECM** – *Erythema Chronicum Migrans (early Lyme disease rash)*

**NCBI** – *National Centre for Biotechnology Information*

**MEGA** – *Molecular Evolutionary Genetics Analysis*

**TYGS** – *Type (Strain) Genome Server*

**BPGA** – *Bacterial Pan-Genome Analysis*

**EDGAR** – *Efficient Database framework for comparative Genome Analyses using BLAST score Ratios*

**RASTtk** – *Rapid Annotation using Subsystems Technology toolkit*

**BLAST** – *Basic Local Alignment Search Tool*

**ITOL** – *Interactive Tree of Life*

**BLASTn** – *Nucleotide BLAST*

**MUSCLE** – *Multiple Sequence Comparison by Log-Expectation*

**CDS** – *Coding Sequence*

**HSPs** – *High-Scoring Segment Pairs*

**JTT** – *Jones-Taylor-Thornton (protein evolution model)*

**CAT** – *Covarion-Auto-Correlated (evolutionary model)*

**LPS** – *Lipopolysaccharide*

**PTX** – *Pertussis Toxin*

**ISEs** – *Insertion Sequence Elements*

# INTRODUCTION

# INTRODUCTION

Since its inception, taxonomy has served as a fundamental discipline in biological research offering a systematic framework for the organization of biological knowledge (Mayr & Bock, 2002). In the field of microbiology, it is essential in understanding microbial diversity, classification, and evolutionary relationships which informs the development of diagnostic methodologies and therapeutic interventions.

Bacterial taxonomy was originally based on phenotypic traits that primarily relied on observable morphological and biochemical characteristics (Tindall et al., 2010a). However, this approach often lacked the resolution required to accurately differentiate closely related species (Rosselló-Mora & Amann, 2001). The incorporation of genome-based techniques significantly advanced this field as they offer deeper insights into evolutionary relationships which improve the precision of taxonomic classifications (Chun, Oren, Ventosa, Christensen, Arahal, Da Costa, et al., 2018; Lalucat et al., 2020; Parks et al., 2018). Today, this field has adopted the Polyphasic approach as the gold standard for an accurate taxonomic delineation. By integrating phenotypic and genomic data, this comprehensive method achieves an exceptionally high resolution enabling precise identification and classification of bacterial taxa (Colwell, 1970; P. Vandamme, Pot, Gillis, de Vos, et al., 1996; P. Vandamme & Peeters, 2014).

The genera *Bordetella* and *Borrelia* comprise pathogens of clinically significant importance that affect both humans and animals. *Bordetella* species are primarily associated with respiratory tract infections, while *Borrelia* species are known for causing Lyme disease and relapsing fever, transmitted mainly through ticks and lice (Barbour & Schwan, 2018c; Mattoo & Cherry, 2005a).

Several genomic studies suggest that some species within these genera may be more closely related than their current classification implies. In particular, the classical *Bordetella* species i.e. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, exhibit a high degree of genetic similarity that challenges their current taxonomical status as distinct species (Cummings, Brinig, Lepp, Van De Pas, et al., 2004; Park et al., 2012; Parkhill et al., 2003).

This study aims to conduct a comprehensive taxonomic reassessment of selected *Bordetella* and *Borrelia* species using the polyphasic taxonomic approach, to clarify the evolutionary relationships within these bacterial groups. By leveraging several modern phylogenomic methods such as Overall Genome Relatedness Indices (OGRIs) analyses, Pan-genome analysis and Subsystems-based functional profiling, we aim to resolve the taxonomical ambiguities within these clinically significant bacterial species.

Refining the taxonomy of these organisms is not only important from an academic perspective, but also has practical implications for understanding pathogen evolution, improving diagnostic accuracy, and guiding the development of targeted control and treatment strategies against their infections.

*CHAPTER 1:*

# LITERATURE REVIEW

# 1. LITERATURE REVIEW

## 1.1. INTRODUCTION TO TAXONOMY

### 1.1.1. The Origins and Evolution of Biological Classification

The term Taxonomy is derived from Greek words *taxis,* meaning "arrangement", and *nomos,* meaning "law" (Johansson & Pettersson, 2002). Taxonomy therefore refers to the scientific discipline of identifying, naming, and classifying living organisms into hierarchical groups based on their shared characteristics (Henke & Tattersall, 2007). It comprises of three closely interconnected aspects: classification, nomenclature, and identification (Tindall et al., 2010).

**Classification** is defined as the systematic organization of organisms into hierarchical groups based on the shared characteristics and evolutionary relationships. **Nomenclature** refers to assigning names to organisms according to standardized rules listed in the International Code of Nomenclature of Prokaryotes (ICNP). **Identification** is the process of determining which established taxonomic group, a particular organism or strain belongs to by analysing its characteristics (Oren et al., 2023; P. Vandamme et al., 1996).

From the dawn of civilization, there have been many attempts to classify living organisms, which were primarily instinctive and not based on scientific criteria. Aristotle, a Greek philosopher, was one of the first to implement a structured scientific approach to classify living things. He based his system mainly on physical traits, grouping plants into trees, herbs, and shrubs, while dividing animals into those with blood and those without. Though his method was a step toward scientific classification, it wasn't entirely consistent and didn't fully reflect the vast complexity of the natural world (Montgomery, 2025).

Later, in 1735 Carl Linnaeus, a Swedish botanist, zoologist, and physician, revolutionized the classification of living organisms and is referred to as the "Father of Taxonomy"(Calisher, 2007). He introduced the binomial nomenclature system, which assigns each species a distinct two-part name, which includes the genus name and an epithet. This system formed the foundation of the nomenclature that is still used today (Mayr, 1982).

Linnaeus also proposed a system for organizing living organisms into hierarchical groups, or taxa, which include Kingdom, Class, Order, Family, Genus, and Species. This system relied entirely on shared morphological characteristics to classify organisms and became the foundation of today's biological classification (Stevens, 1994).

However, Linnaeus' system was based on the concept of special creation and thus grouped organisms under the assumption that species were unchanging i.e., immutable, and had been created in their current form. This idea was later challenged by Charles Darwin's theory of evolution which proposed that organisms were not fixed but rather changing over time through the process of natural selection and shared common ancestry. Hence, Darwin's work fundamentally shifted the basis of classification from static traits to evolutionary relationships (Darwin, 1859).

Darwin's discovery also laid the foundation for the emergence of Phylogenetics, a field that initially relied on morphological features to construct the early types of phylogenetic trees i.e. cladograms.

This further signified a major advancement beyond the Linnaean system (Hull, 1988; Podani, 2010).

Gregor Mendel's later work on genetics further strengthened Darwin's theory by providing the genetic explanation for Darwin's proposed evolutionary relationships. By explaining how traits are inherited, Mendel's work laid the groundwork for the integration of genetics with evolutionary theory, resulting in what is now termed the Modern Synthesis or Neo-Darwinism (Stenseth et al., 2022).

The significant advancements in molecular biology in the mid-20th century, especially the development of DNA sequencing technology, further transformed classification. These tools allowed scientists to more accurately assess evolutionary relationships through the comparative analysis of genetic material of several organisms (Montgomery, 2025). This also gave rise to the Molecular phylogenetics field which relies on genetic sequence data to trace the evolutionary relationships, and has become a crucial approach in the modern taxonomy (Avise, 2000).

In the 21st century, the introduction of high-throughput sequencing technologies and progress in genomics, proteomics, and bioinformatics fields, has revolutionized taxonomy further. Researchers can now perform comprehensive whole-genome analyses, producing vast datasets which significantly increases the precision of the taxonomic position of organism (Montgomery, 2025).

## 1.2. HISTORY OF BACTERIAL TAXONOMY

### 1.2.1. Early Historical Foundations of Bacterial Taxonomy

Bacterial taxonomy is concerned with the classification, identification and determination of evolutionary relationships among bacteria.

Microorganisms were first discovered in the 17th century by Antonie van Leeuwenhoek (Father of Microbiology). While using simple microscopes , he observed and described tiny mobile organisms in water, dental plaque, and other samples, referring to them as "***animalcules***" (Gest, 2004). This discovery, among others, laid the foundation for understanding infectious diseases and the role of microbes in natural ecosystems.

In the 18th century, all microorganisms were grouped under a **two-kingdom system** proposed by Carl Linnaeus, which classified life into ***Plantae*** and ***Animalia*** (Calisher, 2007). However, this system did not account for microorganisms as they exhibited both plant-like and animal-like characteristics and also differed on organizational level, from plants and animals. This limitation prompted the development of more complex alternative classification systems.

In the 19th century, several increasingly advanced and refined classification systems were introduced. Firstly in 1817, Georg August Goldfuss introduced the term ***Protozoa*** ("first animals") to describe primitive simple animal-like organisms. Later, Carl Theodor von Siebold of Germany categorized these organisms into a phylum of invertebrate animals within Kingdom *Animalia* (Scamardella, 1999). Meanwhile, Christian Gottfried Ehrenberg introduced the term ***"Bacteria"*** to describe rod-shaped

microbes of a single genus within the Vibrionia family, which later became the name for the entire group of these organisms (Osorio, 2017).

In 1860, the British naturalist John Hogg proposed a new kingdom "***Protoctista***" for the "lower organisms" that didn't fit well within kingdom Plantae or Animalia. It included two main groups: ***Protophyta*** (lower plant-like organisms), and ***Protozoa*** (lower animal-like) organisms and other ambiguous microorganisms including bacteria. (Scamardella, 1999). Then in 1866, Ernst Haeckel refined Hogg's system by establishing a **three-kingdom system** i.e. **Plantae**, **Animalia** and **Protista.** The new kingdom Protista included all microorganisms not fitting into the plant and animal kingdom and the bacteria were classified under the "phylum **Monera**" within the Protista kingdom (Scamardella, 1999).

### 1.2.2. The Institutionalization of Bacterial Classification with Bergey's Manual

The development of **pure-culture techniques** by Robert Koch and Friedrich Loeffler revolutionized bacterial systematics. By isolating and analysing individual microbial species, scientists gained the ability to conduct more detailed studies of bacterial morphology (shape, motility), physiology (growth conditions, metabolism), and biochemistry (enzyme activity, fermentation profiles) (Blevins & Bronze, 2010; Jiao et al., 2024). The introduction of the Gram stain technique by Hans Christian Gram in 1884 also further improved bacterial differentiation. This method grouped bacteria based on their cell wall structure into either Gram-positive (thick peptidoglycan layer) or Gram-negative (thin peptidoglycan with an outer membrane) (Moyes et al., 2009).

The advancement of the Bacterial classification beyond morphology characteristics to incorporate physiological and biochemical characteristics, led to the creation of the **first Bergey's Manual of Determinative Bacteriology in 1923**. This manual established a standardized, systematic framework for the identification, classification, and description of bacteria. In its early editions, bacteria were still classified under Kingdom *Plantae*, Phylum ***Protophyta***, and Class ***Schizomycetes*** and this classification persisted through to the $^{7th}$ edition (Breed et al., 1957). By the mid-$20^{th}$ century, many microbiologists advocated for recognizing bacteria as a kingdom separate from plants, as discussed earlier.

### 1.2.3. The Emergence of the Five-Kingdom Model in Biological Taxonomy

In 1925, the French protistologist Édouard Chatton introduced the terms **Prokaryote** and **Eukaryote** to classify different types of protists, but this concept initially went unrecognized. Later, Lwoff helped convince R. Stanier and C.B. van Niel to formalize this distinction in 1962 as a widely accepted system. According to this categorisation, Eukaryotic organisms (plants, animals, fungi and Protozoa) have membrane bound nuclei and organelles while Prokaryotic organisms (bacteria) possess freely floating genetic material in the cytoplasm and no organelles (Stanier & Niel, 1962).

Building on Chatton's distinctions, Herbert F. Copeland proposed a **four-kingdom system** of classification (**Kingdom *Monera, Protoctista, Plantae, Animalia***). He excluded bacteria and the "blue-

green algae" (cyanobacteria) from Haeckel's Kingdom *Protista* into a separate kingdom he named ***Monera*****,** because he regarded them to be so different in organisation from nucleated cells. Now Kingdom *Monera* Included only prokaryotes (bacteria and cyanobacteria) and Kingdom *Protoctista* contained all eukaryotic microorganism such as algae, protozoa, and slime moulds (Scamardella, 1999).

In his 1959 article, "On the Broad Classification of Organisms," Robert H. Whittaker re-evaluated Copeland's four-kingdom system based on the three primary modes of nutrition in natural communities (absorption, ingestion, and autotrophy). Based on this, he proposed his own version of a four-kingdom system, dividing life into *Protista, Fungi, Plantae,* and *Animalia*. In this model, bacteria were placed under Kingdom *Protista*, and all algae types (green, brown, and red) were included in Kingdom *Plantae*(Whittaker, 1959).

A decade later, in 1969, Whittaker expanded his system to **five kingdoms**, restoring ***Monera*** as a separate kingdom for bacteria, similar to Copeland's original idea. This revision also emphasized the major distinction between prokaryotic and eukaryotic life forms, from Chatton's earlier concepts (Whittaker, 1969). This led to the well-known five-kingdom classification of Kingdom ***Monera, Protista, Fungi, Plantae,*** and ***Animalia***, which still in use till today.

### 1.2.4. The Integration of Chemotaxonomy and Numerical Methods in Bacterial Classification

In the 1950s, chemotaxonomy became a commonly used method for classifying organisms based on the chemical composition of their cellular structures. It employed techniques such as Fatty Acid Profiling, Isoprenoid Quinone Analysis, Cytochrome Analysis, Cell Wall Composition analysis, etc (Busse et al., 1996). These laid the groundwork for the modern microbial identification techniques such as **Gas** Chromatography-Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC), and mass spectrometry (MS), that are now routinely used to analyse these chemotaxonomic markers (Bridge et al., 2021).

Then numerical taxonomy was formally introduced in the 1960s by Robert R. Sokal and Peter H. A. Sneath. This method applied mathematical and statistical techniques to classify organisms based on the quantitative analysis of a wide range of their phenotypic traits such as their morphology, physiology, and biochemistry (Bridge et al., 2021). By converting descriptive features into numerical data, this approach enabled computer-assisted comparative analysis of phenotypic traits across multiple organisms. This marked a shift from traditional, subjective classification systems to a more objective, standardized, and data-driven approach (Peter H. A. Sneath, 1973).

### 1.2.5. The Molecular Revolution in Bacterial Taxonomy

The earliest development in molecular techniques was the **G+C content analysis,** introduced in the 1950s following Chargaff's discoveries (Bohlin et al., 2010). This method involves measuring the proportion of guanine (G) and cytosine (C) in an organism's DNA to estimate its overall genomic

composition. Comparative analysis of G+C allows taxa delineation as a variation of no more than 3 % in G+C content typically indicates species-level similarity, while a 10 % range was used for genus-level differentiation. This method however lacked the sufficient resolution to distinguish closely related species (Lee et al., 1956).

This limitation led to the introduction of **DNA-DNA Hybridization** (**DDH**) technique in the 1960s. DDH assesses genetic relatedness by measuring the extent of binding / hybridisation of single-stranded DNA from two organisms. A hybridization of 70 % or more is considered evidence that the organisms belong to the same species (Richter & Rosselló-Móra, 2009).

Later in 1977, Carl Woese pioneered the use of **16S ribosomal RNA** (**16S rRNA)** sequences to establish evolutionary relationships among microorganisms. Using this technique, he observed that some prokaryotic organisms initially classified as bacteria were fundamentally different at this genetic level and did not fit within the traditional classification of Bacteria. As a result, he assigned these organisms to a new category called **"*Archaea*".** His molecular comparisons further revealed that life on Earth is divided into three primary evolutionary lineages: ***Eubacteria, Archaebacteria***, and ***Eukaryotes***, which wasn't reflected by the existing Whittaker's five-kingdom system and the prokaryote-eukaryote division. Therefore, Woese proposed a new hierarchical taxon above the kingdom level called a "**domain**" consisting of ***Bacteria*** (true bacteria), ***Archaea*** (methanogens, halophiles, thermophiles) and ***Eukarya*** (animals, plants, fungi, protists) (Woese et al., 1990).

### 1.2.6. The Polyphasic Taxonomical approach

Early taxonomists often relied entirely on individual techniques for classification of bacteria. While helpful to some extent, each of the techniques presented certain limitations which often led to inconsistencies in bacterial systematics. Therefore, in the later 20$^{th}$ century, a more comprehensive approach known as "**Polyphasic Taxonomy**" was introduced to address this concern.

The concept was first proposed by Rita Colwell through her research on marine microbiology and *Vibrio* species. She realised that bacterial classification should not be based solely on morphology or biochemical properties. Rather, she proposed a multidimensional classification system that integrates molecular, ecological, and other relevant data and she termed this approach the "Polyphasic taxonomy" (Colwell, 1970). This approach was later formalized and standardized in microbiology by Vandamme in 1996 (P. Vandamme et al., 1996).

Polyphasic taxonomy is a comprehensive approach to microbial classification that simultaneously integrates multiple sources of data, i.e., Genotypic, Phenotypic, and Chemotaxonomic information, to provide a more robust and accurate system for defining microbial identities and evolutionary relationships (Colwell, 1970; Raina et al., 2019). Phenotypic methods examine an organism's **morphological, physiological,** and **metabolic** characteristics. Chemotaxonomic methods analyse unique chemical markers specific of microbes such as cellular fatty acids and isoprenoid quinones. Genotypic methods utilize molecular techniques to analyse the genetic composition of an organism, providing insights into its evolutionary relationships and taxonomic classification (Bridge et al., 2021).

In the earlier polyphasic taxonomy, Genotypic methods were primarily limited to Phylogenetic techniques such as to 16sRNA analysis, DNA-DNA Hybridization (DDH) and % G+C content analysis, which were used in combination with the Chemo-taxonomical and Phenotypic methods. These methods however had significant limitations, as they were labour intensive, lacked consistency and were ineffective in classifying unculturable microbes (Raina et al., 2019).

With advancements in technologies, Genotypic methods have now become the cornerstone of microbial classification in the modern polyphasic approach due to the development of techniques and tools with better accuracy and resolution.

The introduction of **Whole-Genome Sequencing** (**WGS**) technology was particularly transformative as it allowed for comprehensive examination of the entire genome of each isolated organism. This provided researchers with a more detailed, comprehensive, and precise understanding of genetic composition of the organisms, significantly enhancing taxonomic resolution especially in identifying new species and clarifying taxonomical boundaries (Coenye et al., 2005; Janssen et al., 2003).

WGS offered a complete genomic perspective that surpassed previous methods in both scope and accuracy. It solved the limitations associated with the Sanger sequencing technique which was in use and paved the way for several developments including genome databases, analytical algorithms, software and other comparative techniques such as Average Nucleotide Identity (ANI), Average Amino Acid Identity (AAI), Digital DNA-DNA Hybridization (dDDH), Maximal Unique Matches Index (MUMi) and Multilocus Sequence Analysis (MLSA) (Raina et al., 2019).

The **Average Nucleotide Identity (ANI)** measures the genetic similarity between two microbial genomes by establishing the average percentage of identical nucleotides between them. The genome of the query organism is divided into fragments and each fragment is compared to the entire genome of the subject organism using a sequence alignment algorithm, such as BLASTn. The percentage of matching nucleotides in each pair of fragments is determined and the final ANI value is calculated. An ANI value **≥95** % indicates that two organisms are likely to belong to the same species (Goris et al., 2007).

The **Average Amino Acid Identity (AAI)** instead compares the amino acid sequences of corresponding proteins from two genomes, to measure the genetic similarity between them. The average cutoff score for AAI of **≥95 %** is well correlated with **≥95** % **ANI**, **≥70** % **dDDH** for microbial organisms of the same species (C. C. Thompson et al., 2021).

**Digital DNA-DNA Hybridization (dDDH)** is the latest bioinformatics technique that is gradually replacing the conventional wet-lab DDH method. It is a computational method that determines the degree of Hybridisation between two microbial genomes, based on their genomic sequence data. Organisms are classified as belonging to the same species if the dDDH value is ≥70 %. dDDH is considered a more reliable and reproducible method for species delineation than traditional DDH because it uses digital genome data, making it less prone to errors, provides high resolution in differentiating species, particularly for closely related organisms (Meier-Kolthoff et al., 2014).

**Maximal Unique Matches Index (MUMi)** is a highly sensitive genomic technique that assesses the genetic distance between two genomes based on unique matching segments, particularly at the species or strain level. Proportion of Maximal Unique Matches (MUMs) between the genomes gives the MUMi value (between 0 & 1) where a MUMi value close to 0 indicates closely related genomes, while a value closer to 1 suggests that they are genetically distant (Konstantinidis & Tiedje, 2005a).

**Multilocus Sequence Analysis** (**MLSA**) is a molecular technique used to examines the genetic relatedness of microorganisms by sequencing and comparing multiple housekeeping genes from their genomes. Common housekeeping genes used in MLSA include 16S rRNA, gyrA, rpoB, and atpD etc. The sequences from each gene are used to create a unique allelic profile for each strain, which is helpful for distinguishing between closely related species or subspecies (Maiden, 2006). MLSA provides higher resolution than single-gene sequencing (e.g., 16S rRNA alone) because it uses multiple genes, which reduces the potential for misclassification due to horizontal gene transfers or conserved regions in a single gene (Raina et al., 2019).

The introduction of **Next-Generation Sequencing** (**NGS**) technologies in the early 21st century marked a quantum leap in bacterial systematics by enabling rapid, accurate, and cost-efficient genome sequencing. This technology greatly facilitated progress of genome-related research projects around the world. Key platforms that transformed the field include: Roche 454 Pyrosequencing (Margulies et al., 2005), Illumina systems (Solexa, HiSeq, MiSeq), Ion Torrent platforms (PGM, Proton), Pacific Biosciences SMRT sequencing, and Oxford Nanopore's MinION technology (Raina et al., 2019).

Today, the modern polyphasic taxonomy framework remains central to bacterial systematics. It continues to evolve by incorporating emerging technologies, ensuring that microbial classification aligns with true evolutionary relationships and has also remained adaptive to emerging scientific developments (Vandamme et al., 1996).

## 1.3. THE GENUS *BORDETELLA*

### 1.3.1. Introduction to Genus *Bordetella*

The genus *Bordetella* belongs to the *Alcaligenaceae* family and consists of small, Gram-negative coccobacilli primarily responsible for respiratory infections in humans and animals (Gerlach et al., 2001). The genus was named after **Jules Bordet,** who along with **Gengou Octave**, first described *B. pertussis* as the type species and a causative agent of whooping cough in 1906 (Bordet & Gengou, 1906). The members of this genus are obligate pathogens with major virulence factors including adhesins (e.g., filamentous hemagglutinin, pertactin), toxins (e.g., pertussis toxin, adenylate cyclase toxin), and lipopolysaccharides. They are also non-fermentative and fastidious, requiring specialized media like Bordet-Gengou agar for isolation (Melvin et al., 2014; Leber, 2014). Most species live in close association with animal hosts, either causing diseases directly or occasionally as opportunistic pathogens (Weiss, 2006).

**Classification of the genus *Bordetella***

**Domain:** *Bacteria*
**Branch:** *Pseudomonadota*
**Class:** *Betaproteobacteria*
**Order:** *Burkholderiales*
**Family:** *Alcaligenaceae*
**Genus:** *Bordetella*. (https://lpsn.dsmz.de/genus/*Bordetella* ; Parte et al., 2020)

## 1.3.2. Description of the Common *Bordetella* Species

Since the initial isolation of *Bordetella pertussis*, the *Bordetella* genus has expanded to include about 16 species i.e., *B. parapertussis*, *B. bronchiseptica*, *B. flabilis, B. sputigena, B. bronchialis, B. muralis, B. tumulicola, B. tumbae, B. petrii, B. trematum, B. holmesii, B. avium, B. hinzii, B. pseudohinzii, and B. ansorpii.* These species vary in host range, pathogenic potential, and virulence factor expression as shown in Table **I**. (Miguelena Chamorro et al., 2023; Goodnow, 1980). *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* are referred to as the "classical" *Bordetella* species because they were the first identified and most extensively studied members of the genus (Hamidou Soumana et al., 2017; Miguelena Chamorro et al., 2023).

*Bordetella pertussis* was originally classified as *Haemophilus pertussis* because it requires blood for growth in laboratory media. However, studies demonstrated it isn't dependent on the X (haematin) and V (NAD) growth factors (Hornibrook, 1940) and therefore in 1952, it was officially reclassified under its own genus, *Bordetella* (Moreno-Lopez, 1952; Krieg & Holt, 1984). *B. bronchiseptica* was isolated in 1910 from dogs with distemper (Ferry, 1912)while *B. parapertussis* was first identified in 1937 by Eldering and Kendrick (Bradford & Slavin, 1937; Eldering & Kendrick, 1938; Bradford & Slavin, 1937)

Although all these species cause upper respiratory tract infections, they infect different hosts. *B.pertussis* is limited to humans causing severe whooping cough, while *B. parapertussis* exists in two distinct strains i.e. one that infects humans causing a mild whooping cough and a strain that infects sheep. *B. bronchiseptica* can infect a broad range of mammals and birds, but can also cause opportunistic infections in immunocompromised humans (Woolfreyt & Moody, 1991; Goodnow, 1980; Cullinane et al., 1987).

***Table I:*** *Species of the Genus Bordetella, their Hosts, and associated Diseases.*

| Species | Described By (Year) | Host/Source | Disease/Association |
|---|---|---|---|
| ***B. avium*** | (Kersters et al., 1984) | Strictly birds | Causes respiratory disease in birds |
| ***B. pseudohinzii*** | (Ivanov et al., 2016 ; Perniss et al., 2018) | Laboratory-raised mice | Respiratory infections |
| ***B. holmesii*** | (Weyant et al., 1995) | Humans | Causes pertussis-like respiratory infections and septicemia in individuals with underlying health conditions. |
| ***B. hinzii*** | (Vandamme et al., 1995 ; (Hamidou Soumana et al., 2017b) | Poultry, rodents, humans | A commensal flora in poultry and recognised as an opportunistic pathogen in humans. |
| ***B. bronchialis*** | (P. A. Vandamme et al., 2015) | Humans | Cause respiratory infections in individuals with underlying conditions. |
| ***B. flabilis*** | | | |
| ***B. sputigena*** | | | |
| ***B. trematum*** | (y Castro et al., 2019) | Humans | An opportunistic pathogen that primarily infects wounds and is not linked to respiratory infections. |
| ***B. ansorpii*** | (Ko et al., 2005) | Humans | An opportunistic pathogen that primarily infects wounds and is not linked to respiratory infections. |
| ***B. petrii*** | (von Wintzingerode et al., 2001; Hamidou Soumana et al., 2017b) | Environmental (dechlorinating bioreactor) | First environmental *Bordetella* species |
| ***B. bronchiseptica*** | (Goodnow, 1980) (Hamidou Soumana et al., 2017) | Colonizes a variety of animals and even humans | Chronic respiratory infections in animals and respiratory infections in immunocompromised individuals. |
| ***B. parapertussis*** | (Linnemann, 1977b; Parkhill et al., 2003; Chen et al., 1988) | Humans, Sheep. | Mild whooping cough in humans and Ovine respiratory bordetellosis in sheep. |
| ***B. pertussis*** | (Melvin et al., 2014; Diavatopoulos et al., 2005) | Strictly human. | Severe whooping cough. |
| ***B. muralis*** | (Tazato et al., 2015) | Environmental species | Have not been shown to cause disease in humans or animals. |
| ***B. tumbae*** | | | |
| ***B. tumulicola*** | | | |

### 1.3.3. Genomic and Evolutionary Relationships within the Classical *Bordetella*

Historically, the classical *Bordetella* species were classified based primarily on host range and disease severity (Weiss, 2006). However, later genomic studies in the 21st century have shown that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are much more genetically similar than previously thought. Comparative genetic analyses show that they share a remarkably high degree of sequence identity and conservation indicating that they likely descended from a "*B. bronchiseptica*-like ancestor" through adaptive specialization. (Parkhill et al., 2003; Weiss, 2006). As a result, some researchers propose that these organisms are better classified as subspecies or host adapted strains rather than presenting them as distinct species (Gerlach et al., 2001; Weiss, 2006)..

The differences observed among these species, particularly in host specificity and virulence factor expression, have been largely attributed to **selective gene silencing** and **gene decay mechanisms** such as pseudogene formation, genome reduction, deletions and rearrangements rather than from extensive horizontal gene transfer (HGT) i.e. acquisition of large amounts of new genes (Parkhill et al., 2003; Weiss, 2006; Gerlach et al., 2001).

### 1.3.3.1. Selective Gene Silencing in *Bordetella* Evolution

Selective gene silencing refers to the phenomenon in which certain genes that are present in the genome, are no longer expressed, usually due to mutations in regulatory regions like promoters rather than complete loss of the gene itself. A prime example of selective gene silencing is in the differential expression of the pertussis toxin (PTX) operon across *Bordetella* species (Weiss, 2006). While all the three species have the PTX genes, only *B. pertussis* expresses the toxin. In *B. parapertussis* and *B. bronchiseptica*, PTX expression is silenced due to point mutations in their promoter region (Aricò & Rappuoli, 1987a; Gross & Rappuoli, 1988; Melvin et al., 2014). This specific gene activation *B. pertussis* is believed to have played a key role in its adaptation to the human host (Miguelena Chamorro et al., 2023).

Another example is the expression of flagellar genes, which occurs only in *B. bronchiseptica* but not in *B. pertussis* or *B. parapertussis*. This has been attributed to gene disruption by multiple **pseudogenes** and **Insertion Sequence Elements** (**ISEs**). Therefore, these species thrive in host environments in which motility is not essential for survival (Akerley & Miller, 1993; Leigh et al., 1993; Parkhill et al., 2003). Similarly, the urease gene is also expressed in most *B. parapertussis* and *B. bronchiseptica* strains, but not in *B. pertussis* due to point mutations in the upstream regulatory region which have led to its inactivation (McMillan et al., 1998). These examples demonstrate how transcriptional silencing facilitates host adaptation while preserving the core genomic architecture of the organisms.

### 1.3.3.2. Genome Decay in *Bordetella* Evolution

**Gene Decay** refers to the evolutionary process by which functional genes progressively accumulate inactivating mutations (e.g., nonsense mutations, frameshifts, or insertions) that over time can render them non-functional i.e. Pseudogenes or ultimately lead to their loss from the genome (Cummings et al., 2004; Ochman & Moran, 2001; Andersson & Andersson, 1999).

Genomic comparisons of the classical *Bordetella* species revealed a significantly higher number of pseudogenes i.e. 358 in *B. pertussis* and 200 in *B. parapertussis* compared to only 19 in *B. bronchiseptica*. The majority of these inactive genes are involved in functions such as transport, metabolism of small molecules, and surface structures. This extensive gene loss has significantly reduced the metabolic flexibility of *B. pertussis*, explaining its strict adaptation to the human respiratory tract, where such functions are less critical for survival (Parkhill et al., 2003; Mattoo & Cherry, 2005).

Additionally, genome decay can result from recombination between repetitive elements, such as Insertion Sequence Elements (ISEs), which cause genome rearrangements and gene deletions (Parkhill et al., 2003). Several regions like those coding for lipopolysaccharide O-antigen, capsule and type IV pilus present in *B. bronchiseptica* are frequently deleted or modified in the human-restricted species (Parkhill et al., 2003; Preston et al., 1999; Middendorf & Gross, 1999; Blay et al., 1997; Banemann et al., 1998).

Genome size comparisons also revealed that the genome of *B. pertussis* (approximately 4000kbp) is significantly smaller than that of *B. parapertussis* (approximately 4400kbp) and *B. bronchiseptica* (approximately 5000kbp). This observed reduction in genome size further reinforces this pattern of reductive evolution and indicates that the significant loss of genetic material over time played a key role in the niche specialisation of *B. pertussis* and *B. bronchiseptica* (Stibitz & Yang, 1997; Locht, 1999; Weiss, 2006; Parkhill et al., 2003).

Phylogenetic analyses have also identified two major lineages within *B. bronchiseptica* species i.e. *B. bronchiseptica* complex I (associated with animal infections) and complex IV (associated with human infections), based on their genetic diversity and host associations. Notably, Complex IV is genetically closer to *B. pertussis* while Complex I show greater similarity to *B. parapertussis* (Diavatopoulos et al., 2005). This observation, in conjunction with all the previously discussed evidence, supports the theory of a potential evolutionary trajectory from a *B. bronchiseptica*-like progenitor leading to *B. pertussis* and B. parapertussis (Diavatopoulos et al., 2005; Parkhill et al., 2003; Van Der Zee et al., 1997).

## 1.4. THE GENUS *BORRELIA*

### 1.4.1. Introduction to genus *Borrelia*

The genus *Borrelia* comprises a diverse group of spirochete bacteria that are primarily responsible for causing Lyme disease and relapsing fever (Margos et al., 2018). This genus named after the French biologist **Amédée Borrel,** was initially described by Swellengrebel in 1907, with *B. anserina* assigned as the type species (Skerman et al., 1989). Members of this genus are gram-negative, microaerophilic, and possess a unique genomic structure that includes a polyploid genome with one linear chromosome and multiple linear and circular plasmids. Their cell bodies are helical with regularly spaced coils and have periplasmic flagella for motility. They primarily ferment glucose and to a lesser extent, fructose and maltose (Barbour & Schwan, 2018). *Borrelia* species are primarily transmitted by arthropod vectors, including ticks and lice. Some species have adapted to survive in a wide range of hosts including mammals, birds, and reptiles, through immune evasion mechanisms such as antigenic variation (Radolf et al., 2012).

### 1.4.2. Classification of the Genus *Borrelia*

**Domain:** *Bacteria*

**Phylum:** *Spirochaetota* (formerly *Spirochaetes*)

**Class:** *Spirochaetia*
**Order:** *Spirochaetales*
**Family:** *Borrelia*ceae
**Genus:** *Borrelia* (https://lpsn.dsmz.de/genus/Borrelia; Parte et al., 2020)

### 1.4.3. Overview of Key *Borrelia* Species and Their Pathogenicity

Since the first *Borrelia* species, ***Borrelia recurrentis*,** was discovered (Barbour, 1986), numerous species and strains within the *Borrelia* genus have been identified and characterised. Members of this genus are now well recognized as the causative agents of major human diseases i.e. Lyme borreliosis (LB) and Relapsing Fever (RF).

Despite their genetic similarity, genospecies associated with LB and RF show significant differences in their clinical presentations, biological behavior, and epidemiological characteristics. As a result, they form independent monophyletic clades that likely evolved from a shared ancestor(Takano et al., 2010). In 2016, new *Borrelia* species were discovered in echidna ticks (*Bothriocroton concolor*) in Australia and Phylogenetic analysis revealed these species belong to neither the LB nor RF clades, and instead form unique lineages that exist as an outgroup and diverged recently from the RF group (Loh et al., 2017).

### 1.4.4. Relapsing Fever (RF) cluster

Relapsing Fever (RF) is a vector-borne bacterial infection caused by spirochetes from the *Borrelia* genus. It is categorised into **Louse-Borne Relapsing Fever (LBRF)**, spread by body lice and **Tick-Borne Relapsing Fever (TBRF)**, transmitted mainly by soft ticks. These pathogens have a wide range of vertebrate hosts affecting both birds and mammals, including humans (Faccini-Martínez et al., 2022).

The disease was first formally recognised by Dr. David Craigie, during the Edinburgh epidemic (1843–1848), coining the term "relapsing fever" to describe its hallmark febrile episodes (Warrell, 2019). Then following the 1868 relapsing fever outbreak in Berlin, Dr. Otto Obermeier identified *Borrelia spirochetes* in the blood of patients as causative agent of this fever. These spirochete species were initially named *Spirochaeta obermeieri,* which is now known as *Borrelia recurrentis* (Wright & Boyce, 2011).

Relapsing fever borreliosis is primarily characterised by recurring episodes of fever alternating with afebrile periods (Nakayima, 2023). Louse-borne relapsing fever infection often presents symptoms like rapid heartbeat (tachycardia), headache, muscle pain (myalgia), and joint pain (arthralgia). Rarely, enlarged liver and spleen (hepatosplenomegaly), nosebleeds (epistaxis), petechial skin rash, and jaundice, can also occur (Elbir et al., 2013). Tick-borne relapsing infection is often misdiagnosed as malaria, tropical fevers and other tick-related infections due to its non-specific symptoms, such as fever, chills, headache, nausea, and muscle pain. In severe cases, it can lead to neurological complications such as meningitis and encephalitis (Jakab et al., 2022). The severity of symptoms is independent of the patient's age or sex and the acquired immunity from prior infection is very short-lived with reinfections starting as soon as six months after recovery (Elbir et al., 2013). Relapsing fever

is also highly associated with an increased risk of maternal and perinatal deaths in pregnant women (Lambert, 2020).

#### 1.4.4.1. Louse Born Relapsing Fever

LBRF is a human-exclusive disease caused by the bacterium *Borrelia recurrentis* and is transmitted solely by a single vector, the human body lice (*Pediculus humanus humanus*), which depends entirely on human blood for feeding (Faccini-Martínez et al., 2022).

The role of the body louse in transmitting LBRF was first identified in 1907 by Nicolle and his team in Tunisia (Mackie, 1907). This finding was further confirmed in 1910 by Sergent and Foley in Algeria, who elaborated on the actual mechanism of louse-borne transmission of LBRF (Nakayima, 2023).

Historically, Louse-Borne Relapsing Fever has been one of the major epidemic diseases, particularly affecting Europe and North America until the early 20th century. It has long been a disease of hardship, spreading in times of war, famine, and poverty, thriving in overcrowded and unsanitary conditions where people are displaced by crises (Kahlig et al., 2021). By the 1940s, LBRF had largely faded from public health concerns due to the widespread use of insecticides like DDT significantly reduced louse infestations. However, it remained endemic in Ethiopia (Kahlig et al., 2021). Today, LBRF remains endemic in the Horn of Africa, with outbreaks reported in Somalia, South Sudan (Rumbek County), and specific districts in Peru, such as Chavin (Ancash Province) and Calca (Urubamba Valley). Additionally, the presence of *Borrelia recurrentis* in head lice among Congolese pygmies suggests there may be other unknown human reservoirs (Warrell, 2019).

Recent updates also indicate that LBRF has re-emerged in Europe, particularly among young male refugees from African nations such as Ethiopia, Eritrea, Somalia, and Libya, arriving in countries like Italy and Germany (Ciervo et al., 2016); (Antinori et al., 2016)(Warrell, 2019).

#### 1.4.4.2. Tick Born Relapsing Fever

Unlike Louse-Borne Relapsing Fever (LBRF), which is caused exclusively by *Borrelia recurrentis* and transmitted only by body lice, Tick-Borne Relapsing Fever (TBRF) is a zoonotic disease caused by several *Borrelia* species and transmitted to humans through tick bites, typically from animal reservoirs. The disease is found on every continent except Australia and Antarctica, with different *Borrelia* species endemic to specific regions where their tick vectors thrive, including Africa, the Americas, Asia, and Europe (Jakab et al., 2022).

The first documented case of a deadly tick-borne fever was reported in 1857 by **Dr. David Livingstone** in Angola. Later, in 1904, researchers Ross (working in Uganda) and **Dutton** (in the Congo) independently confirmed that this fever was caused by spirochetes transmitted by soft ticks bites (Dutton & Todd, 1905; Ross & Milne, 1904).

These spirochetes primarily cycle between soft ticks of the *Ornithodoros* genus and small animals, especially rodents, with various vertebrates acting as natural reservoirs. Humans are typically accidental hosts, except in cases of *Borrelia duttonii* in Africa, which appears to exclusively infect humans (Cutler, 2010). Over time, additional tick species have been identified as carriers of different *Borrelia* strains, broadening the known range of TBRF transmission (Jakab et al., 2022). For decades, TBRF was believed to be transmitted exclusively by soft ticks. However, this view changed in 2011 with the discovery that *Borrelia miyamotoi* could also be transmitted by hard ticks of the *Ixodes* genus (Talagrand-Reboul et al., 2018); (FUKUNAGA et al., 1995).

TBRF remains an important public health concern in certain regions, particularly in Africa accounting for approximately 13 % of febrile illnesses in West Africa while in East Africa, it has one of the highest fatality rates among children (Talbert et al., 1998).

***Table II:*** *Borrelia Species responsible for Tick-Borne Relapsing Fever (TBRF), their Vectors, and Reservoirs.*

| Species | Arthropod Vectors | Reservoirs |
|---|---|---|
| ***B. crocidurae*** | *O. erraticus, O. sonrai* | Mammals |
| ***B. duttonii*** | *O. moubata complex* | |
| ***B. hermsii*** | *O. hermsi* | |
| ***B. hispanica*** | *O. erraticus* | |
| ***B. miyamotoi*** | *Ixodes species (hard ticks)* | |
| ***B. parkeri*** | *O. parkeri* | |
| ***B. persica*** | *O. tholozani* | |
| ***Candidatus B. kalaharica*** | *O. savignyi* | |
| ***B. latyschewii*** | *O. tartakovskyi* | |
| ***B. mazzottii*** | *O. talaje* | |
| ***B. venezuelensis*** | *O. rudis* | |
| ***B. turicatae*** | *O. turicata* | Rodents; wild & domesticated pigs |
| ***B. baltazardii*** | *Unknown* | Unknown |
| ***B. brasiliensis*** | *O. brasiliensis* | |
| ***B. caucasica*** | *O. asperus* | Possibly rodents |
| ***B. dugesii*** | *O. dugesii (O. talaje)* | Not known |
| ***B. graingeri*** | *Possible bats* | Possible bats |
| ***B. harveyi*** | *Possible monkeys* | Possible monkeys |

Reference : (Barbour & Schwan, 2018) modified

### 1.4.5. Lyme Disease Cluster

Lyme disease, also known as Lyme borreliosis, is a complex tick-borne infection caused by *Borrelia* species, primarily ***Borrelia burgdorferi*** (Bamm et al., 2019).

The first recognized cases of Lyme disease were reported in the late 19th century in Europe (Weber, 2001), but its causative agent remained unknown until the 1980s, when researchers confirmed *Borrelia burgdorferi* as the pathogen responsible (Barbour & Benach, 2019).

These bacteria are primarily vectored by hard ticks of the *Ixodidae* family, particularly those within the *Ixodes ricinus* complex including the *Ixodes ricinus* (Europe), *Ixodes persulcatus* (Europe and Asia), *Ixodes pacificus* and *Ixodes scapularis* (North America) (Rudenko et al., 2011). While all three life stages of *Ixodes* ticks can transmit the bacteria, nymphs are the most common source of human infections due to their small size and high activity (Kurtenbach et al., 1998). The global persistence of Lyme disease has been sustained by its wildlife reservoirs, particularly rodents and birds, which harbour *Borrelia* asymptomatically, enabling continuous transmission cycles (Halsey et al., 2018; Mannelli et al., 2012).

Clinically, Lyme borreliosis typically presents with an erythema migrans (EM) rash, also known as a bull's-eye rash, alongside flu-like symptoms such as fever, fatigue, muscle aches, and headaches. If left untreated, the infection can spread to various organs and systems including the skin, joints, heart, nervous system, endocrine glands, and gastrointestinal tract, leading to complications such as arthritis, neurological disorders (e.g., facial palsy, meningitis), and heart problems (Lyme carditis) (Wills et al., 2018).

### 1.4.6. Taxonomic Delineation of *B. burgdorferi* Strains

*Borrelia burgdorferi* was first documented by Willy Burgdorfer in 1981 as the first known cause of Lyme disease. While investigating ticks for *Rickettsia*, he unexpectedly observed these spirochetes in *Ixodes dammini* (now *Ixodes scapularis)* and suspected a link to Erythema Chronicum Migrans (ECM). Further studies confirmed this link by detecting these bacteria in ticks from Lyme-disease endemic regions and by also finding corresponding specific antibodies in the blood of infected individuals (Burgdorfer, 1993). By 1984, this spirochete bacteria was officially named *Borrelia burgdorferi* and classified as a new species within the *Borrelia* genus (Johnson et al., 1984).

As research progressed, researchers isolated additional Lyme-associated *Borrelia* strains from ticks from other parts of the world e.g. Europe and Asia. Scientists initially assumed these strains were identical to the North American *B. burgdorferi*. However, molecular analyses revealed notable genetic and phenotypic differences between the European/Asian isolates and the North American ones. This led to the establishment of the ***Borrelia burgdorferi sensu lato* (s.l.) complex,** which is a group of genetically distinct, but closely related *Borrelia* species that cause Lyme borreliosis. Despite their genetic diversity, members of this complex are morphologically similar and belong to a single evolutionary lineage (Wang et al., 1999). Within this complex, the term ***Borrelia burgdorferi sensu stricto*** was specifically designated to the first species isolated by Burgdorfer et al. (1982) in North America, from *Ixodes dammini*, now reclassified as *Ixodes scapularis.* And *Borrelia burgdorferi sensu lato* was designated to all the genetically distinct isolates from the other different parts of the world,

as shown in **Table III:** Currently Known Spirochete Species in the *Borrelia burgdorferi Sensu Lato* Complex. (Baranton et al., 1992; Oliver et al., 1993).

The *B. burgdorferi sensu lato* complex is genetically diverse and includes at least 18 recognized species, with additional unnamed variants such as "genomospecies 2" proposed by Postic et al. (2007). The total number continues to evolve with ongoing research (Rudenko et al., 2011d).

### 1.4.7. Evolutionary Relationships within the *Borrelia* species of interest

For our study, we focused on two groups of borrelia species within the *Borrelia burgdorferi* sensu lato complex, a group of spirochetes responsible for Lyme borreliosis i.e. a group of *Borrelia garinii* and *Borrelia bavariensis* and a group of *Borrelia bissettii*, *Borrelia carolinensis*, and *Borrelia kurtenbachii* (Rudenko et al., 2011; Baranton et al., 1992).

#### 1.4.7.1. Relationship Between Borrelia garinii and Borrelia bavariensis

*Borrelia garinii* and *Borrelia bavariensis* are two closely related species within the *Borrelia burgdorferi sensu lato* complex, a group of spirochetes responsible for Lyme (Baranton et al., 1992). Initially, several strains that are now assigned to *B. bavariensis* were grouped under *B. garinii*. However, subsequent molecular studies led to their reclassification as a distinct species.

Early classification efforts relied on outer surface protein A (OspA) typing, which grouped *B. garinii* strains into multiple serotypes. OspA serotypes 3, 5, 6, and 7 were typically bird-associated and widely distributed geographically (Hanincová et al., 2003; Kurtenbach et al., 1998) while serotype 4 was mainly associated with rodents, especially *Apodemus* mice in Europe (Hu et al., 2022; Huegli et al., 2002)

With the advent of more robust molecular tools, i.e. Multilocus Sequence Analysis (MLSA) provided new insights. MLSA is a molecular technique that analyzes sequences of several housekeeping genes (genes essential for basic cellular functions), to differentiate and classify bacterial species (Glaeser & Kämpfer, 2015; Joshi et al., 2022).

By analysing conserved housekeeping genes, outer surface proteins, and the 5S–23S rRNA intergenic spacer, MLSA revealed significant genetic divergence between bird and rodent-associated strains. This ultimately supported the elevation of OspA serotype 4 to species level, resulting in the designation of *Borrelia bavariensis* sp. nov. (Margos et al., 2009).

While MLSA is a practical tool particularly in the absence of whole-genome data, it has limitations. It samples only a small fraction of the genome, which means certain cases of species divergence (e.g., due to genomic rearrangements, horizontal gene transfer, or variations outside the selected genes) may be missed. Moreover, MLSA also lacks a universal threshold for species delineation, mainly because the choice of the analysed housekeeping genes can vary across studies.(Hu et al., 2022; Jain et al., 1999; Liu et al., 2017)

More comprehensive approaches such as Average Nucleotide Identity (ANI) and digital DNA–DNA hybridization (dDDH) have since become the gold standards for species delineation. These methods assess genome-wide similarity, offering higher resolution than gene-based techniques like MLSA.

In light of these developments, our study revisits the taxonomic conclusions of Margos et al. (2009) using updated phylogenomic tools to evaluate the current placement of *B. bavariensis*.

#### 1.4.7.2. Relationship Between B. bissettiae, B. carolinensis and B. kurtenbachii

The earliest delineation among these three species dates back to 1998, when *Borrelia bissettii* was identified as a distinct genospecies. This classification was based on restriction fragment patterns and sequences of the rrf-rrl intergenic spacer, as well as 16S rDNA sequences. The species was primarily associated with *Ixodes* ticks particularly *I. pacificus* and *I. neotomae* (now *I. spinipalpis*) and rodent hosts in California (Bissett & Hill, 1987; Brown & Lane, 1992; Postic et al., 2007).

Among the isolates included in *B. bissettii* was strain 25015. However, in 2010, Multilocus Sequence Analysis (MLSA) revealed that this strain was phylogenetically distinct from other *B. bissettii* strains. This led to its reclassification as a new species, *Borrelia kurtenbachii* sp. nov. (Lin et al., 2003; Mathiesen et al., 1997; Postic et al., 2007).

In a separate development, a novel genospecies was isolated in 2009 from rodents such as *Peromyscus gossypinus* and *Neotoma floridana* in the southeastern United States. While these isolates exhibited some genetic similarity to *B. bissettii* and *B. andersonii*, phylogenetic analysis based on 16S rRNA, *ospA*, and the rrf-rrl intergenic spacer region demonstrated sufficient divergence to warrant classification as a new species: *Borrelia carolinensis* sp. nov. (Rudenko et al., 2009, 2011).

Our current study revisits the taxonomic relationships among *B. bissettiae*, *B. kurtenbachii*, and *B. carolinensis* using more comprehensive whole-genome approaches. In particular, we apply Average Nucleotide Identity (ANI) and digital DNA–DNA hybridization (dDDH), which are now widely accepted as the gold standards for bacterial species delineation due to their ability to capture overall genomic similarity with high resolution.

***Table III:*** *Currently Known Spirochete Species in the Borrelia burgdorferi Sensu Lato Complex.*

| ***Borrelia* species** | **Vector** | **Hosts/reservoirs** | **Geographical distribution** | **Reference** |
|---|---|---|---|---|
| ***B. afzelii*** | *I. ricinus, I. persulcatus* | Rodents | Asia, Europe | Canica et al. (1993) |
| ***B. americana*** | *I. pacificus, I. minor* | Birds | United States | Rudenko et al. (2000c) |
| ***B. andersonii*** | *I. dentatus* | Cotton tail rabbit | United States | Marconi et al. (1995) |
| ***B. bavariensis*** | *I. ricinus* | Rodents | Europe | Margos et al. (2009) |
| ***B. bissettiiae*** | *I. pacificus, I. minor* | Rodents | Europe, United States | Postic et al. (1998) |
| ***B. burgdorferi sensu stricto*** | *I. ricinus, I. scapularis, I. pacificus* | Rodents, birds, lizards, big mammals | Europe, United States | Baranton et al. (1992) |
| ***B. californiensis*** | *I. pacificus, I. jellisonii, I. spinipalpis* | Kangaroo rat, mule deer | United States | Postic et al. (2007) |
| ***B. carolinensis*** | *I. minor* | Rodents, birds | United States | Rudenko et al. (2009) |
| ***B. garinii*** | *I. ricinus, I. persulcatus, I. hexagonus, I. nipponensis* | Birds, lizards, rodents | Asia, Europe | Baranton et al. (1992) |
| ***B. japonica*** | *I. ovatus* | Rodents | Japan | Kawabata et al. (1993) |
| ***B. kurtenbachii*** | *I. scapularis* | Rodents | Europe, United States | Margos et al. (2010) |
| ***B. lusitaniae*** | *I. ricinus* | Rodents, lizards | Europe, North Africa | Le Fleche et al. (1997) |
| ***B. sinica*** | *I. ovatus* | Rodents | China | Masuzawa et al. (2001) |
| ***B. tanukii*** | *I. tanuki* | Unknown (possibly dogs and cats) | Japan | Fukunaga et al. (1996) |
| ***B. turdi*** | *I. turdus* | Birds | Japan | Fukunaga et al. (1996) |
| ***B. spielmanii*** | *I. ricinus* | Rodents | Europe | Richter et al. (2006) |
| ***B. valaisiana*** | *I. ricinus, I. granulatus* | Birds, lizards | Asia, Europe | Wang et al. (1997) |
| ***B. yangtze*** | *Haemaphysalis longicornis, I. granulatus* | Rodents | China | Chu et al. (2008) |
| ***Genomospecies 2*** | *I. pacificus* | Unknown | United States | Postic et al. (2007) |

*CHAPTER 2:*

# MATERIAL AND METHODS

# 2. MATERIAL AND METHODS

## 2.1. Objective of the study

This study aims to conduct a comprehensive taxonomic reassessment of selected pathogenic species from the genera *Bordetella* and *Borrelia* by leveraging modern genomic tools such as phylogenomic analyses, overall genomic relatedness indices (OGRIs), and core-genome phylogenomic analyses.

The primary goal is to refine and clarify the existing taxonomic ambiguities surrounding these selected pathogenic species through a polyphasic taxonomic approach.

Within the genus *Bordetella*, our study focuses on reviewing taxonomic relationship of *B. pertussis*, *B. parapertussis and B. bronchiseptica*. While in genus *Borrelia* we focused on *B. bavariensis, B. garinii, B. bissettiae, B. carolinensis, B. kurtenbachii.*

We gathered genomic and phenotypic data for our target genera from trusted databases i.e., LPSN, NCBI, and BacDive. Then following a polyphasic taxonomic approach, we performed a comprehensive data analysis on our obtained datasets which included Phylogenetic analysis, Phylogenomic analysis, Core genome analyses and Phenotypic data analysis. Visualization tools were also employed throughout to enhance interpretation and clearly communicate the results.

This analysis workflow allows for a thorough assessment of the evolutionary relationships and genomic characteristics of the studied strains which helps resolve existing taxonomic ambiguities. This, in turn, facilitates more accurate identification of pathogenic species which is essential for clinical diagnostics, epidemiology, and public health.

## 2.2. Methods

### 2.2.1. Preliminary Taxonomic Analysis

The identification of species with taxonomical ambiguities in the genus *Bordetella* was mainly based on a thorough review of existing scientific literature. This review consistently emphasized the close evolutionary relationships among the classical *Bordetella* species, which led to their selection for our analysis.

For the genus *Borrelia*, species were mainly selected based on an initial analysis performed through the Type (Strain) Genome Server (TYGS), utilizing genome sequences obtained from the List of Prokaryotic Names with Standing in Nomenclature (LPSN) database.

This TYGS analysis generated two phylogenetic trees. In these trees, each strain is normally marked with a unique colour in the metadata cluster columns. However, in our results, some strains shared the same colour in the species cluster column but displayed distinct colours in the subspecies cluster column.

This pattern suggests that these strains may represent different subspecies within the same species rather than separate species. Based on this finding, we selected specific groups for detailed analysis,

including *B. garinii* with *B. bavariensis*, and a cluster comprising *B. bissettiae*, *B. carolinensis*, and *B. kurtenbachii*.

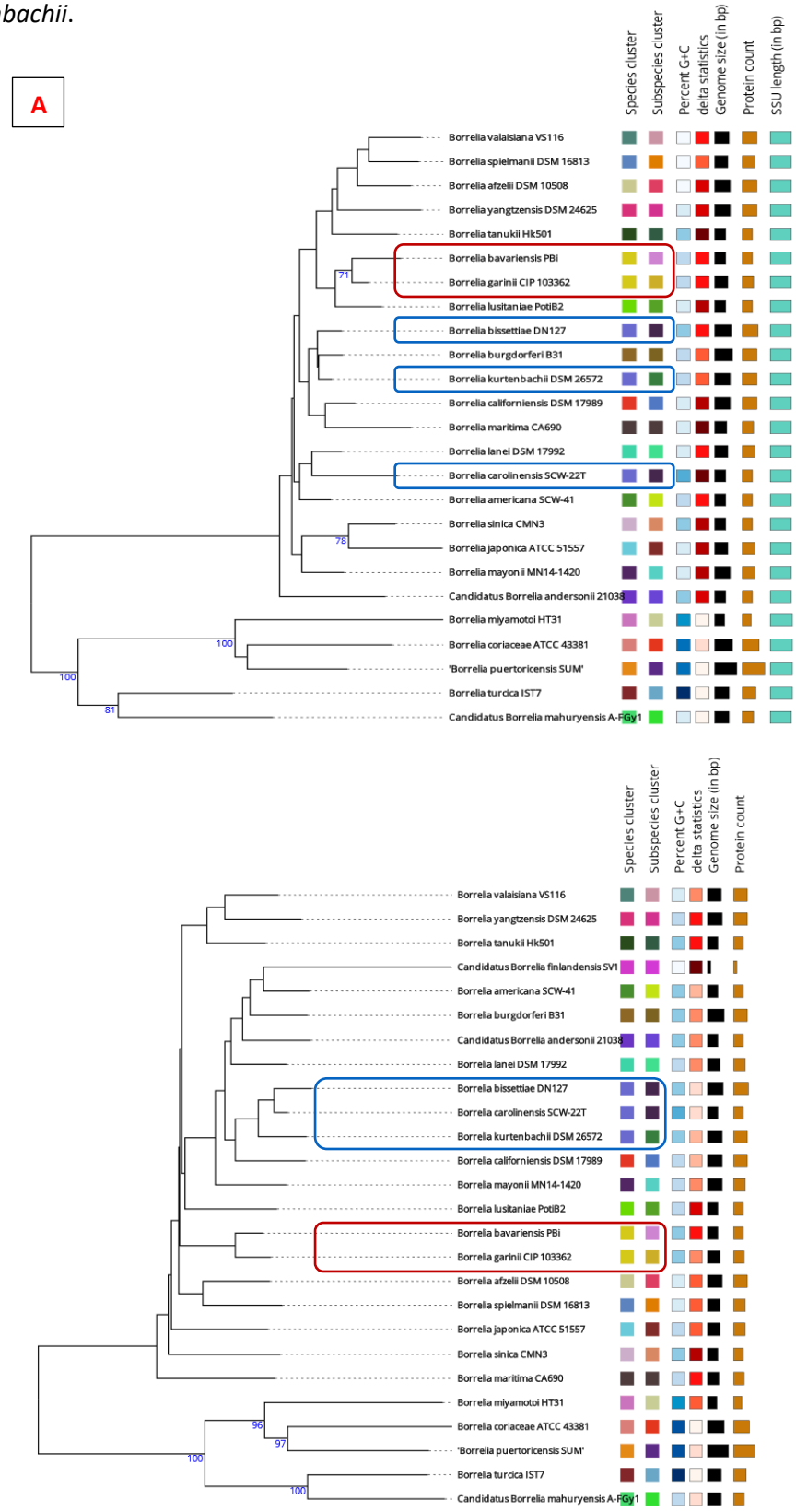


*Figure 1:* ***Preliminary Taxonomic Analysis:*** *(A) Phylogenetic tree based on the 16S rRNA. (B) Phylogenetic tree based on Whole genome. The red and blue rectangles correspond to the two groups of the Borrelia species chosen for our analysis.*

## 2.2.2. Selection and Download of *Bordetella* and *Borrelia* Genome Sequences

### 2.2.2.1. Introduction of the NCBI Genome Database

**Principle:** The datasets used was downloaded from the NCBI Genome Database. The NCBI Genome Database is a comprehensive collection of genomic data maintained by the National Centre for Biotechnology Information (NCBI), part of the National Library of Medicine (NLM) at the National Institutes of Health (NIH). It is a vital resource for researchers serving as a central repository for genome sequences from various organisms, including bacteria, viruses, fungi, plants, animals, and humans. This platform can be accessed via https://www.ncbi.nlm.nih.gov/genome/ on the NCBI platform.

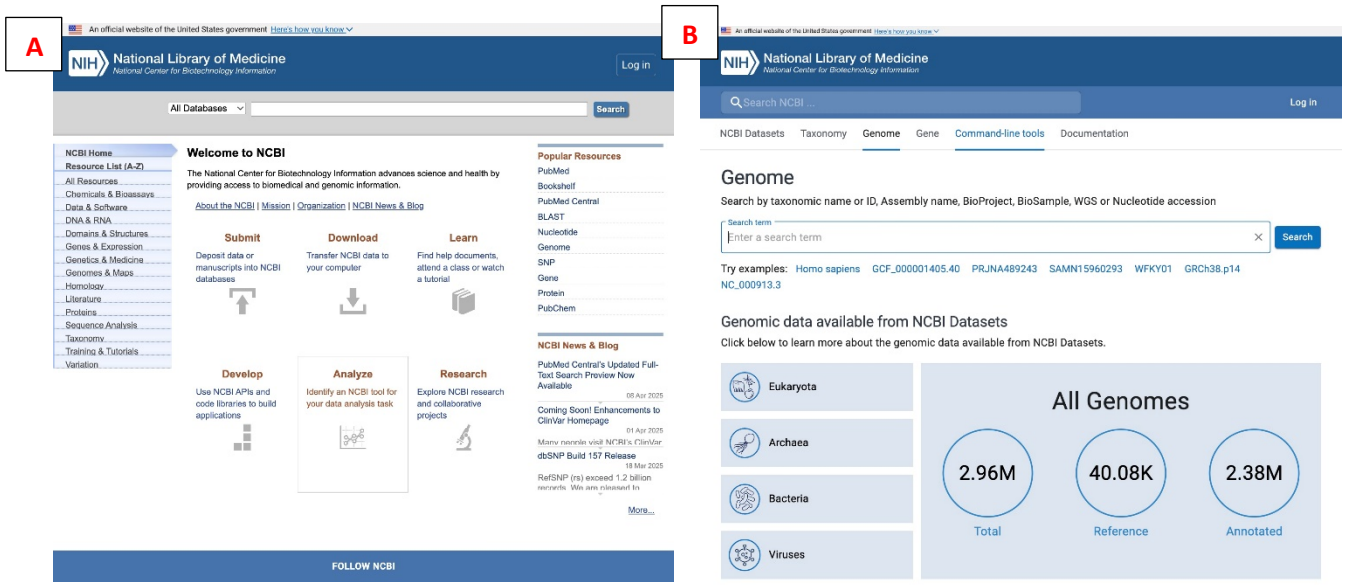


*Figure 2:* ***The NCBI Platform.*** *(A) Homepage of the NCBI platform showing the various tools and services including data submission, downloads, analysis tools, research access, and educational resources. (B) The NCBI genome database interface: It allows users to search for and access genomic data using taxonomic names, accession numbers, and more. It highlights genome statistics and provides categorized access to genomic data from viruses, bacteria, archaea, and eukaryotes through the NCBI Datasets platform.*

### 2.2.2.2. Selection and Download of Bordetella Genome Sequences

A total of 22 genome sequences were analysed, including 20 *Bordetella* genomes (11 type material genomes and 09 additional genomes) and 2 outgroup sequences.

The selection process of the type material genomes began by querying the NCBI genome database for all available *Bordetella* sequences, which retrieved 2392 genomes. This dataset was then filtered to include only type material and exclude atypical genomes, narrowing the dataset to 26 sequences. From these, 11 genomes representing distinct *Bordetella* species were selected. This dataset included all available reference genomes (8) along with other 3 strains identified as type strains in their respective species as per the LPSN database (https://lpsn.dsmz.de/genus/*Bordetella*).

The 09 additional genomes consisted of 03 genome sequences from each of the three *Bordetella* species under investigation i.e. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. These were obtained by querying the NCBI Genome Database for all available sequences of each species. Then among the results, reference genomes not classified as type material were prioritized. The

remaining genomes were chosen by sorting the sequences based on CheckM completeness (Parks et al., 2015), selecting those with the highest completeness and low contamination scores that were also not from type material.

For the outgroup, two species were selected i.e. *Achromobacter xylosoxidans* and *Bacillus subtilis*. The genome sequences chosen from NCBI were *Achromobacter xylosoxidans* NCTC10807 and *Bacillus subtilis* NCIB 3610, both of which represent the type strains of their respective type species as per the LPSN (https://lpsn.dsmz.de/species/achromobacter-xylosoxidans and https://lpsn.dsmz.de/species/bacillus-subtilis).

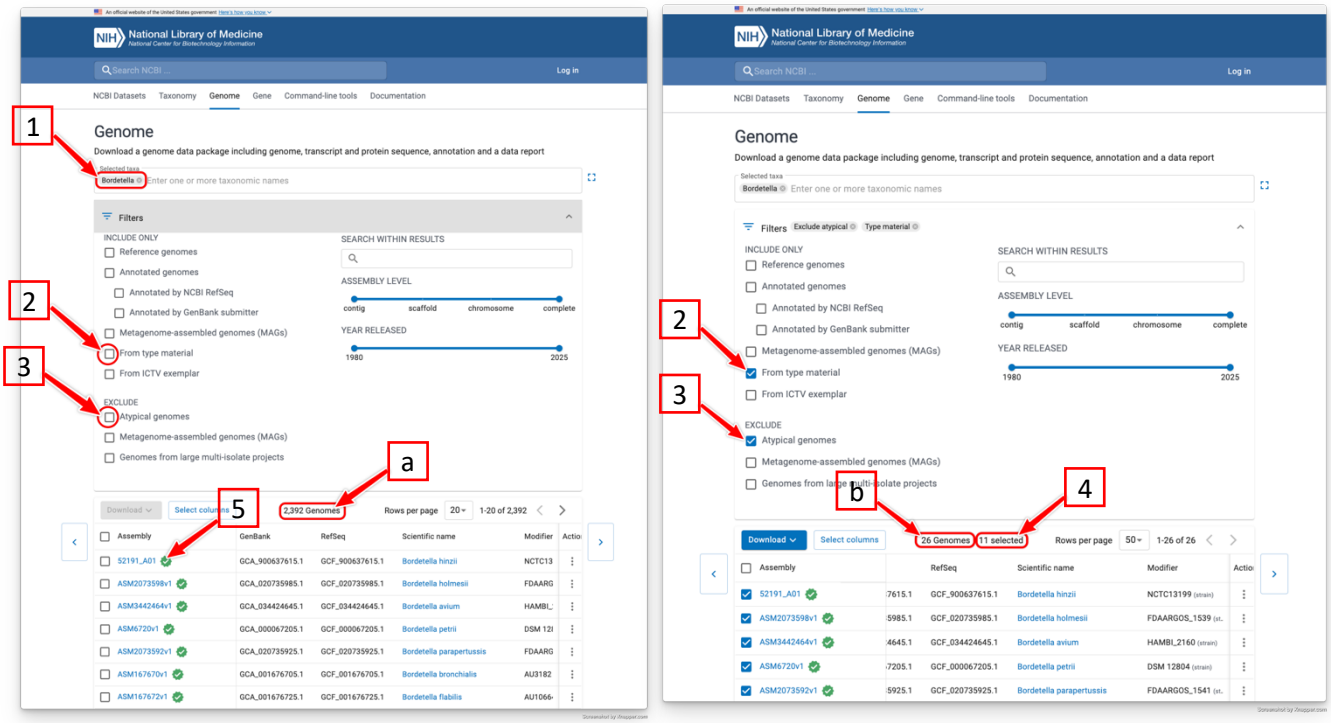


*Figure 3:* ***Search and Selection of Bordetella type material genome sequences.*** *(1) Searching for Genus Bordetella returned (a) 2392 genomes. Filtering for type material (2) and excluding atypical genomes (3) returned (b) 26 genomes from which 11 genomes of distinct species were selected (4). Green tick indicating reference genomes (5).*

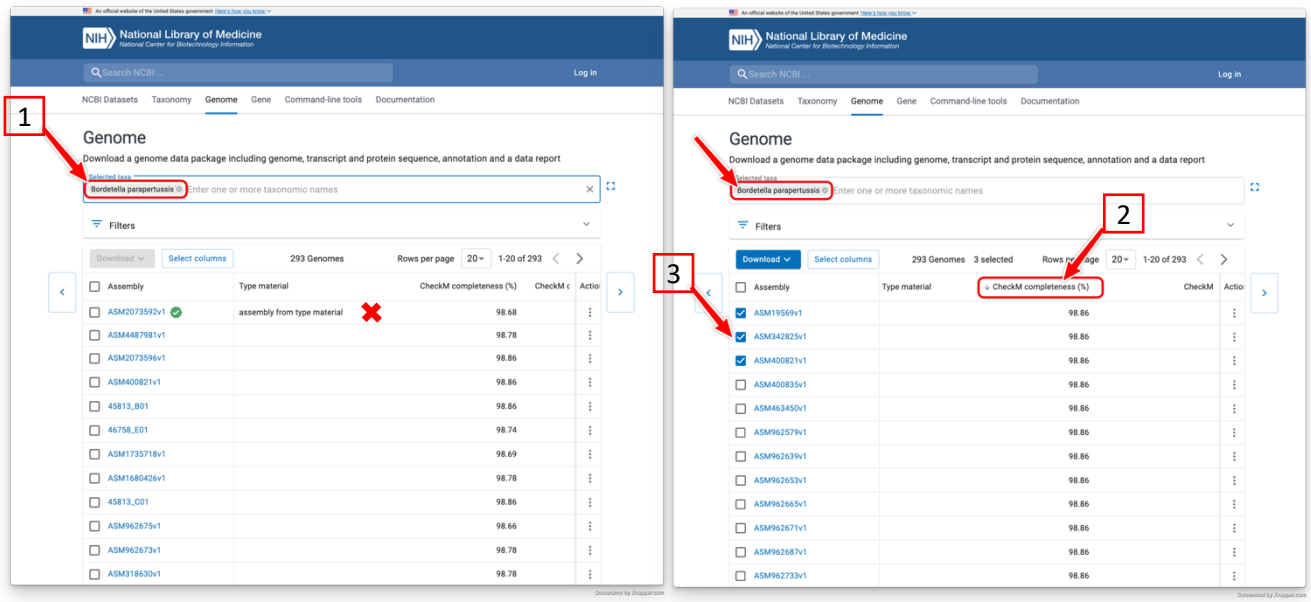

*Figure 4:* ***Search and Selection of Additional Genome sequences.*** *(1) Genome sequences for each of the species with taxonomic ambiguities were searched individually e.g., Bordetella parapertussis. (2) The resulting genomes were then sorted based on their CheckM completeness and contamination scores (Parks et al., 2015), and the top three genomes were selected (3). In this selection, the reference genome was excluded as it originated from type-material.*

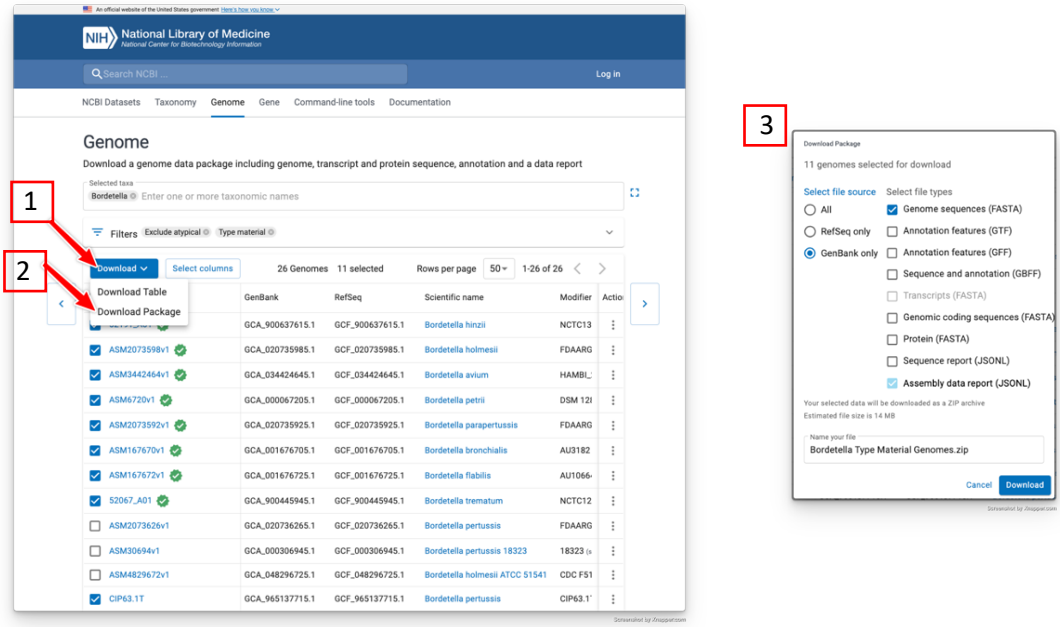


*Figure 5:* ***Downloading of the Datasets****. (1) The selected genome sequences were downloaded by clicking the Download button, the Download Package option was selected (2), GenBank only was chosen as the database selected to download, then followed by clicking Download to complete the process (3). The data was assembled into a Error! Reference source not found..*

### 2.2.2.3. Selection and Download of *Borrelia* Genome Sequences

A total of 35 genome sequences were analysed, including 34 *Borrelia* genomes (all type strains) and one outgroup. The selection and downloading procedure followed the same steps previously described for *Bordetella*.

## 2.2.3. Phylogenetic Analysis

### 2.2.3.1. Analysis of the 16S rRNA

#### 2.2.3.1.1 *Bordetella* 16S rRNA Sequence Similarity Computation using Ez BioCloud

**Principle:** EzBioCloud is a genome-based platform specifically designed for microbiome identification and discovery of *Bacteria* and *Archaea*. It provides a curated database of 16S rRNA gene sequences and whole-genome assemblies of type strains, allowing researchers to perform species identification, pairwise sequence alignments, phylogenetic analysis, and genome-based classifications. It is accessible at (https://www.ezbiocloud.net/) (Chalita et al., 2024; Yoon et al., 2017).

**Process:** In our study, we calculated the percentage similarities among the 16S rRNA sequences of *Bordetella pertussis* ATCC 9797$^T$, *Bordetella parapertussis* ATCC 15311$^T$, and *Bordetella bronchiseptica* NBRC 13691$^T$ in our *Bordetella* dataset, using the Pairwise Sequence Alignment Tool (https://www.ezbiocloud.net/tools/pairAlign) available on the EzBioCloud online platform.

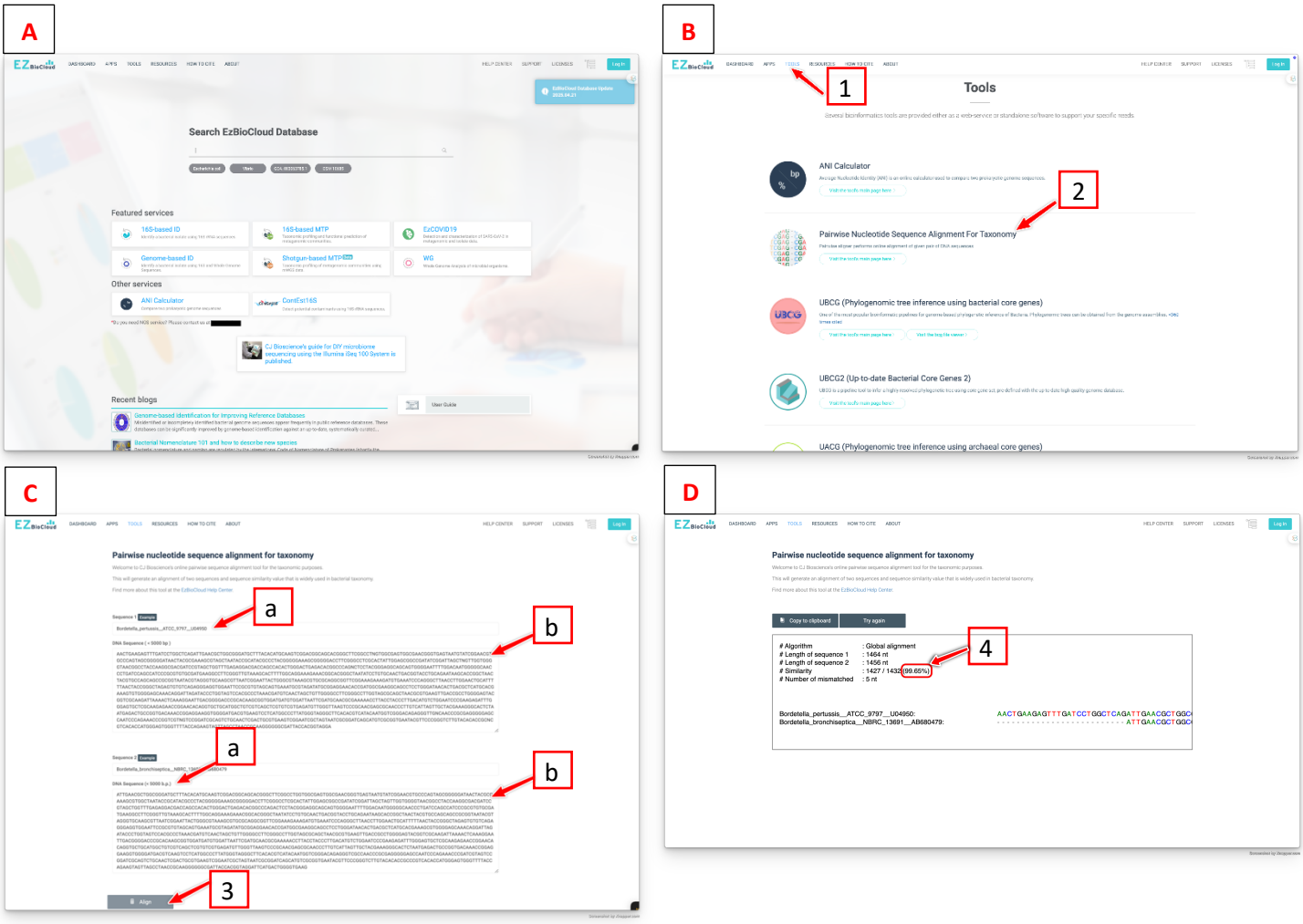


*Figure 6:* ***Calculation of Bordetella 16S rRNA Similarity on EzBioCloud. (A)*** *Homepage of the EzBioCloud platform showing the various tools and services.* ***(B)*** *For the 16S rRNA sequence similarity analysis, the "Tools" tab is selected (1), followed by the "Pairwise Sequence Alignment Tool" (2). The Sequence names (a) and their corresponding DNA sequences (b) are then entered into their input fields, after which the alignment is performed (3), and the percentage similarity between the sequences is displayed (4).* .

#### 2.2.3.1.2 *Borrelia* 16S rRNA Sequence Similarity Computation using Ez BioCloud

The percentage similarities of the 16S rRNA sequences among the two *Borrelia* strain groups with taxonomic ambiguities i.e. among *B. bavariensis* and *B. garinii*, as well as *B. bissettiae*, *B. carolinensis*, and *B. kurtenbachii,* were calculated using the same procedure as previously applied to *Bordetella*.

#### 2.2.3.1.3 *Bordetella* 16S rRNA Phylogeny using MEGA

**Principle: MEGA** (Molecular Evolutionary Genetics Analysis) is a software application designed for conducting statistical analysis of molecular evolution, constructing phylogenetic trees, and analysing molecular data, such as DNA or protein sequences (Kumar et al., 2016).

It offers several key tools and functionalities for exploring the genetic relationships between species and conducting phylogenetic analyses. Its key features include: Sequence Alignment by tools like ClustalW (J. D. Thompson et al., 1994) and MUSCLE (Edgar, 2004) which are essential for identifying conserved regions across different sequences of DNA, RNA, and protein sequences. It also supports evolutionary distance calculation using different methods (like Jukes-Cantor, Kimura, or P-distance) and Phylogenetic Tree Construction through methods like the Neighbor-Joining (Saitou & Nei, 1987), Maximum Parsimony (Nei & Kumar, 2000), Maximum Likelihood (Tamura et al., 2004) and Bayesian Inference (Zhang & Matsen IV, 2024), among other features.

The construction of phylogenetic trees typically follows four main steps. Firstly, the homologous DNA or protein sequences to be analysed must be selected and gathered from the public database. These sequences are then aligned to ensure proper comparison, and a phylogenetic tree is inferred from the aligned sequences using appropriate computational methods. Finally, the trees are presented in a way that effectively communicates the evolutionary relationships among the sequences studied (Hall, 2013).

**i. Selection and Collection of the 16s RNA sequences**

In our study, the MEGA7 version was employed to analyse the 16S rRNA gene sequences of the *Bordetella* species and *Achromobacter xylosoxidans* NCTC10807$^{T}$ as the outgroup. Their 16S rRNA gene sequences were downloaded as FASTA files from the LPSN (List of Prokaryotic names with Standing in Nomenclature) platform https://lpsn.dsmz.de/.

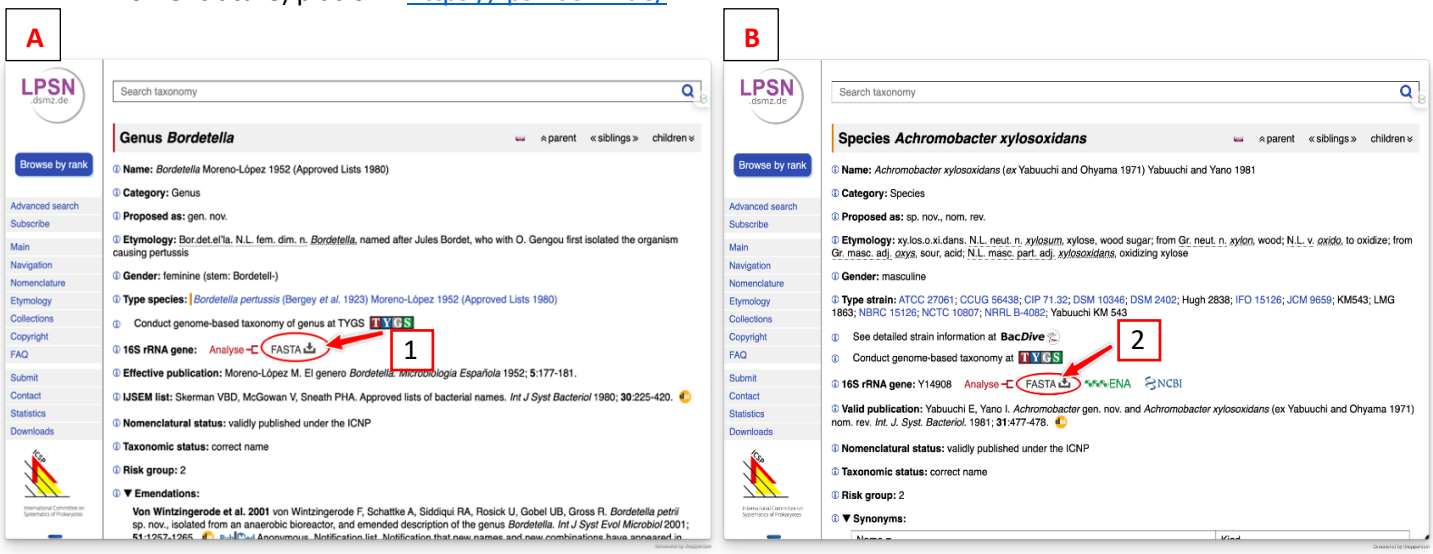

*Figure 7:* ***Selection and Collection of the 16S rRNA sequences.*** *(A) Downloading of FASTA files of the Bordetella species (1) and (B) Achromobacter xylosoxidans* NCTC10807$^{T}$ *from the LPSN platform (2).*

### ii. 16S RNA sequence Alignment

In our study, we conducted a multiple sequence alignment of the 16S rRNA sequences from both downloaded FASTA files using the **MUSCLE algorithm** (Edgar, 2004) within the MEGA software. The resulting alignment session was used in the subsequent phylogenetic tree reconstruction.

The MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm aligns the 16S rRNA sequences through an iterative three-step process. Initially, it performs a rapid draft alignment based on k-mer similarities between the sequences followed by the creation of a rough guide tree using UPGMA clustering (P. H. A. Sneath & Sokal, 1973). Sequences are then progressively aligned according to the initial tree. Then, an improved progressive alignment, in which the initial alignment is used to calculate more accurate pairwise distances based on the Kimura model, is performed (Kimura, 1985). This alignment is used to reconstruct a refined guide tree and then the sequences are selectively realigned based on where tree topology differs from the initial tree. Finally, this refined guide tree is iteratively partitioned at selected edges, and the resulting sequence profiles are realigned retaining only those modifications to make the overall alignment better (overall sum-of-pairs (SP) score). Through this approach, MUSCLE is able to manage large datasets while minimizing gaps and misalignments (Edgar, 2004).

*Figure 8: **16S rRNA sequence Alignment in MEGA**. (A) The dataset containing 16S rRNA sequences of Bordetella and Achromobacter xylosoxidans is first uploaded into the MEGA application (1), where the sequences initially appear unaligned(a). (B) All the entries are then selected, and the MUSCLE algorithm (2) is employed to perform a multiple DNA sequence alignment (3). (C) Following the alignment process, the sequences are displayed in a properly aligned format, ready for further phylogenetic or comparative analysis (4).*

### iii. Phylogenetic analysis and Phylogenetic Tree reconstruction

Following the 16S rRNA sequence alignment, a phylogenetic analysis is performed in which the MEGA computes **pairwise genetic distances** between every pair of aligned sequences using various mathematical models of evolution such as the Jukes-Cantor model, Kimura 2-parameter model and P-distance.

These genetic distances are organised into a distance matrix which then serves as the basis for constructing phylogenetic trees using methods like Neighbor-Joining, Maximum likelihood and Maximum Parsimony Method.

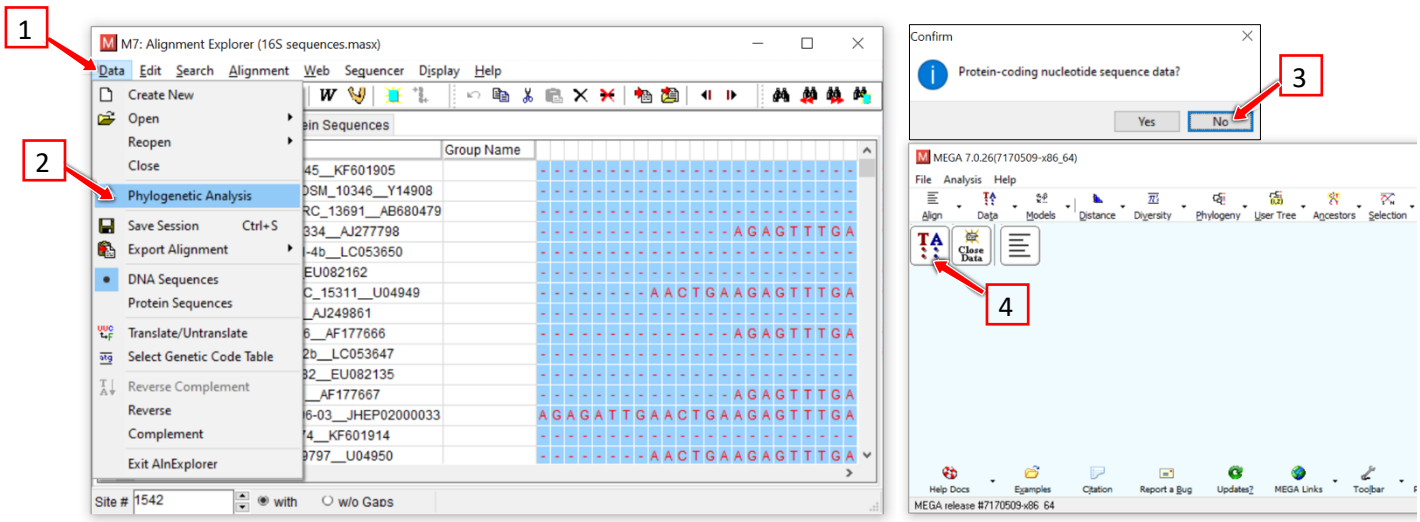


*Figure 9: **Performing the Phylogenetic Analysis in MEGA**. This is initiated by selecting the Data tab (1) and choosing Phylogenetic Analysis from the dropdown menu (2). In the confirmation pop-up window, the "No" option is chosen, indicating that the sequences are not protein-coding (3). The analysed data is then loaded into the main analysis interface (4) for subsequent evolutionary tree reconstruction.*

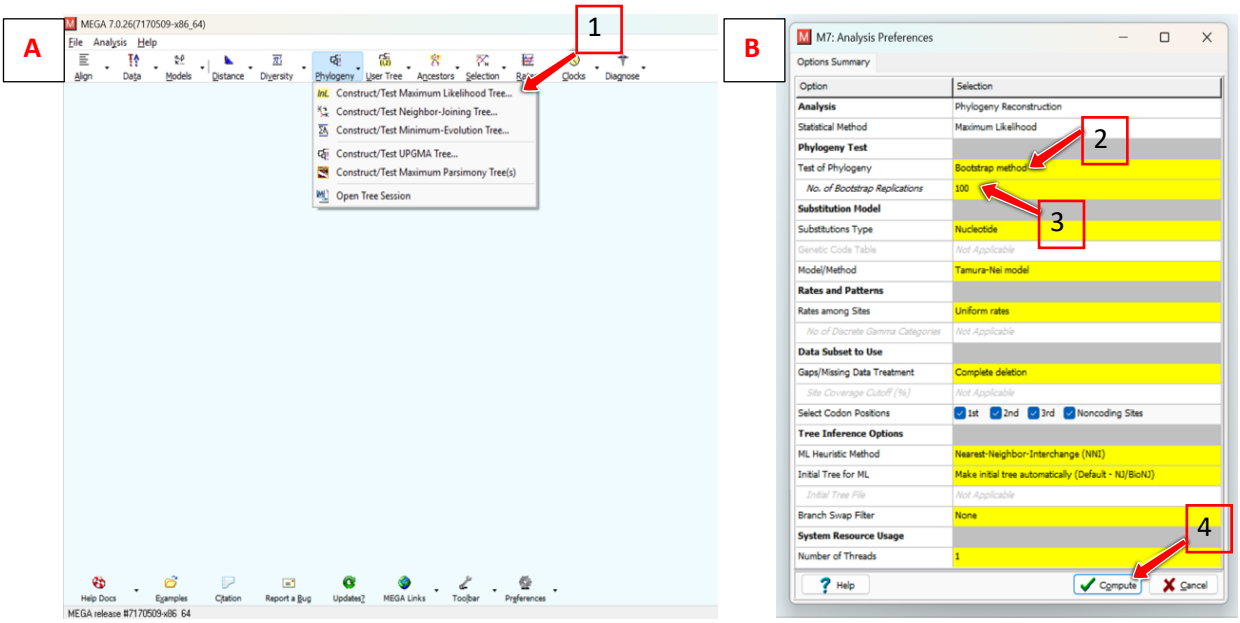


*Figure 10:* ***Construction of the Maximum likelihood Phylogenetic tree****. (A) Under the Phylogeny tab, the Maximum Likelihood Method is selected as the approach for tree construction. (B) Subsequently, the Bootstrap option is enabled (2), and the number of replications is set to 100 to evaluate the robustness of the tree branches (3) and the tree is computed.*

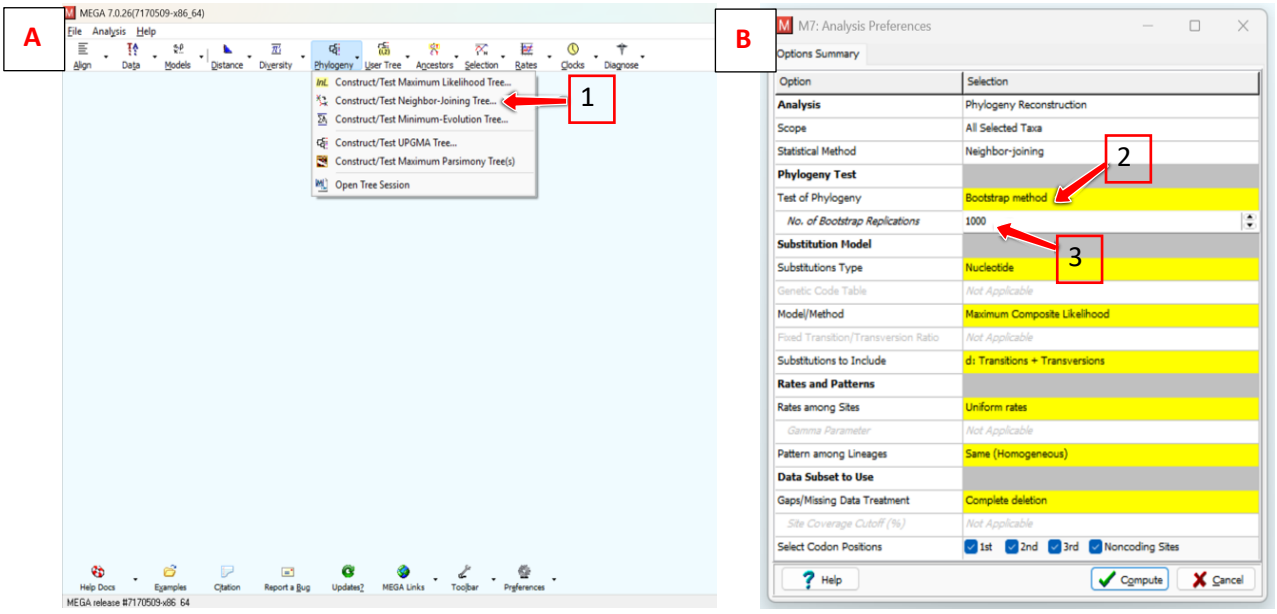


*Figure 11:* ***Construction of the Neighbor-Joining Phylogenetic tree****. (A) Under the Phylogeny tab, the Neighbor-Joining Method is selected as the approach for tree construction. (B) Subsequently, the Bootstrap option is enabled (2), and the number of replications is set to 1000 to evaluate the robustness of the tree branches (3) and the tree is computed.*

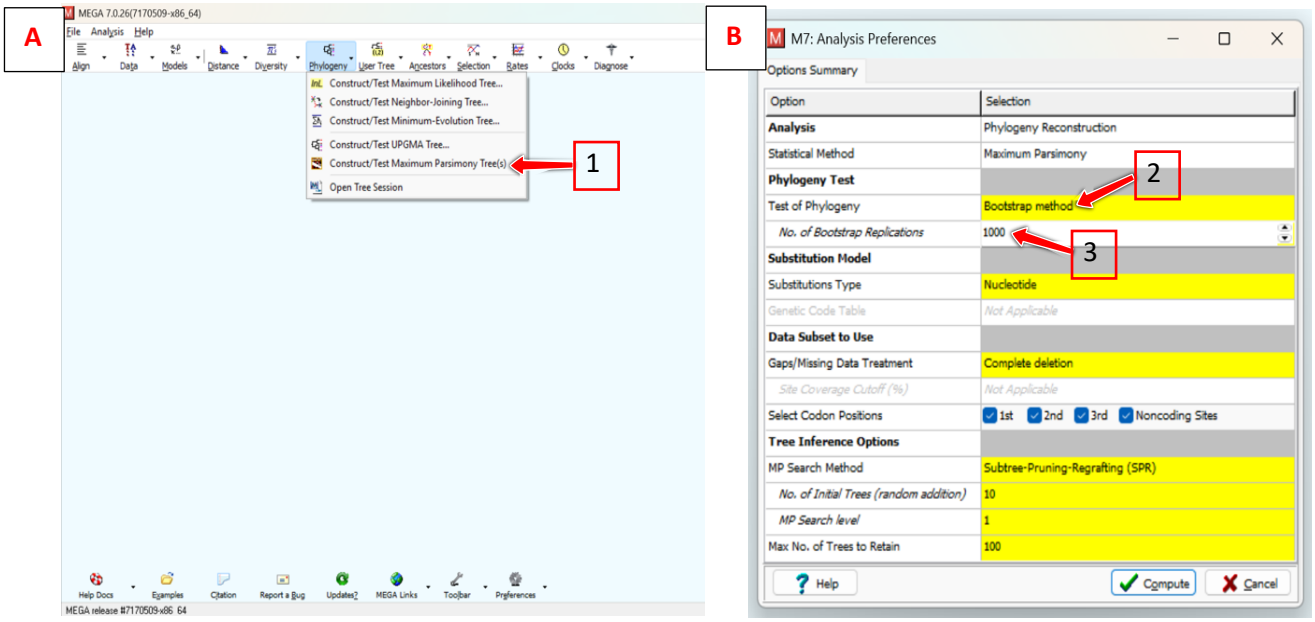


*Figure 12:* ***Construction of the Maximum Parsimony Phylogenetic tree****. (A) Under the Phylogeny tab, the Maximum Parsimony Method is selected as the approach for tree construction. (B) Subsequently, the Bootstrap option is enabled (2), and the number of replications is set to 1000 to evaluate the robustness of the tree branches (3) and the tree is computed.*

#### 2.2.3.1.4 *Borrelia* 16S rRNA Phylogeny using MEGA

The 16S rRNA phylogenetic trees for *Borrelia* were reconstructed using 25 gene sequences available on LPSN, along with *Breznakiella homolactica* RmG30$^T$ as the out group. This analysis was carried out following the same procedure previously used for *Bordetella*.

## 2.2.4. Phylogenomic Analyses and OGRIs calculations

### 2.2.4.1. *Bordetella* Genomic Analysis using the TYGS platform

**Principle:** The **Type (Strain) Genome Server (TYGS)** is a web-based, high-throughput platform designed to facilitate genome-based taxonomy of prokaryotes by simplifying the intricate computational techniques usually necessary for microbial classification (Meier-Kolthoff & Göker, 2019). This platform enables researchers to classify microorganisms based on whole-genome sequence comparisons, which is now regarded as a leading approach in microbial taxonomy and its accessible at https://tygs.dsmz.de/.

It offers several features, which include: **Digital DNA-DNA hybridization (dDDH)** (Auch et al., 2010), **Genome Blast Distance Phylogeny (GBDP)** analysis (Meier-Kolthoff et al., 2013), Phylogenetic tree construction based on intergenomic distances, among others. These enable researchers to efficiently perform high-quality genome-based taxonomic assignments (Meier-Kolthoff & Göker, 2019).

In this study, the TYGS platform was employed to carry out a genome-based phylogenomic analysis and to compute the dDDH values for the selected *Bordetella* and *Borrelia* datasets.

This platform employed the GBDP (Genome Blast Distance Phylogeny) method to compute intergenomic distances. The process involves the 'trimming' algorithm and utilizes the $d_5$ distance formula for pairwise comparisons of the chosen genome sequences (Meier-Kolthoff et al., 2013a). Based on these calculated distances, an evolutionary tree is built using FASTME 2.1.6.1 application, which implements the balanced minimum evolution approach to infer the most likely tree topology. To improve precision, subtree pruning and regrafting (SPR) optimizations are applied (Lefort et al., 2015a). The reliability of branches is assessed through 100 pseudo-bootstrap replicates. Finally, the tree is midpoint-rooted (Farris, 1972) and graphically represented using PhyD3 (Kreft et al., 2017).

**Process:** The dDDH values were calculated using the Genome-to-Genome Distance Calculator (GGDC) 4.0, which estimates intergenomic relatedness based on pairwise comparisons of genome sequences within our dataset. This process involves fragmenting the genomes and identifying high-scoring segment pairs (HSPs) through BLAST+ and applying three distance formulas (d0, d4, d6) to calculate intergenomic distances. These distances were then converted into dDDH values using statistical models that replicate laboratory DNA-DNA hybridization results (Meier-Kolthoff et al., 2013a). The resulting dDDH values were then utilized to generate a detailed heatmap using Morpheus, an online tool offered by the Broad Institute (https://software.broadinstitute.org/morpheus), by performing hierarchical clustering based on the Euclidean distance metric (Danielsson, 1980).

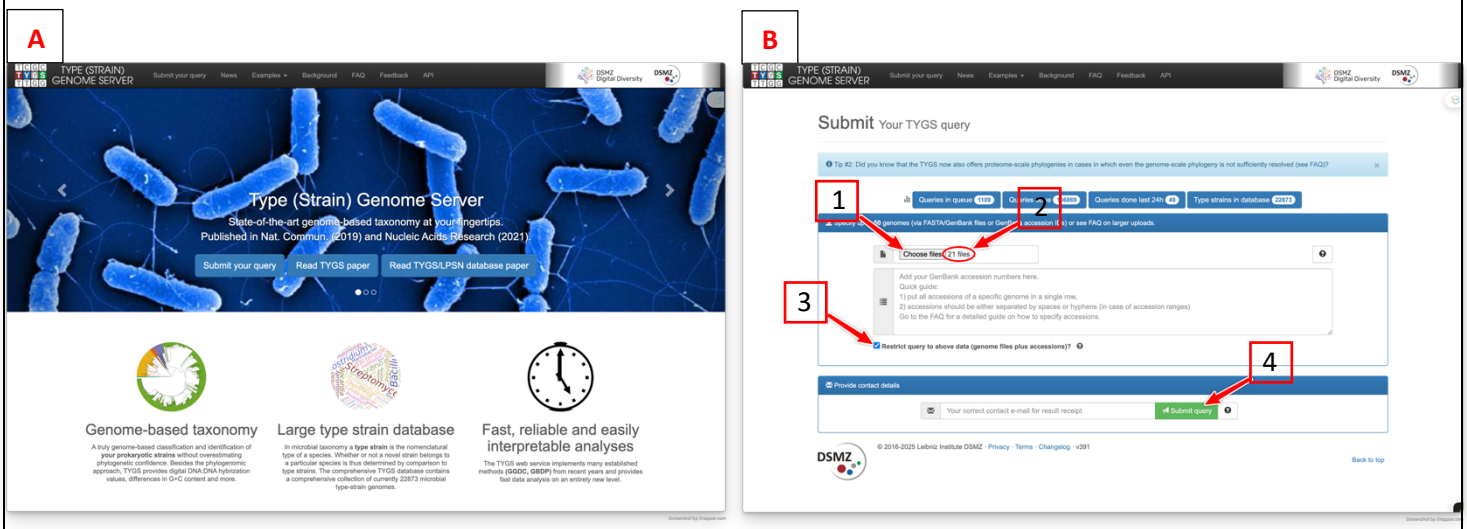


*Figure 13:* ***TYGS Platform Workflow for Taxonomic Analysis of Genomic Data****. (A) Homepage of the TYGS platform displaying the main interface and the various services it offers. (B) TYGS platform submission page used to upload genome data and run taxonomic analyses. (1). Our final dataset, consisting of 21 FNA files (2), was uploaded. The query was restricted to the uploaded genomes only (3). A valid email address was entered to receive the result notification, and the query was then submitted (4).*

#### 2.2.4.2. *Bordetella* Genomic Analysis using the TYGS platform

The TYGS platform was equally employed to carry out a genome-based phylogenomic analysis and to compute the dDDH values for our *Borrelia* dataset.

#### 2.2.4.3. Genome Based Similarity Indices (ANI & AAI) calculations

**Average Nucleotide Identity** (**ANI**) and **Average Amino Acid Identity** (**AAI**) are genome-based metrics commonly used to assess genetic relatedness among prokaryotic species.

ANI calculates the average percentage of nucleotide similarity between two microbial genomes (Konstantinidis et al., 2006) while AAI evaluates the similarity between the amino acid sequences of shared proteins across genomes (Konstantinidis & Tiedje, 2005b). AAI ≥95 % typically align with ANI values of ≥95 % and digital DNA-DNA hybridization (dDDH) values of ≥70 %, indicating organisms likely belong to the same species (C. C. Thompson et al., 2013).

##### 2.2.4.3.1 Calculation of the *Bordetella* Average Nucleotide Identity (ANI) using the FastANI tool

**Principle:** Galaxy Europe (https://usegalaxy.eu,) is an open-source web-based platform designed to support accessible, reproducible, and transparent computational research in life sciences and other scientific domains. Hosted primarily by the Freiburg Galaxy Team, it provides free access to thousands of bioinformatics tools and workflows through a user-friendly interface which enable researchers to run complex bioinformatics analyses without needing to write code or install software locally.

**Process:** For our study, we accessed the Galaxy Europe website via https://usegalaxy.eu and created a user account. We then uploaded our dataset files containing all the downloaded genome sequences of *Bordetella* species along with the outgroup and we created a dataset collection list to manage them together. The FastANI tool (Fast Alignment-Free Computation of Whole-Genome Average Nucleotide Identity) was then ran to calculate the ANI values among all the genomes within our dataset. The resulting pairwise ANI values were downloaded and used as input to generate informative heatmaps using Morpheus, an online tool provided by the Broad Institute (https://software.broadinstitute.org/morpheus/). The heatmap generation process involved hierarchical clustering based on the Euclidean distance metric (Danielsson, 1980).

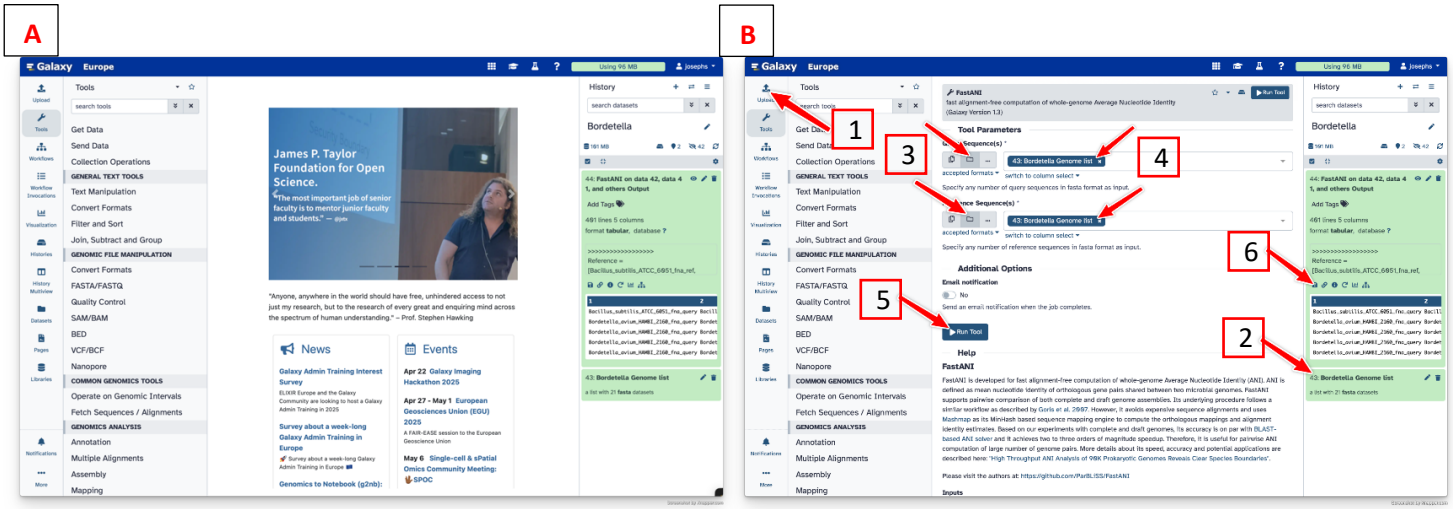


*Figure 14:* ***Genome Sequence Analysis Using Galaxy Europe's FastANI Tool.*** *(A) Homepage of the Galaxy Europe platform the main interface and the various tools and feature it offers. (B) we uploaded our dataset of genome sequences via the upload panel (1) from which a Dataset Collection list was created (2). The FastANI tool was selected and then our Dataset Collection list was selected as both the Query and as the Reference genome sequences (3,4). After setting the parameters, the analysis was launched by clicking the "Run Tool" button (5) and once the computation was complete, the output table displaying pairwise ANI values among the genomes was downloaded from the history panel (6).*

#### 2.2.4.3.2 Calculation of the *Borrelia* Average Nucleotide Identity (ANI) using the FastANI tool

The average nucleotide identity (ANI) values for the *Borrelia* dataset were calculated using the FastANI tool on the Galaxy platform. Then these values were used to create an informative heatmap matrix with Morpheus (Broad Institute) (https://software.broadinstitute.org/morpheus/) (Danielsson, 1980). This analysis was also done following the same steps as previously outlined for *Bordetella*.

#### 2.2.4.3.3 Calculation of the Average Amino Acid Identity (AAI) using the EDGAR Platform

**Principle:** EDGAR (Efficient Database framework for comparative Genome Analyses using BLAST score Ratios) is a comprehensive online software platform designed for the comparative analysis of prokaryotic genomes (Blom et al., 2009). This platform is accessible via (http://edgar.computational.bio).This platform provides various features, including Comparative Genomics (e.g., calculation of the core genome, pan-genome, and singleton genes), Phylogenetic analysis (such as generating phylogenetic trees and calculating AAI and ANI matrices), and Visualization tools like synteny plots and Venn diagrams.

**Process:** In our study, we used the EDGAR platform to perform an Average Amino Acid Identity (AAI) analysis on our *Bordetella* genome dataset. To begin, we reached out to the EDGAR support team via email and provided our genomic data. The EDGAR team then set up a private project with our dataset, allowing us to use the platform's phylogenetic toolkit to generate the AAI matrix for our *Bordetella*

dataset. The resulting AAI values were then utilized to generate a detailed heatmap using Morpheus, an online tool offered by the Broad Institute (https://software.broadinstitute.org/morpheus), by performing hierarchical clustering based on the Euclidean distance metric (Danielsson, 1980).

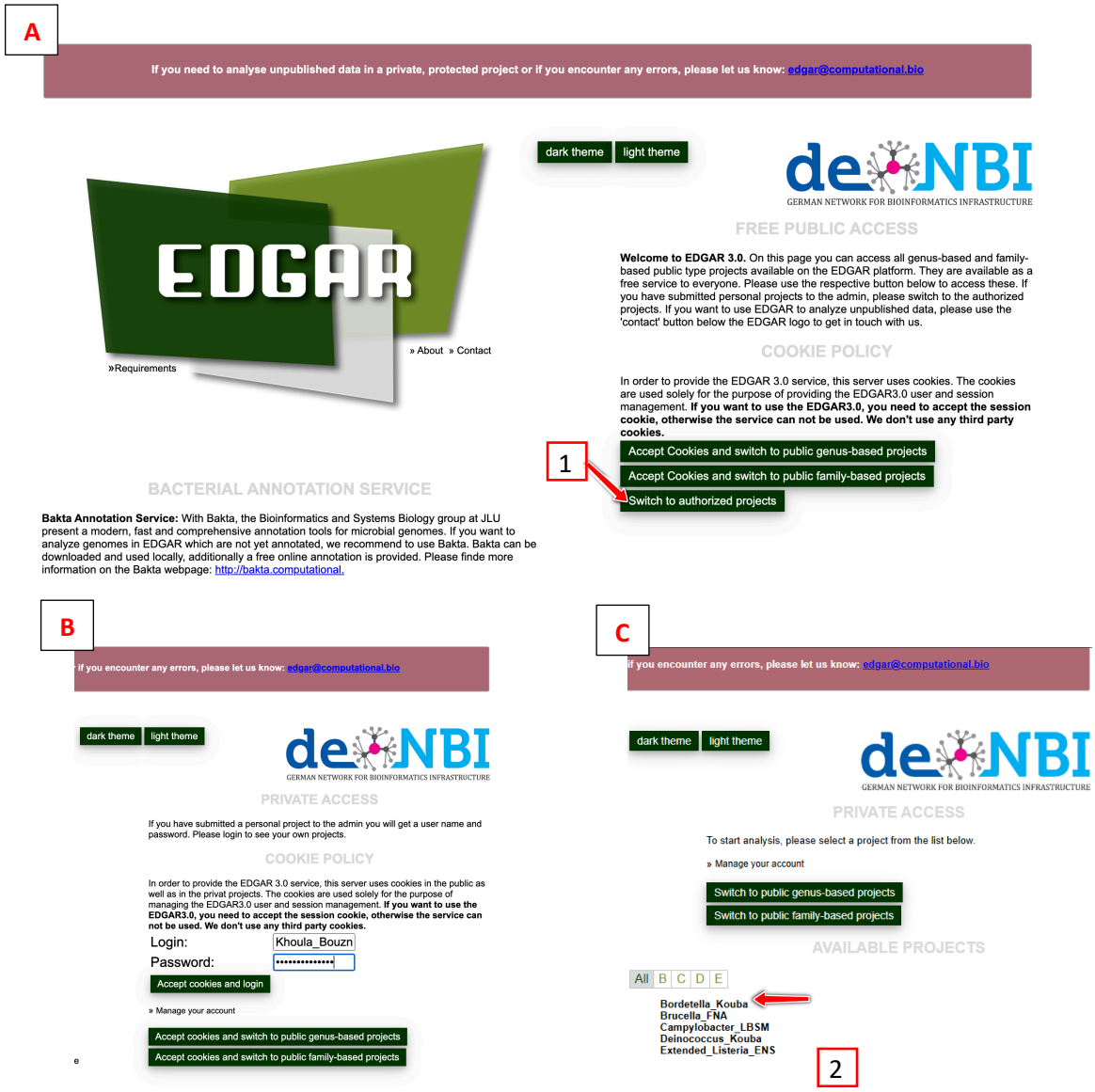


*Figure 15:* ***Setting up the EDGAR platform.*** *(A) Home page of the EDGAR platform: on which we accessed the authorized projects section (1). After logging-in (B), we selected our specific project titled Bordetella_Kouba (2) on which we carried out the AAI analyses (C).*

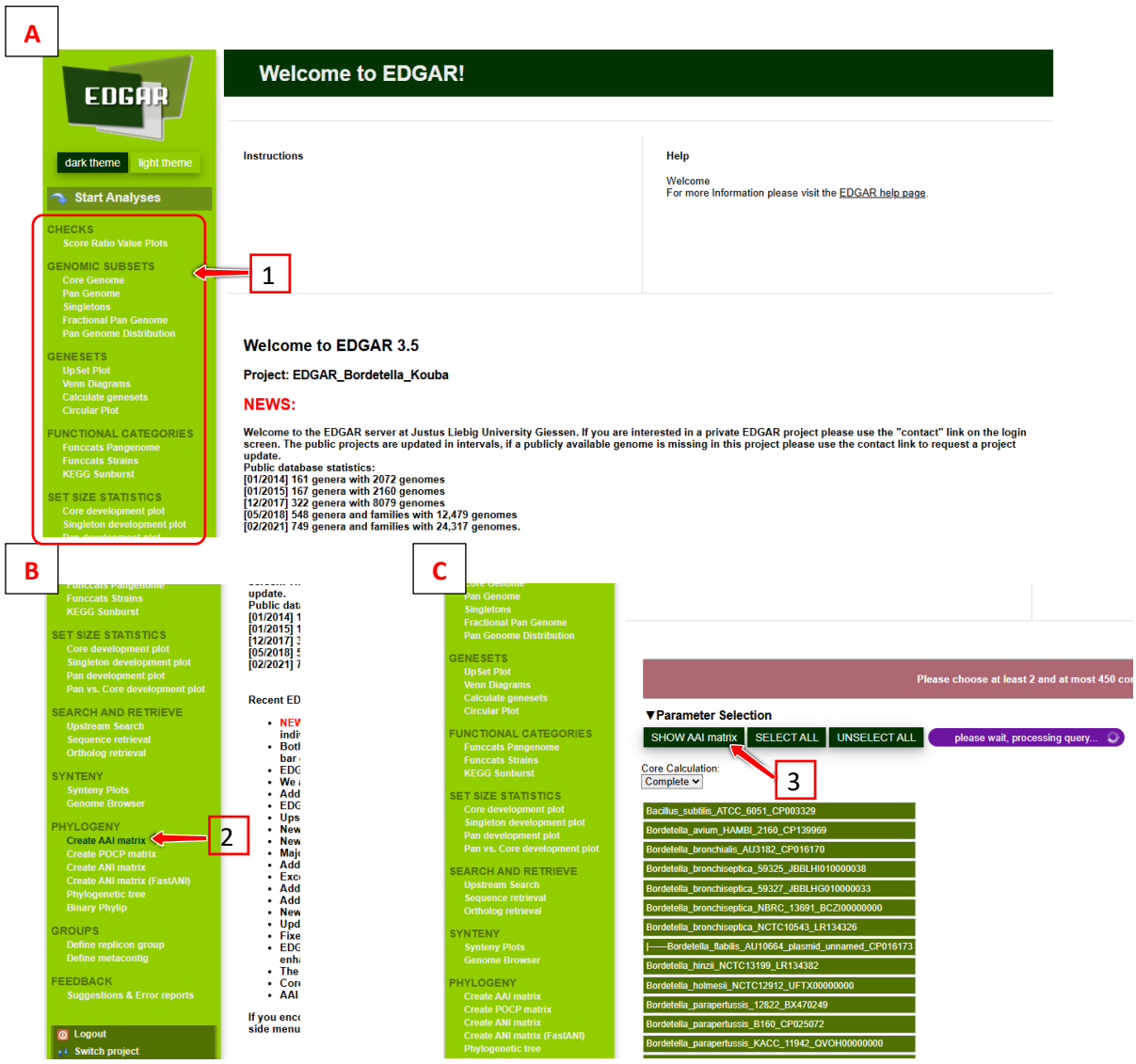


*Figure 16: **AAI Calculation using the EDGAR platform**. (A) The homepage of the EDGAR platform's Private project section showing the various features and tools provided by the platform (1). (B) To generate the AAI matrix, we chose the AAI matrix tool under the Phylogeny category (2), selected all the dataset genomes and executed the tool to show the AAI matrix (3).*

### 2.2.4.4. Core Genome Phylogenomic Analysis

**Principle:** Core Genome Phylogenomics is the study of evolutionary relationships by analysing the set of genes shared by all members of a group of organisms, such as a bacterial species i.e. the Core genome. This analysis was conducted, to further elaborate on the findings of the 16S rRNA and whole genome analyses.

**Process:** This analysis begins with performing a de novo annotation of the DNA sequences within our dataset using the Prokka tool. Then the resulting annotated files are fed into the BPGA pipeline which performs the Core Genome analyses.

#### 2.2.4.4.1 Genome Annotation with the Prokka tool

The **Prokka (Prokaryotic Genome Annotation) tool** available on the Galaxy platform (https://usegalaxy.eu/?tool_id=prokka), is a tool that quickly annotates prokaryotic genomes by identifying the positions and functions of genomic features such as coding sequences (CDSs), tRNAs, rRNAs, and other elements within genome sequences (Seemann, 2014).

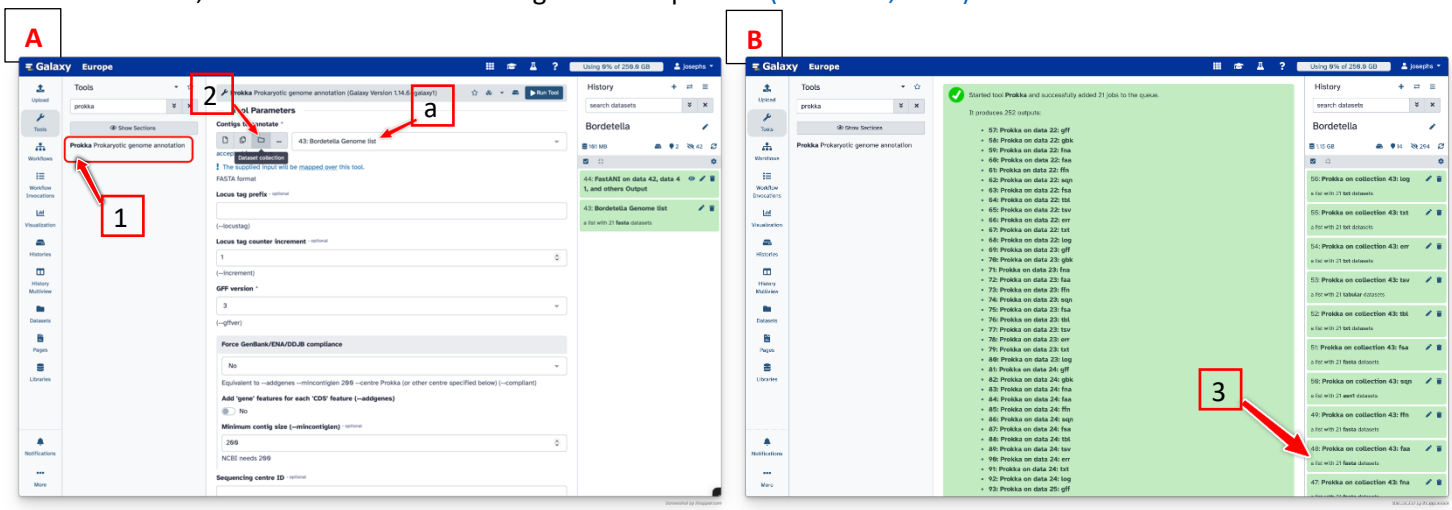


*Figure 17:* ***Prokka tool Workflow for Genome Annotation.*** *(A) In the Galaxy Platform, the Prokka tool is chosen (1), and the Data collection list (a) is entered into the tool (2). The tool is then executed using the default settings to perform the annotation. From the results, the .faa file is downloaded for later use in the BPGA analysis.*

#### 2.2.4.4.2 Genome Analysis by using BPGA

**Principle:** The Bacterial Pan Genome Analysis (BPGA) is a software tool that provides a range of detailed pan-genome analyses for bacterial species. The BPGA pipeline accepts input formats such as protein FASTA files, GenBank files, or binary matrices, and employs clustering tools like OrthoMCL, USEARCH, or CD-HIT to generate orthologous gene clusters. These clusters are then used for various analyses, including pan-genome profiling, functional annotation (COG/KEGG), extraction of core and accessory genes, and phylogenetic tree construction (Chaudhari et al., 2016).

The pan-genome refers to the full set of genes found within a species, consisting of the core genome (genes common to all strains), the dispensable genome (accessory genes found in multiple strains), and unique genes (specific to individual strains) (Chaudhari et al., 2016). Analysing the pan-genome has become an essential technique for understanding genetic diversity and evolutionary dynamics.

**Process:** In our study, we used the *.faa* annotated file generated by Prokka and ran it through the BPGA pipeline with default settings to identify core, accessory, and unique genes. This gene clustering was carried out using the USEARCH algorithm at a 50 % sequence similarity threshold across 30 dataset combinations. A core genome phylogenetic tree was then generated using the Maximum Likelihood method by the FastTree2 program which applies the JTT (Jones-Taylor-Thornton) + CAT (Covarion-AutoCorrelated) protein evolution models (Price et al., 2010), available at the Galaxy Europe platform

(Goecks et al., 2010). The resulting phylogenetic tree was visualized and edited using the Interactive Tree of Life (iTOL) platform (Letunic & Bork, 2019a) accessible at https://itol.embl.de/.

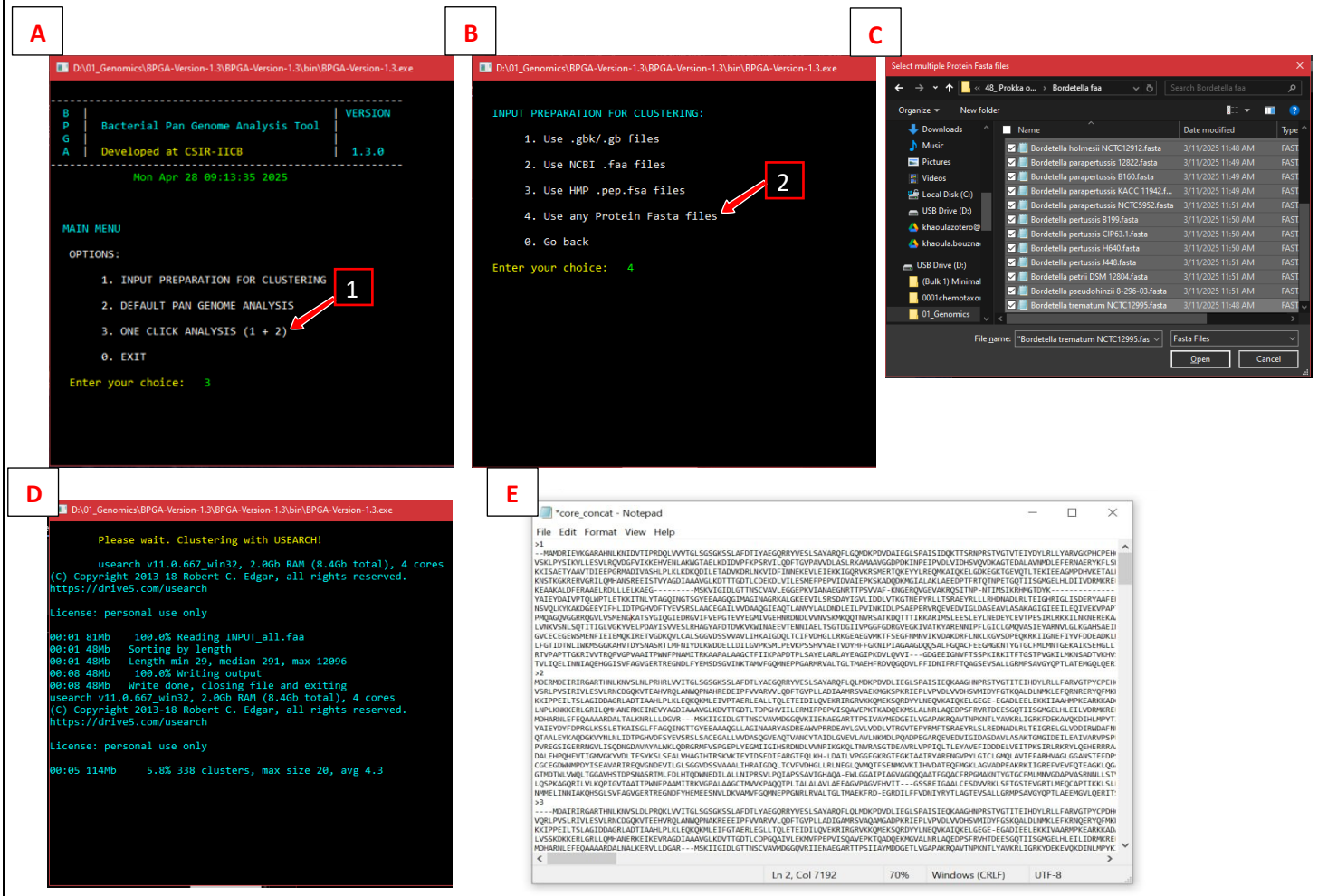


*Figure 18:* ***BPGA Pipeline Workflow.*** *(A) The BPGA program is launched, and the main menu appears, where the "One click Analysis (1+2)" option is selected (1) to perform both clustering and pan-genome analysis sequentially. (B) Next, the input preparation menu for clustering genomic data pops up, where the "Protein Fasta files" option is chosen (2), enabling the upload of .faa files generated by the Prokka tool. (C) The .faa files are uploaded, and then the clustering process is executed using the USEARCH algorithm (D). The generated concatenated .fasta file is saved for the subsequent steps(E).*

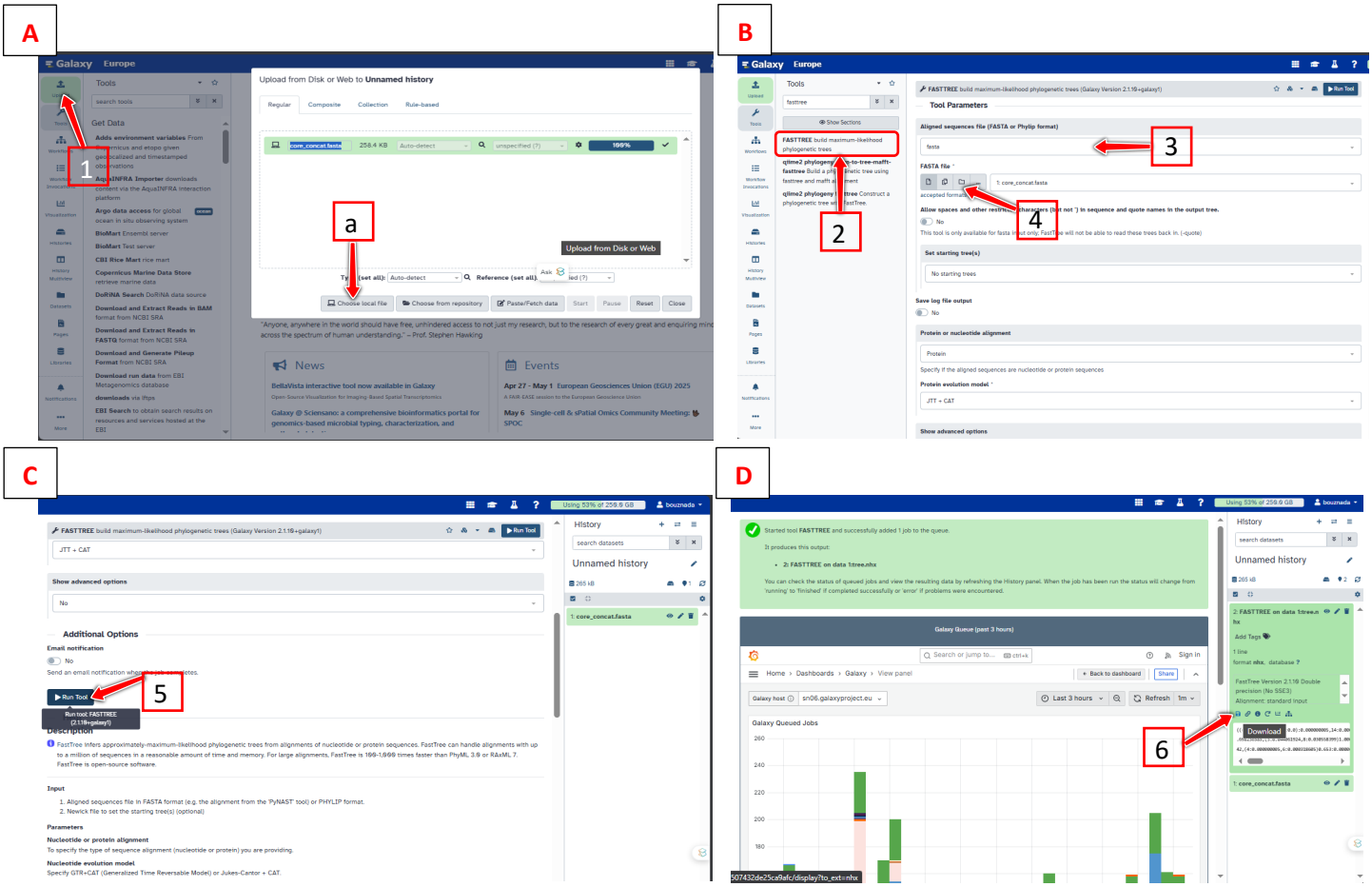


*Figure 19:* ***Phylogenetic tree construction using the FastTree tool.*** *(A) The concatenated .fasta file from the BPGA is uploaded to the Galaxy platform (1) by selecting the option to upload from a local file (a). (B) The FastTree tool is chosen from the Galaxy tool list, and fasta is selected as the format of the aligned sequence file to be used (3). The uploaded concatenated .fasta file is chosen (4) and the tool is ran (5). The resulting .nhx file is downloaded for use in the next step of tree visualization and editing by the iTOL platform. (6).*

#### 2.2.4.4.3 Phylogenomic Tree Visualisation by the iTOL platform.

**Principle:** iTOL (Interactive Tree Of Life) is a web-based platform for visualizing, annotating, and management of phylogenetic trees. It allows users to display trees in various organised formats, add interactive annotations, and export high-quality tree images for publication purposes (Letunic & Bork, 2019b). it is accessible at https://itol.embl.de/.

**Process:** In our study, we uploaded the *.nhx* file from the FastTree tool onto the iTOL platform for visualisation, editing and annotation. The resulting phylogenetic tree was further refined by using the Inkscape application to enhance the visual appeal of the phylogenetic tree.

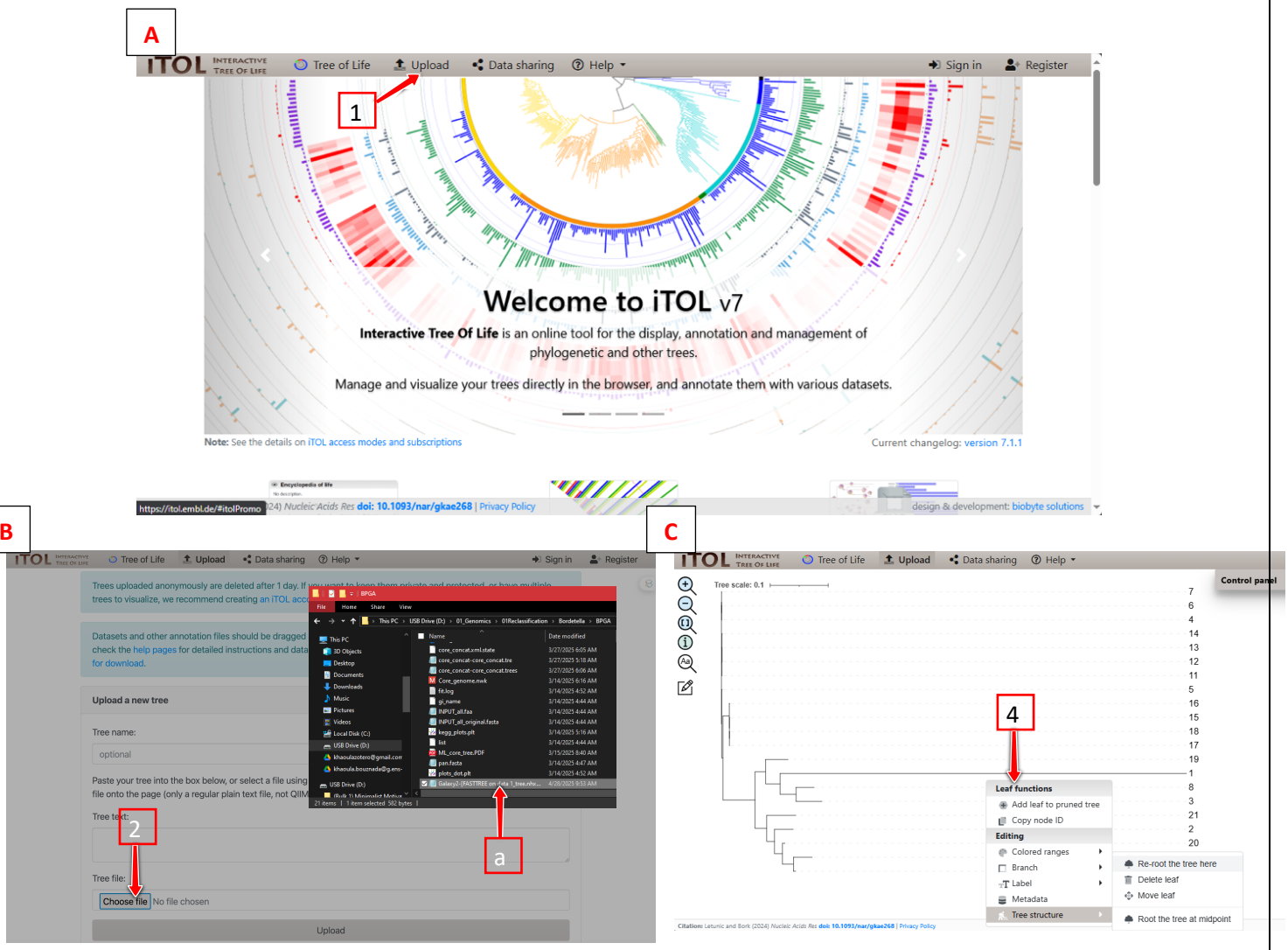


*Figure 20:* ***Phylogenomic Tree Visualisation on the iTOL platform. (A)*** *The iTOL tool homepage is accessed through [https://itol.embl.de/](https://itol.embl.de/). The .nhx file (a) generated by the FastTree tool was uploaded by clicking the Upload button on the homepage (1) and selecting the Choose File option (2). Various features within the iTOL interface were then utilized for editing and annotating the tree (4).*

### 2.2.4.5. Orthologous Gene Clustering using OrthoVenn

The **OrthoVenn3** tool ([https://orthovenn3.bioinfotoolkits.net/](https://orthovenn3.bioinfotoolkits.net/)) is a web-based platform designed for comparative genomics and orthologous gene cluster visualisation. It helps researchers identify shared and unique genes across different species or strains using Venn diagrams and functional annotations, by clustering proteins based on sequence similarity (Sun et al., 2023).

**Process:** In this study, we employed the platform to conduct a comparative genomic analysis of the *Bordetella* and *Borrelia* species that exhibit taxonomic ambiguities within our respective datasets.

Prokka annotated fasta files of each species were uploaded onto the platform and the analysis was performed using default parameters, applying the OrthoMCL algorithm with an E-value threshold of $1\times10^{-5}$, and an inflation value of 1.5. The resulting visualizations were then downloaded for further interpretation.

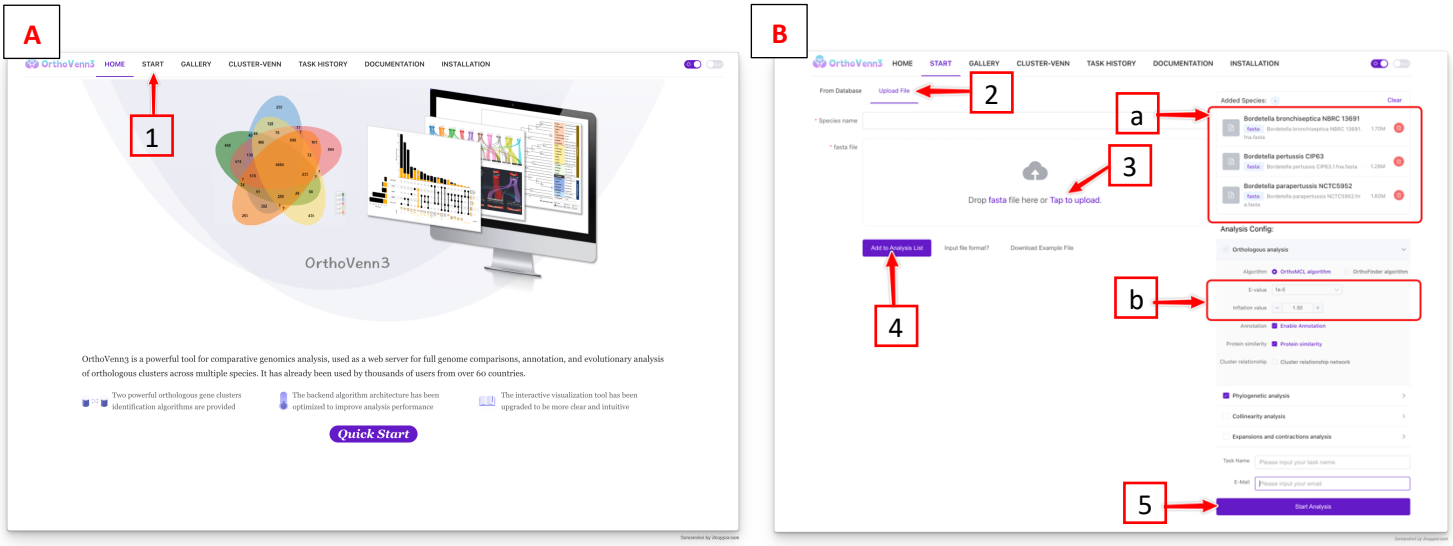


*Figure 21:* ***OrthoVenn Platform Workflow for Orthologous gene clustering.*** *(A) The Homepage of the OrthoVenn3 web Platform. The analysis is initiated by switching to the Start tab (1) . (B) The fasta files of the three Bordetella species (a) are individually uploaded (2, 3, 4) and with the E-value cutoff and an inflation value set to 1e-5 and 1.5 respectively(b), the analysis is initiated (5).*

### 2.2.4.6. Subsystem-Based Functional Gene Annotation using RAST Toolkit

**Principle:** The **RASTtk** (Rapid Annotation using Subsystems Technology) toolkit on the **BV-BRC** platform (https://www.bv-brc.org/) is a modular extension of the RAST server that is used to systematically analyse and annotate bacterial genomes (Aziz et al., 2008; Brettin et al., 2015).

It assists researchers in organizing and annotating bacterial genomes into gene clusters known as **Subsystems**. These subsystems categorize genes into functional groups based on their roles in specific biological processes or pathways, such as metabolism, stress response, or DNA replication. This categorisation provides a structured framework for analysing gene functions which facilitates the understanding of genomic diversity and evolutionary relationships among bacteria (Aziz et al., 2008; Brettin et al., 2015).

**Process:** In our study, we used the RAST toolkit to perform a subsystem-based functional annotation of genomes of the *Bordetella* and *Borrelia* species that exhibit taxonomic ambiguities within our respective datasets, to further expound on and validate our prior analyses.

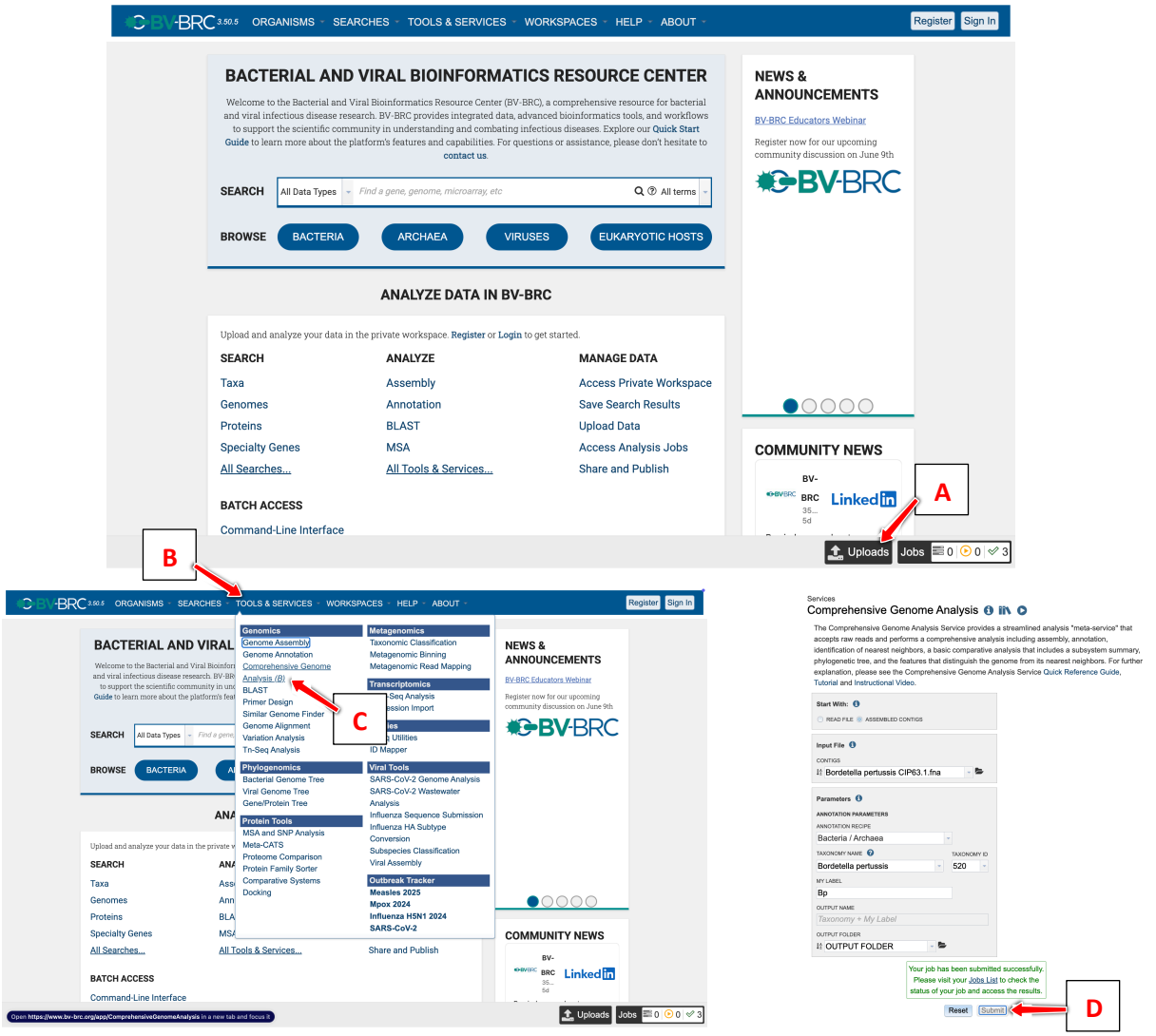


*Figure 22:* ***Functional Gene Annotation based on Subsystems using the BV-BRC Platform.*** *The BV-BRC platform is accessible at via https://www.bv-brc.org and the Genome sequences are uploaded onto the platform (A). Under the "Tools & Services" tab (B), the "Comprehensive Genome Analyses service" is selected (C). Relevant information is then entered, and the job is submitted for processing (D).*

#### 2.2.4.6.1 Phenotypic Analysis review using BacDive Platform

**Principle:** The Bacterial Diversity Meta database (**BacDive**) is a platform dedicated to the collection, standardization, and dissemination of bacterial and archaeal strain-level data. It brings together carefully curated information on taxonomy, morphology, cultivation, metabolism, genomic sequences, and isolation sources, drawn from species descriptions and major culture collections such as DSMZ, CIP, CCUG, and CABI (Reimer et al., 2021).

BacDive is used by researchers to systematically compare and identify bacterial strains with specific characteristics, based on a combination of phenotypic, genotypic, and ecological data. This is enabled by its advanced search tools allowing precise querying of its database. BacDive is hosted by the Leibniz Institute DSMZ and is accessible at https://bacdive.dsmz.de.

**Process:** This platform was used in our study to obtain data for a comparative analysis of the phenotypic characteristics the *Bordetella* and *Borrelia* strains that exhibit taxonomic ambiguities within our respective datasets.

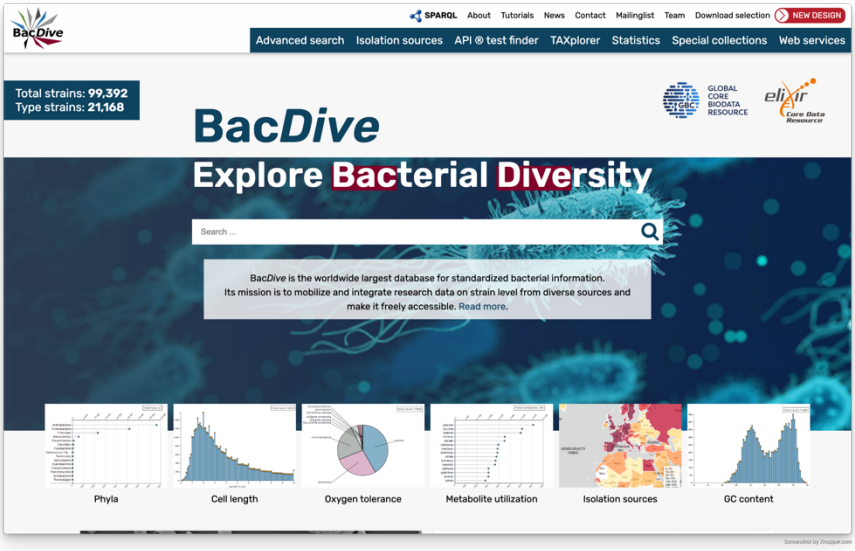


*Figure 23 :* ***BacDive Platform homepage.*** *The homepage interface displaying key functionalities such as search tools, data statistics, and access to strain-level bacterial diversity resources.*

*CHAPTER 3:*

# RESULTS AND DISCUSSION

# 3. RESULTS AND DISCUSSION

## 3.1. *Bordetella* Analysis

### 3.1.1. Phylogenetic Analysis Based on 16S rRNA Gene Sequences

To examine the evolutionary relationships and reevaluate the taxonomic classification of *Bordetella pertussis* ATCC 9797$^T$, *Bordetella parapertussis* ATCC 15311$^T$, and *Bordetella bronchiseptica* NBRC 13691T, we carried out a phylogenetic analysis of their 16S rRNA gene sequences using MEGA software and determined their pairwise percentage gene similarities through EzBioCloud web-based platform.

This comparative analysis of the 16S rRNA gene sequences revealed that *Bordetella pertussis* ATCC 9797$^T$ *Bordetella parapertussis* ATCC 15311$^T$ and *Bordetella bronchiseptica* NBRC 13691$^T$, shared a high level of sequence similarity, with their percentage similarities ranging from **99.63** % to **99.95** % (**Table IV**)**.** Also, the phylogenetic analyses conducted using the Maximum Likelihood (ML), Neighbor-Joining (NJ), and Maximum Parsimony (MP) methods, generated phylogenetic trees in which the three species consistently clustered together within a single, well-supported clade, with a stable Bootstrap value of **99** % (**Figure 24, Figure 25, Figure 26**).

Given that the 16S rRNA gene similarity values are above the 98.65% threshold for species delineation (Kim et al., 2014; Stackebrandt & Goebel, 1994), and that these strains consistently cluster together in phylogenetic trees with strong bootstrap support, their close taxonomic relationship is evident.

***Table IV:*** *16S rRNA Pairwise percentage gene similarity values among the Classical Bordetella.*

| | ***B. pertussis*** | ***B. parapertussis*** | ***B. bronchiseptica*** |
|---|---|---|---|
| ***B. pertussis*** | 100 % | 99.73 % | 99.65 % |
| ***B. parapertussis*** | 99.73 % | 100 % | 99.95 % |
| ***B. bronchiseptica*** | 99.65 % | 99.95 % | 100 % |

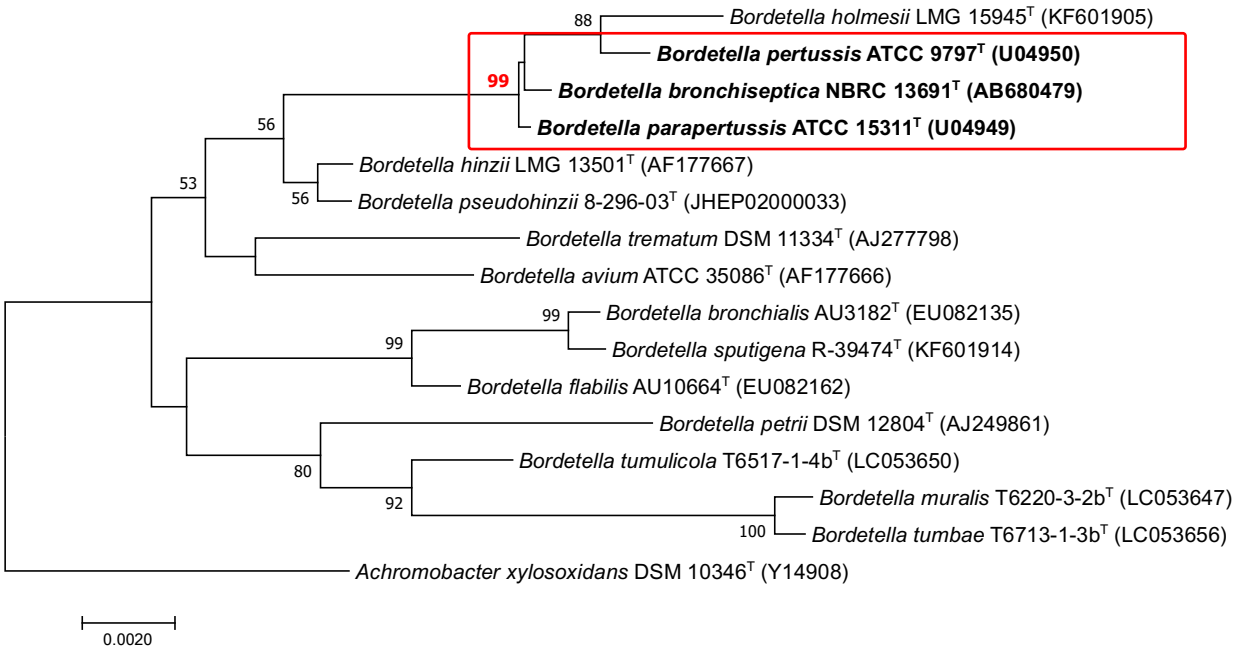


*Figure 24:* ***The 16S rRNA Phylogenetic Tree based on the Neighbor Joining Method.*** *The tree was inferred for the near-complete 16S rRNA gene sequences using Neighbor Joining method (Saitou & Nei, 1987) where the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). This phylogenetic tree illustrates the close evolutionary relationship between B. pertussis ATCC 9797$^T$, B. bronchiseptica NBRC 13691$^T$and B. parapertussis ATCC 15311$^T$ and their taxonomic position among the other closely related type strains in the Bordetella Genus. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values >50 % are shown) (Felsenstein, 1985). Achromobacter xylosoxidans NCTC10807$^T$ was used as an outgroup. Bar 0.002 nucleotide substitution per site. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).*

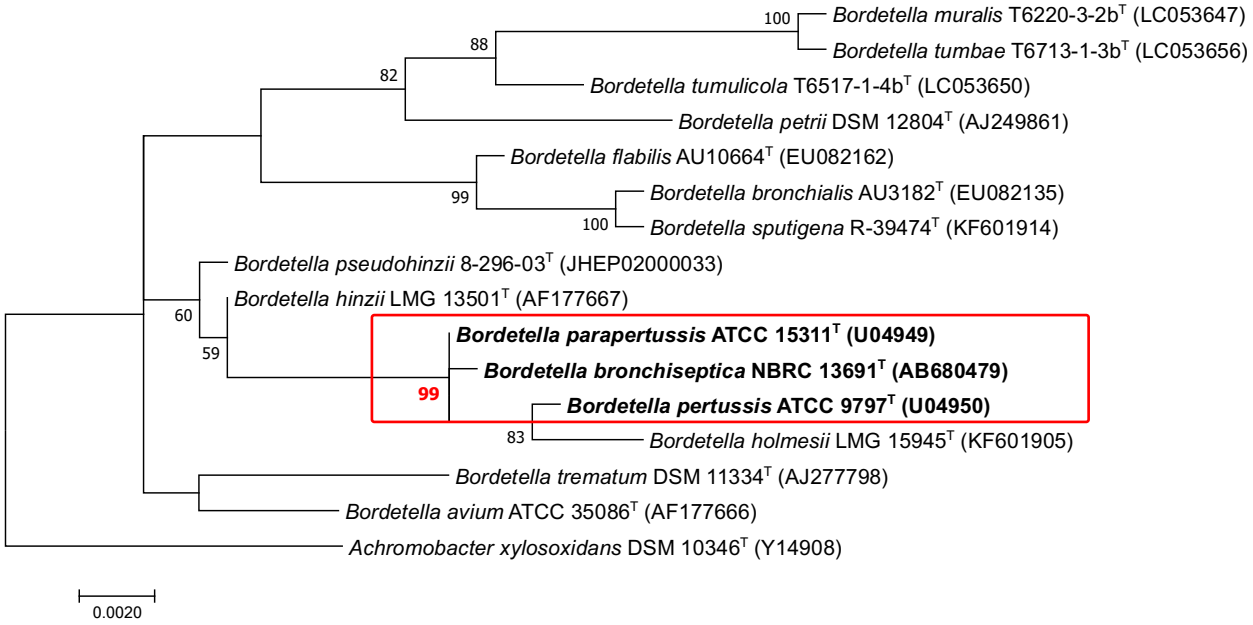


*Figure 25:* ***The 16S rRNA Phylogenetic Tree based on the Maximum Likelihood Method.*** *The tree was inferred for the near-complete 16S rRNA gene sequences using the Maximum Likelihood Method and Tamura-Nei Model (Tamura & Nei, 1993) where the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). This phylogenetic tree illustrates the close evolutionary relationship between B.*

*pertussis ATCC 9797$^T$, B. bronchiseptica NBRC 13691$^T$and B. parapertussis ATCC 15311$^T$ and their taxonomic position among the other closely related type strains in the Bordetella Genus. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values >50 % are shown) (Felsenstein, 1985). Achromobacter xylosoxidans NCTC10807$^T$ was used as an outgroup. Bar 0.002 nucleotide substitution per site. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).*

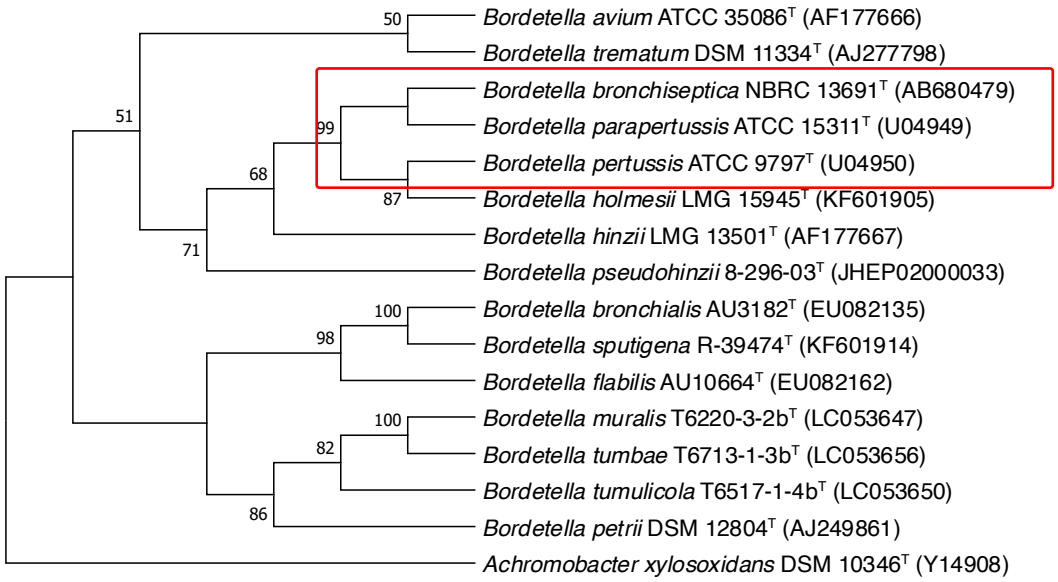


Figure 26: **The 16S rRNA Phylogenetic Tree based on the Maximum Parsimony Method.** The tree was inferred for the near-complete 16S rRNA gene sequences using the Maximum-Parsimony method (Nei & Kumar, 2000)**.** The Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. This phylogenetic tree illustrates the close evolutionary relationship between *B. pertussis* ATCC 9797$^T$, *B. bronchiseptica* NBRC 13691$^T$and *B. parapertussis* ATCC 15311$^T$ and their taxonomic position among the other closely related type strains in the *Bordetella Genus*. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 resamplings (only values >50 % are shown) (Felsenstein, 1985). *Achromobacter xylosoxidans* NCTC10807$^T$ was used as an outgroup.

## 3.1.2. Phylogenomic Delineation and Comparative Genomic Analysis

### 3.1.2.1. Pairwise Genomic Comparisons and dDDH Estimation

To further examine these taxonomic relationships within our *Bordetella* dataset, pairwise comparisons among the genomes were conducted using the GBDP approach via the Type (Strain) Genome Server (TYGS). Digital DDH values and their confidence intervals were also calculated using the GGDC 4.0 (Meier-Kolthoff et al., 2013, 2022).

The obtained heatmap matrix of pairwise dDDH values (Figure 27) shows that all analysed strains of *Bordetella parapertussis*, *Bordetella pertussis*, and *Bordetella bronchiseptica* (type and additional strains) cluster together in the same dendrogram, with high dDDH percentages ranging between 85–

95%. These values are well above the 70% DDH threshold commonly used for species delineation (Meier-Kolthoff et al., 2013; Moore et al., 1987).

The phylogenomic analysis conducted using the GBDP approach yielded a 16S rRNA-based phylogenetic tree **(**

*Figure 28***)** and a Whole genome based phylogenetic tree **(Figure 29)**. In both trees, all the classical *Bordetella* species (type and additional) consistently cluster together in a single monophyletic clade with a branch support of 100. Additionally, all Bordetella parapertussis, *Bordetella pertussis*, and *Bordetella bronchiseptica* strains display similar colours in the species and subspecies cluster columns of the tree's metadata, indicating their grouping within the same genomospecies.

Therefore, the high dDDH values (85–95) and the formation of a single monophyletic clade with robust bootstrap support across both 16S rRNA and whole-genome phylogenies indicate that these strains share a close genomic relatedness. Furthermore, their uniform clustering in species and subspecies cluster columns of the tree's metadata (same colour) reinforces the notion that they constitute a single species rather than distinct species.

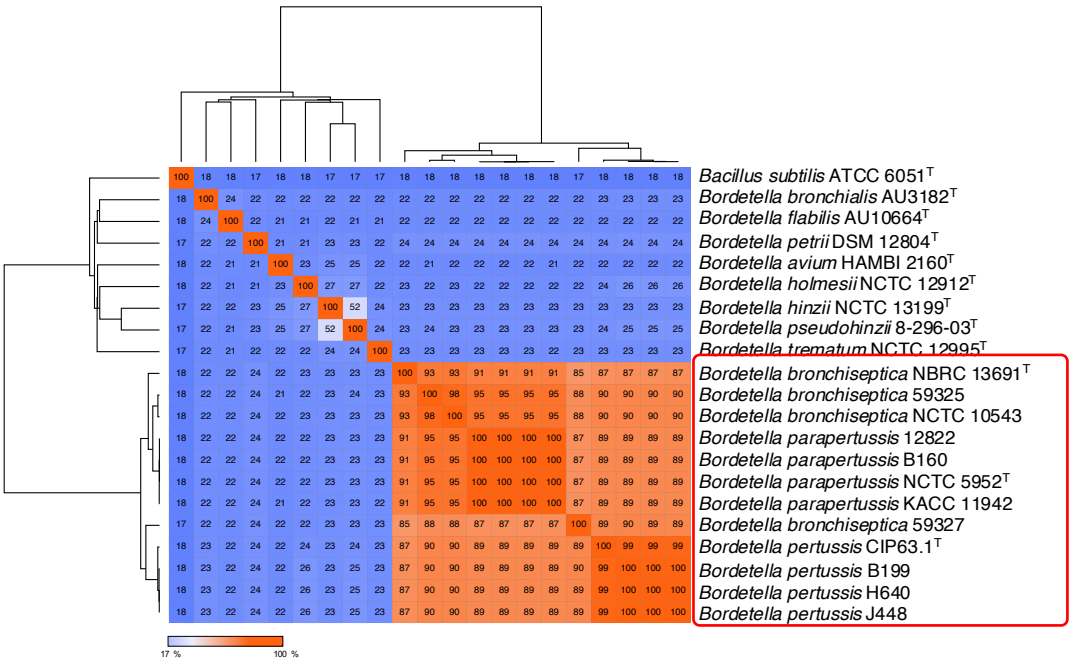


*Figure 27:* ***Heatmap of Digital DNA–DNA Hybridization (dDDH) values.*** *This matrix shows pairwise genomic similarities among the Bordetella strains with* Bacillus subtilis *ATCC 6051$^T$ included as an outgroup. The dDDH values were computed using the (GGDC) 4.0 (Meier-Kolthoff et al., 2013) available on the TYGs platform. A standard threshold of 70 % dDDH for species delineation was applied and the resulting values were used to generate dDDH matrix using the Morpheus tool. Colour intensity reflects the degree of genomic similarity, with warmer tones (orange) showing higher dDDH values (≥70 %), which suggests that those strains are likely of the*

*same species. Notably, B. parapertussis, B. bronchiseptica, and B. pertussis form a distinct cluster (orange dendrogram) with high dDDH values ranging from (85 % to 95 %) indicating a close genetic relationship.*

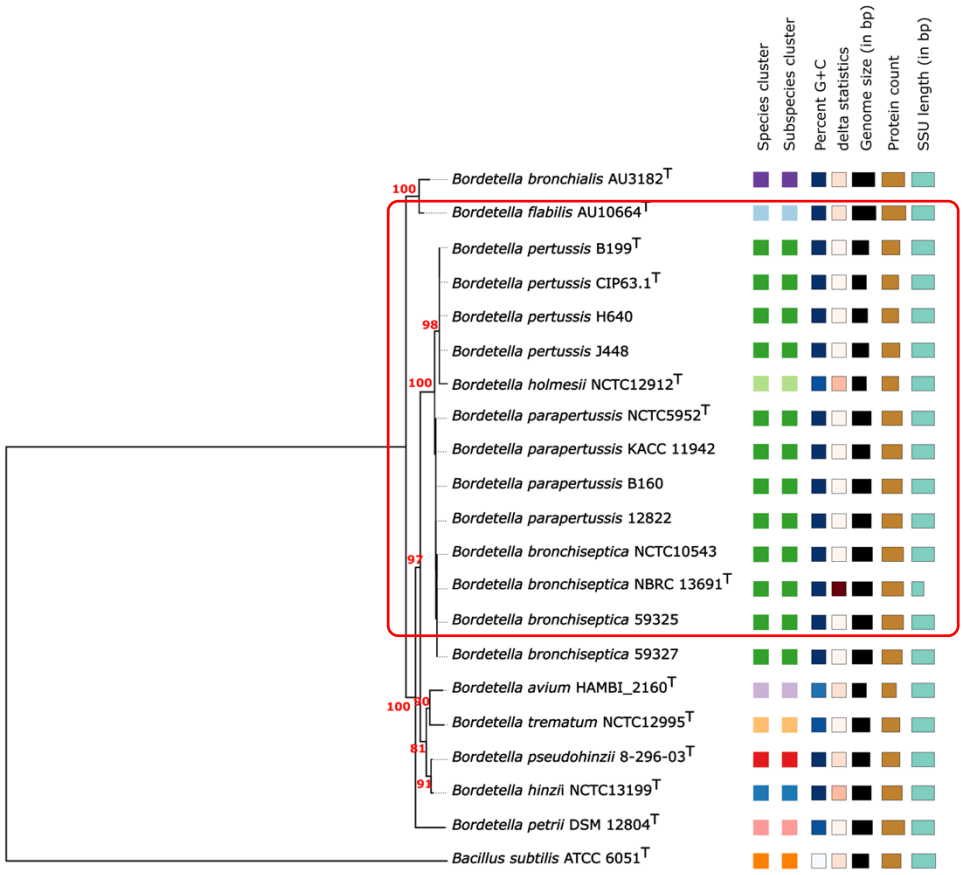


*Figure 28:* ***The 16S rRNA Phylogenetic Tree based on the GBDP Approach.*** *This phylogeny was* inferred *with FastME 2.1.6.1 (Lefort et al., 2015) based on the GBDP approach (Meier-Kolthoff et al., 2013). It illustrates the evolutionary relationship among strains of taxonomically debated Bordetella species and their closest relatives with Bacillus subtilis ATCC 6051$^T$ used as an outgroup. The numbers above the branches are GBDP pseudo-bootstrap support values from 100 replications (only values >50% are shown). Color-coded metadata columns indicate key genomic features such as species/subspecies groupings, G+C content, genome size, and protein count. Clusters of the same species or subspecies are highlighted in matching colours.*

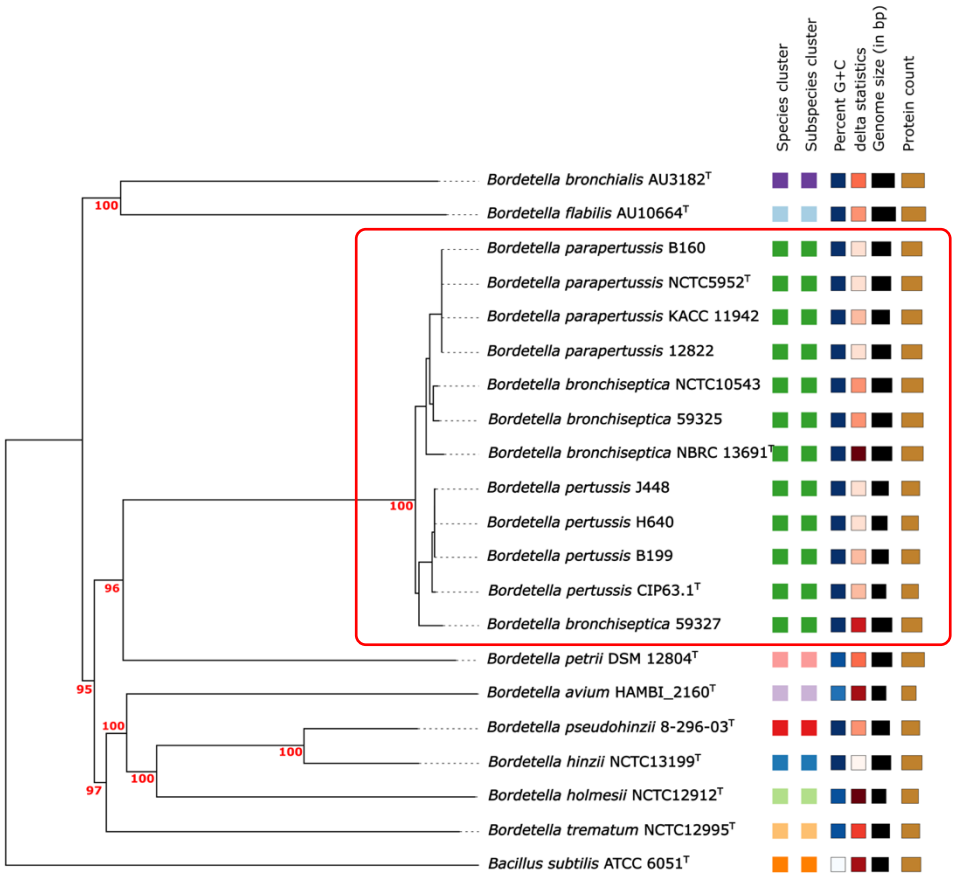


*Figure 29:* ***Whole-genome Phylogenomic Tree.*** This phylogeny was *inferred with FastME 2.1.6.1 (Lefort et al., 2015) based on the GBDP approach (Meier-Kolthoff et al., 2013). It illustrates the evolutionary relationship among strains of taxonomically debated Bordetella species and their closest relatives with Bacillus subtilis ATCC 6051$^T$ used as an outgroup. The numbers above the branches are GBDP pseudo-bootstrap support values from 100 replications (only values >50 % are shown). Color-coded metadata columns indicate key genomic features such as species/subspecies groupings, G+C content, genome size, and protein count. Clusters of the same species or subspecies are highlighted in matching colours.*

### 3.1.2.2. Overall Genome Relatedness Indices (OGRIs)

In modern taxonomy, a combination of 16S rRNA gene sequence analysis and OGRIs calculation is frequently employed to achieve more precise species delineation (Chun, Oren, Ventosa, Christensen, Arahal, da Costa, et al., 2018). Thus, to enhance our taxonomic resolution, we performed the Average Nucleotide Identity (ANI) and Average Amino Acid Identity (AAI) analyses on our *Bordetella* dataset, utilizing the Galaxy Europe and EDGAR platforms, respectively. The resulting pairwise similarity values were then used to generate informative heatmaps using the Morpheus tool (http://biodev.cea.fr/morpheus/).

The generated ANI **(Figure 31)** and AAI **(Figure 30)** matrices, show that all the examined strains of *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* (including both type and additional strains) cluster closely together within the dendrogram (coloured in orange) with values

ranging from (**98–99%**) for ANI and **(97–99 %)** for AAI, all of which are significantly higher than the standard 95% threshold for species delineation.

These observations provide strong evidence that *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* exhibit a high degree of gnenomic similarity, suggesting that these traditionally recognized species may, in fact, represent a single species due to their close evolutionary relationship.

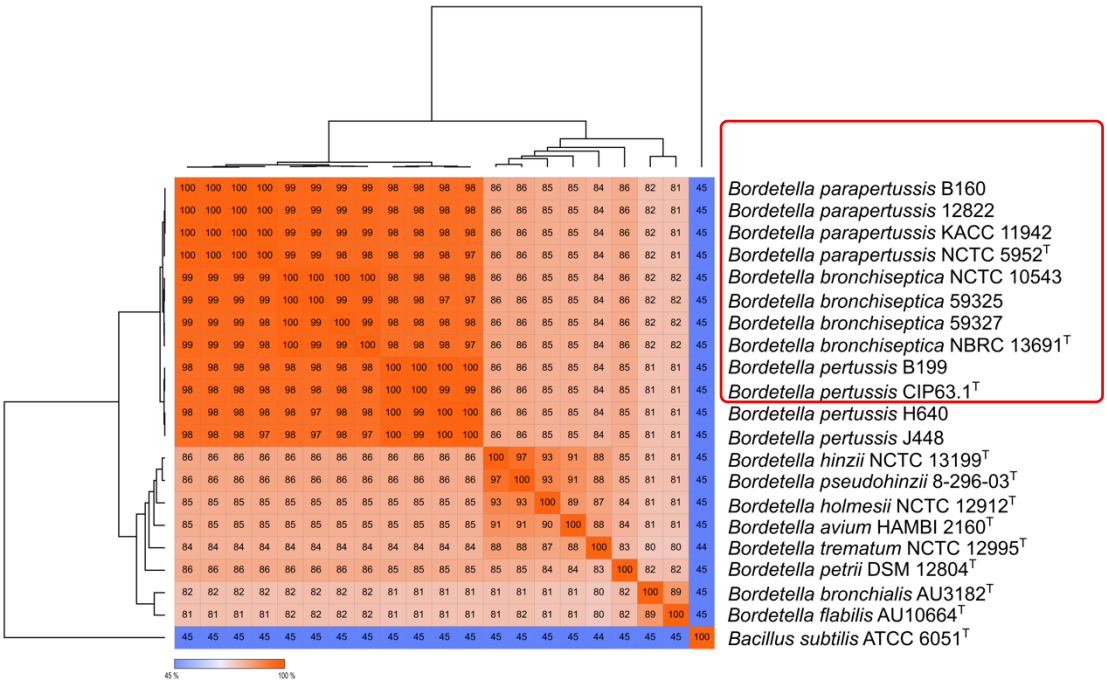


*Figure 30:* ***Heatmap of Average Nucleotide Identity (AAI) values.*** *This represents proteomic relatedness among strains of Bordetella species and their closest relatives with Bacillus subtilis ATCC 6051$^T$ used as an outgroup. AAI values were computed using the EDGAR 3.0 platform (Blom et al., 2009), with the heatmap constructed through hierarchical clustering based on the Euclidean distance metric metric (Danielsson, 1980) via Morpheus software (Broad Institute). Color gradients indicate AAI percentages, from 65% (blue) to 100 (orange) of similarity. B. parapertussis, B. bronchiseptica, and B. pertussis display the highest ANI values (orange dendrogram) indicating a close genetic relationship.*

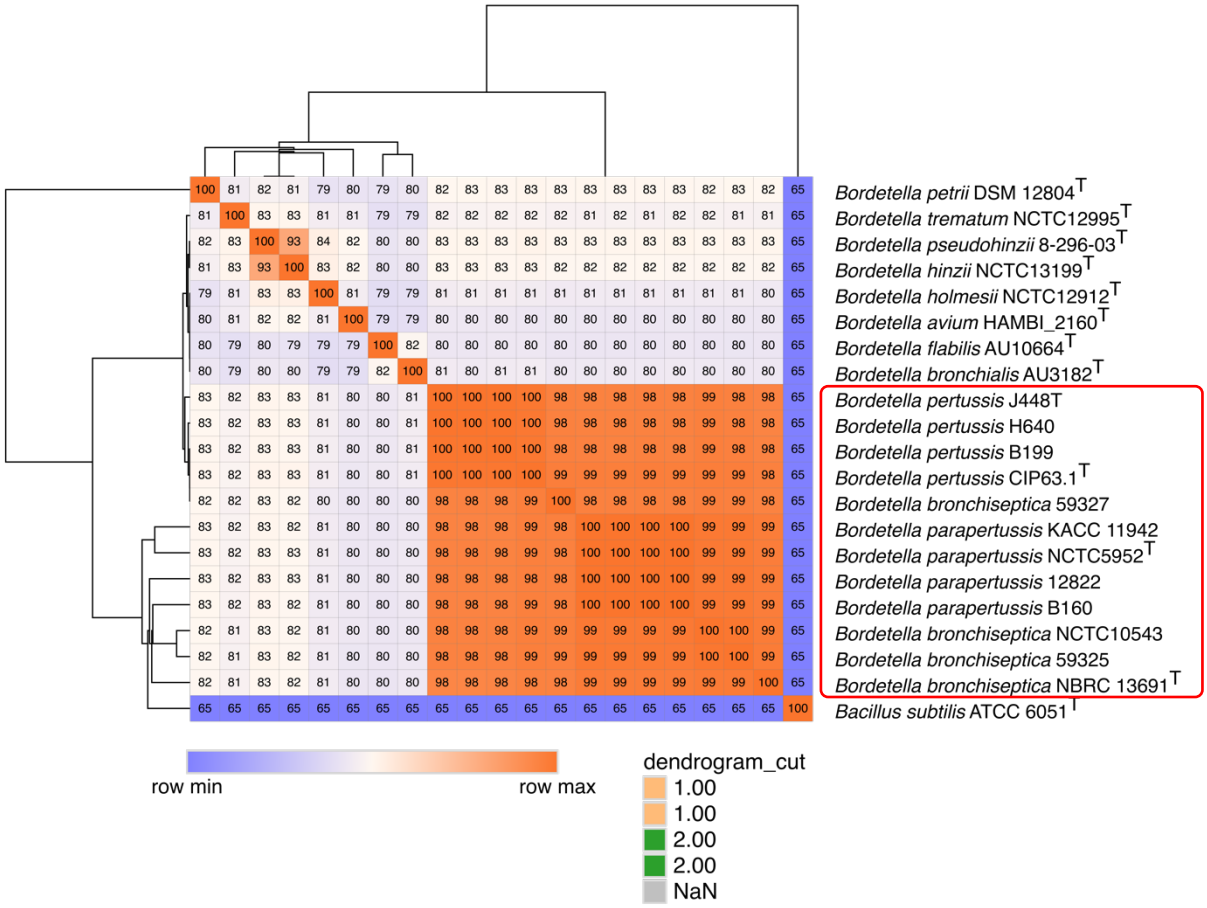


*Figure 31 :* ***Heatmap of Average Nucleotide Identity (ANI) values.*** *This illustrates genomic relatedness among strains of Bordetella species and their closest relatives with Bacillus subtilis ATCC 6051$^T$ used as an outgroup. ANI values were calculated using FastANI v1.3, with the heatmap generated via hierarchical clustering based on the Euclidean distance metric (Danielsson, 1980) using Morpheus software (Broad Institute). Color gradients reflect ANI percentages, ranging from 45 % (blue) to 100 % (orange) of similarity. B. parapertussis, B. bronchiseptica, and B. pertussis display the highest ANI values (orange dendrogram) indicating a close genetic relationship.*

### 3.1.2.3. Core genome-based phylogeny

A Core genome-based phylogeny analyses genes shared by all members of a species to understand their evolutionary history and thus avoids confusion due to horizontal gene transfer. This allows for clearer view of evolutionary relationships (Segata & Huttenhower, 2011).

It is widely applied in modern microbial taxonomy, often alongside ANI and dDDH, to define or revise species boundaries. It has facilitated reclassifications in genera such as *Mycobacterium* (Riojas et al., 2018), *Klebsiella pneumoniae* complex (Bialek-Davenet et al., 2014) and in the family of *Listeriaceae* (Bouznada et al., 2025). In our study, we conducted this core genome analysis on our *Bordetella* dataset using the BPGA pipeline and subsequently generated a Maximum Likelihood (ML) phylogenomic tree (**Figure 32**).

On this ML tree, all examined “classical” *Bordetella* strains (both type strains and additional) form a distinct and well-supported monophyletic cluster, with a bootstrap support value of 100. This robust

clustering and strong statistical support reinforce their close taxonomic relatedness and emphasizes the need for a thorough re-evaluation of their current classification.

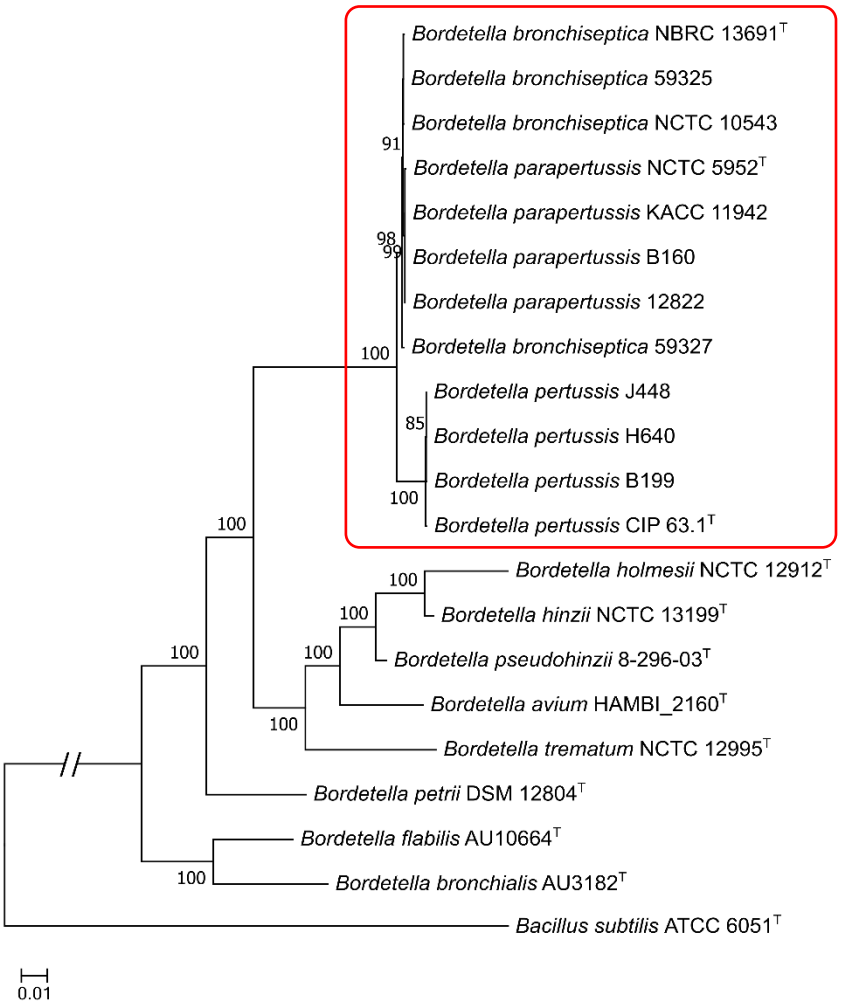


*Figure 32:* ***Maximum Likelihood Core-genome phylogenomic tree****. The phylogeny is built from the proteome of concatenated core genes of the Bordetella species inferred by the BPGA program (Meier-Kolthoff et al., 2013c). This illustrates the evolutionary relationships among strains of taxonomically debated Bordetella species and their closest relatives. Bacillus subtilis ATCC 6051$^T$ was used as an outgroup. Number above branches are bootstrap values (only values >50 % are shown). Bar 0.01 nucleotide substitution per site.*

### 3.1.2.4. Ortho Venn Gene Cluster Analysis

The Ortho Venn analysis provided a clear identification and visualization of orthologous gene clusters across the classical *Bordetella* species. This analysis revealed a total of 4341 gene clusters in *B. bronchiseptica*, 4251 in *B. parapertussis*, and 3402 in *B. pertussis*. Among these, 3220 orthologous gene clusters were shared across all three species, representing 94.65 % of the gene clusters in *B. pertussis*, 75.75 % in *B. parapertussis*, and 74.18 % in *B. bronchiseptica* (**Figure 33**).

The significant overlap of shared gene clusters clearly indicates a common ancestral genomic content, emphasizing the high evolutionary relatedness among these species. Furthermore, the observed reduction in the number of gene clusters from *B. bronchiseptica* to *B. parapertussis* (a 2.07 % decrease) and from *B. parapertussis* to *B. pertussis* (a 19.97 % decrease) reflects a divergent evolutionary trend characterized by gene decay. This pattern is likely linked to host restriction, a phenomenon well-documented in the literature supporting the idea of the evolution of *B. bronchiseptica , B. pertussis*, and *B. parapertussis*, from a "*Bronchiseptica- like ancestor*"(Cummings, Brinig, Lepp, Van De Pas, et al., 2004; Park et al., 2012; Parkhill et al., 2003).

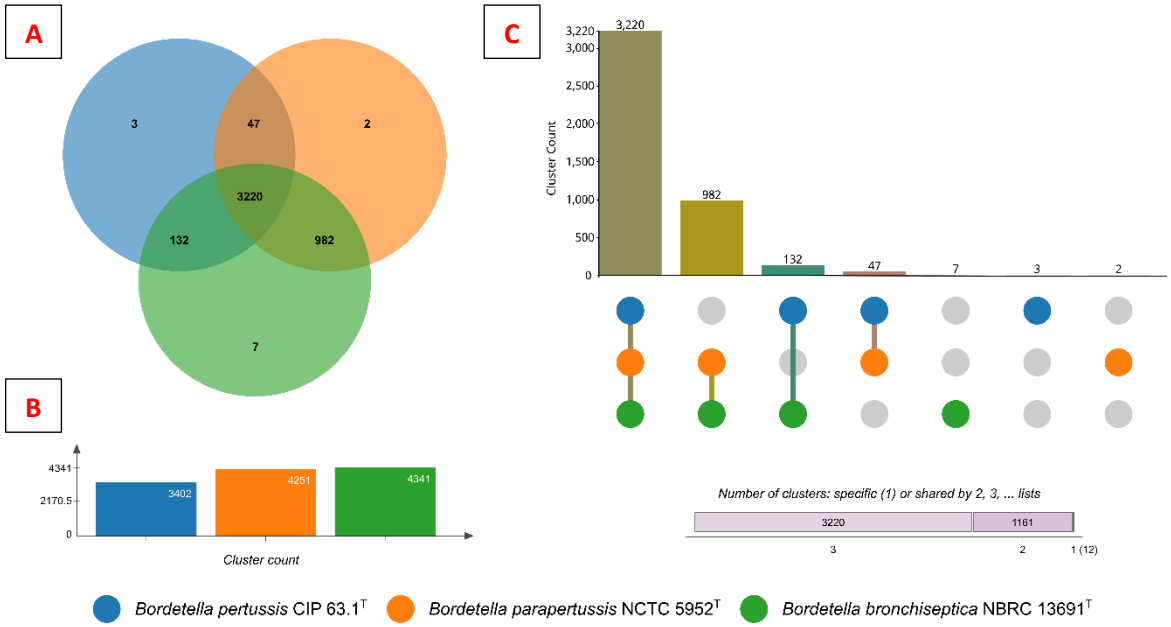


*Figure 33:* ***Distribution of Orthologous Gene Clusters Among the Classical Bordetella Species****. (A) The Venn diagram displays orthologous gene clusters shared among all three strains, those shared between two strains, and strain-specific singletons. (B) Total number of cluster counts in each species (C) Bar graphs showing the distribution of shared cluster counts among these three strains.*

### 3.1.2.5. Subsystem-Based Functional Gene Annotation Analysis

To better understand the genomic diversity among the classical *Bordetella* species, we conducted a subsystem analysis on the type strains *B. pertussis* CIP 63.1$^T$, *B. parapertussis* NCTC 5952T, and *B. bronchiseptica* NBRC 13691$^T$. This analysis identified the functional gene clusters in each species genome i.e. the number of subsystems and genes associated, as shown in **Figure 34**.

The results indicate that the three species exhibit a significant functional similarity, as evidenced by the near-equal number of subsystems and genes particularly in the energy, protein processing, DNA and RNA processing, and pathways. This high degree of similarity strongly suggests a close phylogenetic relationship and a common evolutionary ancestry.

The results also highlight a progressive increase in the number of subsystems and gene counts related to metabolism, membrane transport, stress response, and virulence pathways from *B. pertussis* to *B. parapertussis*, and then to *B. bronchiseptica*, with *B. bronchiseptica* exhibiting the highest number. This variation in gene content is primarily attributable to the differences in host specificity and adaptation among these strains.

*B. bronchiseptica* NBRC 13691T, capable of surviving in various hosts and environmental conditions, exhibits the highest number of subsystems and gene counts, granting it the highest metabolic flexibility. This flexibility enables it to infect a wide range of mammals (Goodnow, 1980; Woolfrey & Moody, 1991). In contrast, *B. parapertussis* NCTC 5952$^{T}$, which is restricted to humans and sheep hosts (Brinig et al., 2006), possesses a slightly lower count of these genes. This decrease is likely as a result of its partial adaptation to more specialized ecological niches, corresponding to its narrower host range compared to *B. bronchiseptica*.

Lastly, *B. pertussis* CIP 63.1T, a strictly human-adapted pathogen (Baroli et al., 2023; Melvin et al., 2014), exhibits the most significant genome reduction, particularly in energy metabolism pathways. This reduction reflects its adaptation to a highly specialized and restricted ecological niche i.e. the human host in which nutrient availability is more controlled and less variable, as well as a strong reliance on these host-derived nutrients.

These functional differences suggest that *B. pertussis* and *B. parapertussis* evolved separately from a *B. bronchiseptica*-like ancestor, gradually losing metabolic functions as they adapted to a parasitic lifestyle within their respective specific hosts (Taylor-Mulneix et al., 2017).

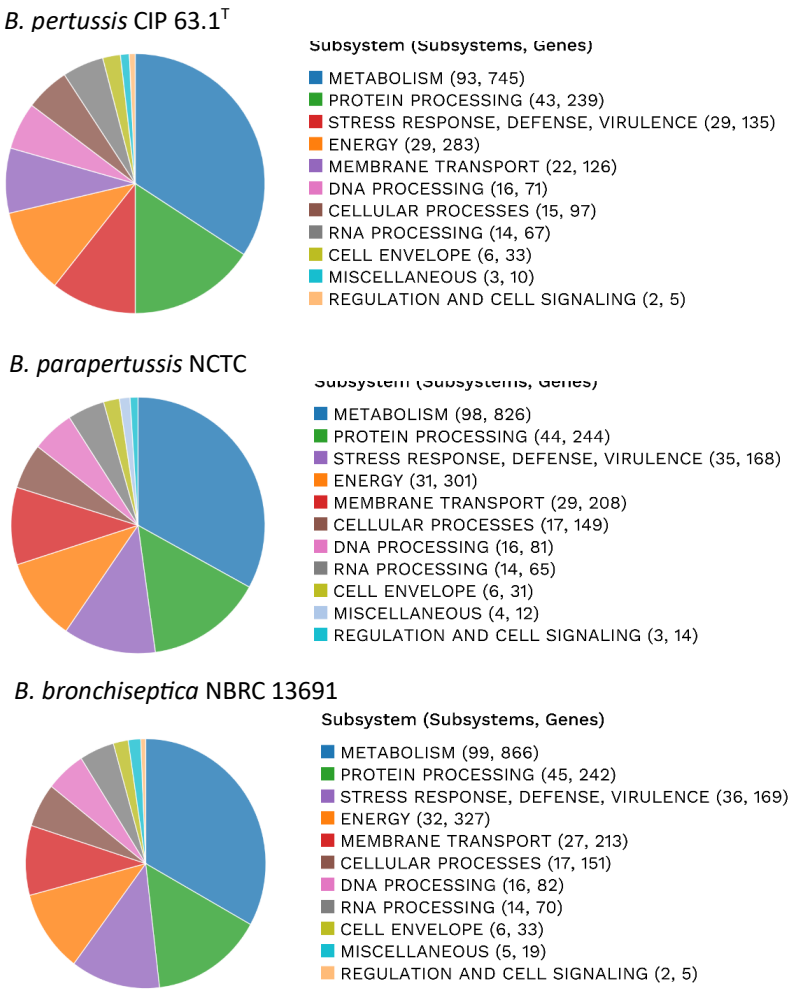


*Figure 34 :* ***Functional Categorization of Annotated Genes in the Bordetella strains by the RAST toolkit.***

### 3.1.3. Phenotypic Feature Analysis

All the prior analyses from 16S rRNA phylogenetics to Ortho Venn analyses of gene cluster distributions, primarily aimed at examining the genetic similarities and differences among the strains. So, for a more comprehensive assessment, we also conducted phenotypic analysis to account for gene expression.

A comprehensive literature review of the phenotypic characteristics of *B. pertussis* ATCC 9797$^T$, *B. parapertussis* ATCC 15311$^T$, and *B. bronchiseptica* NBRC 13691$^T$ shows that all these strains share common characteristics such as being Gram-negative, rod-shaped, and aerobic (**Table V**). However, notable differences were observed in traits such as motility, temperature tolerance, and enzyme activity, reflecting their distinct ecological adaptations and host-specific evolutionary paths.

*B. bronchiseptica* possesses peritrichous flagella for motility, exhibit the highest salt tolerance and can grow across a wide temperature range (10–41°C). These characteristics support its survival in diverse environments and hosts (domestic animals to wildlife) unlike the immotile, host restricted *B. pertussis* (humans) and *B. parapertussis* (humans and sheep) (Heininger et al., 2002).

While none of the three *Bordetella* strains ferment carbohydrates, *B. bronchiseptica* and *B. parapertussis* are capable of nitrate reduction and have urease activity, which contribute to their survival in a wider range of environments and hosts. In contrast, *B. pertussis*, which is limited to human hosts, shows a streamlined metabolism, reflecting its evolutionary adaptation to the specific conditions of the human respiratory tract (Rivera et al., 2020).

When cross-referencing these biochemical and physiological differences with genomic data, it becomes evident that these differences are more likely the result of pathoadaptive evolution rather than significant genetic divergence (Parkhill et al., 2003). While *B. bronchiseptica* is highly versatile, infecting a broad range of mammals, *B. pertussis* and *B. parapertussis* are more specialized to their respective hosts and the loss of certain phenotypic traits, reflects a pathoadaptive pattern of evolution for optimal survival within their host environments over time. This reinforces the hypothesis that these strains are likely ecotypes of a single species.

**Table V:** Phenotypic characteristics of *B. pertussis* CIP 63.1T, *B. bronchiseptica* NBRC 13691T, and B. *parapertussis* NCTC 5952T.

| Characteristic / Test | *B. pertussis* CIP 63.1T | *B. bronchiseptica* NBRC 13691T | B. parapertussis NCTC 5952T |
|---|---|---|---|
| **Basic characteristics** | | | |
| Gram stain | Negative | Negative | Negative |
| Cell shape | Rod-shaped | Rod-shaped | Rod-shaped |
| Motility | – | + (peritrichous) | – |
| Growth temperature range | 30 – 41 °C | 10 – 41 °C | 25 – 37 °C |
| Salt tolerance (NaCl) | 0–2 % | 0–4% | 0–2 % |
| Haemolysis type | Alpha haemolysis (1–2 days) | Gamma haemolysis (Clear zone) | ND |
| Oxygen Tolerance | ND | Obligate aerobe / aerobe | Obligate aerobe |
| **Carbohydrates & related compounds** | | | |
| D-glucose | – | – | – |
| Esculin | – | – | – |
| D-mannitol | – | – | – |
| D-sorbitol | – | – | – |
| Maltose | – | – | – |
| Mannose | – | – | – |
| Sucrose | – | – | – |
| Palatinose | ND | – | – |
| Ribitol | ND | – | – |
| Potassium 5-ketogluconate | ND | – | – |
| L-arabitol | ND | – | – |
| D-galacturonic acid | ND | – | – |
| Cellobiose | ND | – | – |
| Trehalose | ND | – | – |
| **Organic acids, amino acids & derivatives** | | | |
| Arginine | + | – | – |
| Adipate | – | ND | – |
| Decanoate | – | + | + |
| L-malate | – | – | – |
| Nitrate Reduction | – | + | + |
| Arabinose | – | – | – |
| Citrate | + | + | – |
| Myo-inositol | – | – | – |
| L-arabinose | – | – | – |

| Characteristic / Test | *B. pertussis* CIP 63.1T | *B. bronchiseptica* NBRC 13691T | B. parapertussis NCTC 5952T |
|---|---|---|---|
| **Enzyme & biochemical activities** | | | |
| Catalase | + | + | + |
| Cytochrome c oxidase | + | + | |
| Oxidase | | + | + |
| Urease | – | + | + |
| Indole production | – | – | – |
| Voges-Proskauer test | – | – | – |
| Methyl Red test | – | – | – |
| Gelatinase | + | – | – |
| DNase | + | – | ND |
| Caseinase | + | – | – |
| Trypsin | + | – | – |
| Leucine arylamidase | + | + | – |
| Valine arylamidase | – | – | – |
| Cystine arylamidase | – | – | – |
| Acid phosphatase | + | + | + |
| Alkaline phosphatase | + | – | – |
| Alpha-galactosidase | ND | – | – |
| Beta-galactosidase | – | – | – |
| N-acetyl-β-glucosaminidase | – | – | – |
| Alpha-mannosidase | – | – | – |
| Alpha-fucosidase | – | – | – |
| Beta-glucuronidase | – | – | – |
| Alpha-glucosidase | – | – | – |
| Beta-glucosidase | – | – | – |
| Esterase (C 8) | + | – | + |
| Esterase (C 4) | + | + | + |
| Lipase (C 14) | + | – | – |
| Lysine decarboxylase | – | – | – |
| Ornithine decarboxylase | – | – | – |
| Phenylalanine ammonia-lyase | – | – | – |
| Tryptophan deaminase | – | – | – |
| Gamma-glutamyltransferase | – | + | + |
| Lecithinase | – | – | – |
| Amylase | – | – | – |

**+, positive reaction; –, negative reaction; ND, not**

## 3.2. *Borrelia* Analysis

### 3.2.1. Phylogenetic Analysis Based on 16S rRNA Gene Sequences

To reassess the evolutionary relationships and taxonomic positions of our selected Borrelia species, we conducted a comparative 16S rRNA gene-based phylogenetic analysis.

*B. garinii* and *B. bavariensis* exhibited a **99.63% sequence similarity** (**Table VI**). Similarly, *B. bissettiae*, *B. kurtenbachii*, and *B. carolinensis* also showed high similarity (99.27%–99.80%) ( **Table VII**). These values are well above the 16S rRNA gene similarity thresholds for species delineation generally range of 97% to 99% (Kim et al., 2014; Stackebrandt & Goebel, 1994). This indicates a very close evolutionary relationship among the species within the respective groups.

Additionally, in all the phylogenetic trees constructed using Maximum Likelihood, Neighbor-Joining, and Maximum Parsimony methods (**Figure 35, Figure 36, Figure 37**), the *Borrelia* strains of the two groups respectively cluster together in two separate monophyletic clades on both phylogenetic trees with exception to *B. carolinensis* in the 16S rRNA-based phylogenetic tree. This further indicates a close evolutionary and taxonomic relationship among the species within each respective group.

***Table VI**: 16S rRNA Pairwise Percentage Gene Similarity Between B. garinii and B. bavariensis*

| | ***B. garinii*** | ***B. bavariensis*** |
|---|---|---|
| ***B. garinii*** | 100 % | 99.63 % |
| ***B. bavariensis*** | 99.63 % | 100 % |

***Table VII:** 16S rRNA Pairwise Percentage Gene Similarity among B. bissettiae, B. caroliniensis and B. kurtenbachii.*

| | ***B. bissettiae*** | ***B. caroliniensis*** | ***B. kurtenbachii*** |
|---|---|---|---|
| ***B. bissettiae*** | 100 % | 99.34 % | 99.80 % |
| ***B. caroliniensis*** | 99.34 % | 100 % | 99.27 % |
| ***B. kurtenbachii*** | 99.80 % | 99.27 % | 100 % |

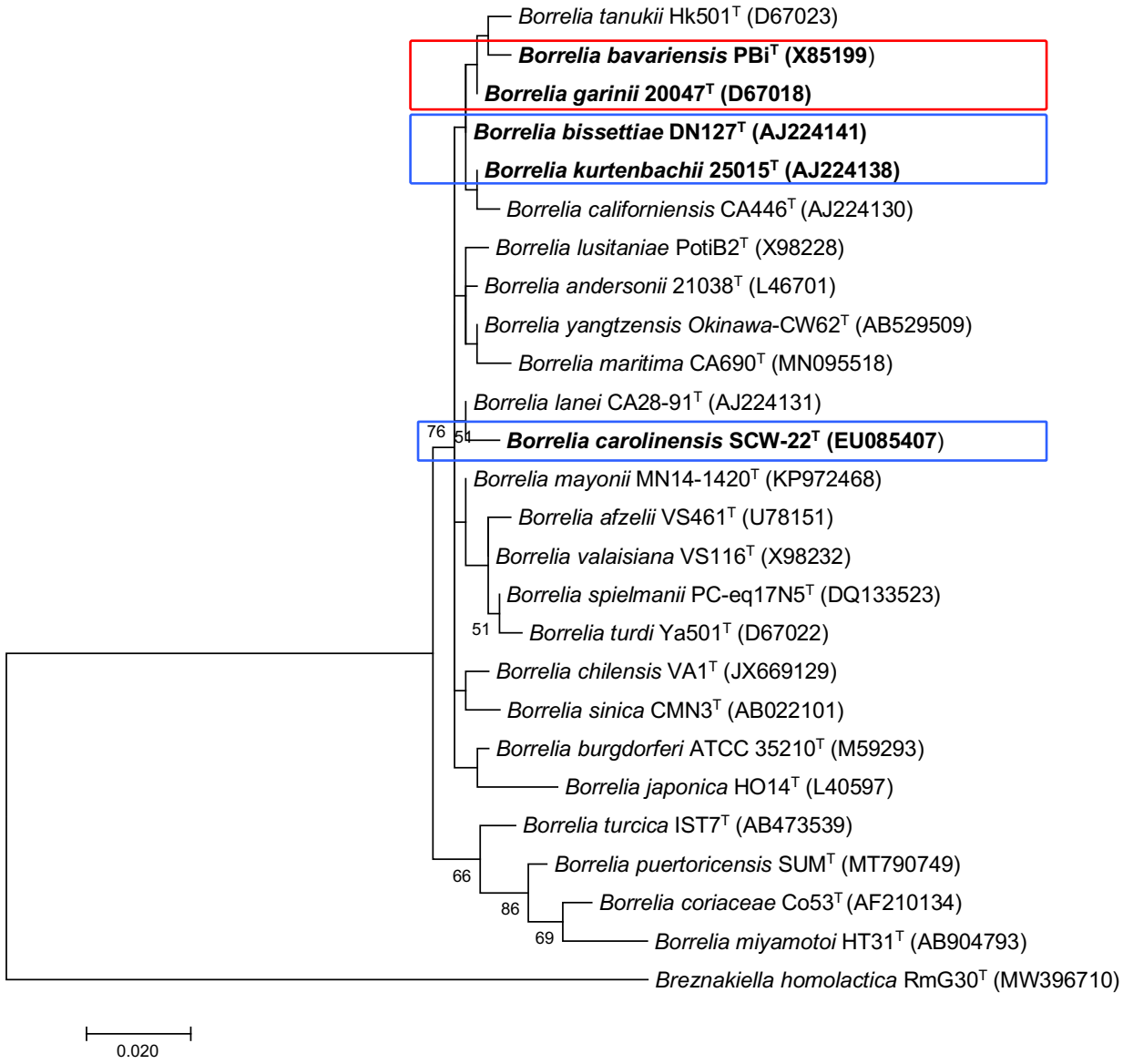


*Figure 35 :* ***The 16S rRNA Phylogenetic Tree based on the Maximum Likelihood Method.*** *The tree was inferred for the near-complete 16S rRNA gene sequences using the Maximum Likelihood Method and Tamura-Nei Model (Tamura & Nei, 1993) where the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). This phylogenetic tree illustrates the close evolutionary relationship between Borreliella bavariensis PBi$^T$ and Borreliella garinii CIP 103362 plus Borreliella bissettiae DN127$^T$, Borreliella caroliniensis SCW 22$^T$, and Borreliella kurtenbachii 25015$^T$, and their taxonomic position among the other closely related type strains in the Borrelia Genus. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values >50 % are shown) (Felsenstein, 1985). Breznakiella homolactica RmG30$^T$ was used as an outgroup. Bar 0.002 nucleotide substitution per site. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016b).*

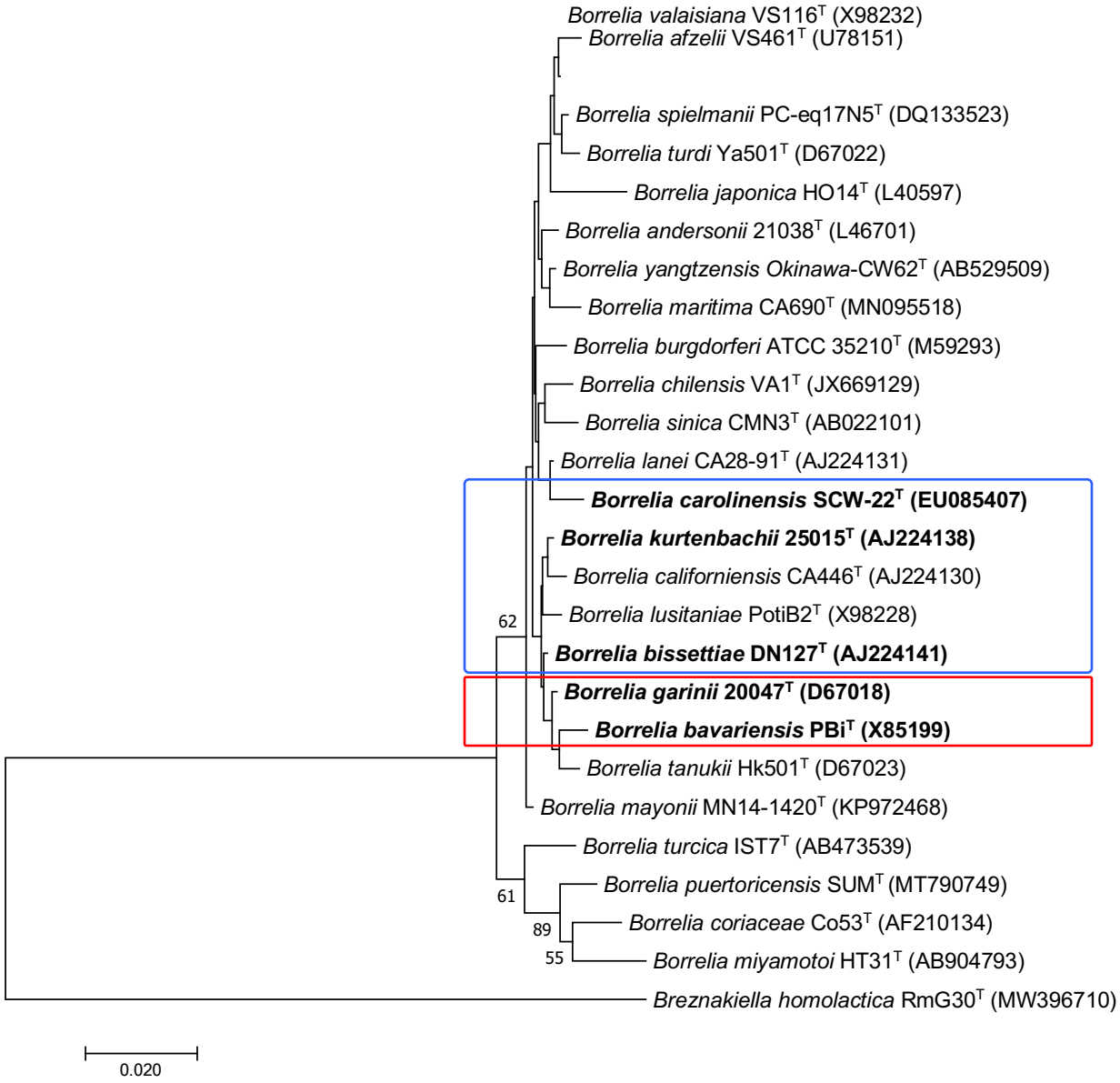


*Figure 36:* ***The 16S rRNA Phylogenetic Tree based on the Neighbor Joining Method.*** *The tree was inferred for the near-complete 16S rRNA gene sequences using Neighbor Joining method (Saitou & Nei, 1987) where the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). This phylogenetic tree illustrates the close evolutionary relationship between Borreliella bavariensis PBi$^T$ and Borreliella garinii CIP 103362$^T$, plus Borreliella bissettiae DN127$^T$, Borreliella caroliniensis SCW 22$^T$ and Borreliella kurtenbachii 25015$^T$, and their taxonomic position among the other closely related type strains in the Borrelia Genus. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values >50 % are shown) (Felsenstein, 1985). Breznakiella homolactica RmG30$^T$ was used as an outgroup. Bar 0.002 nucleotide substitution per site. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).*

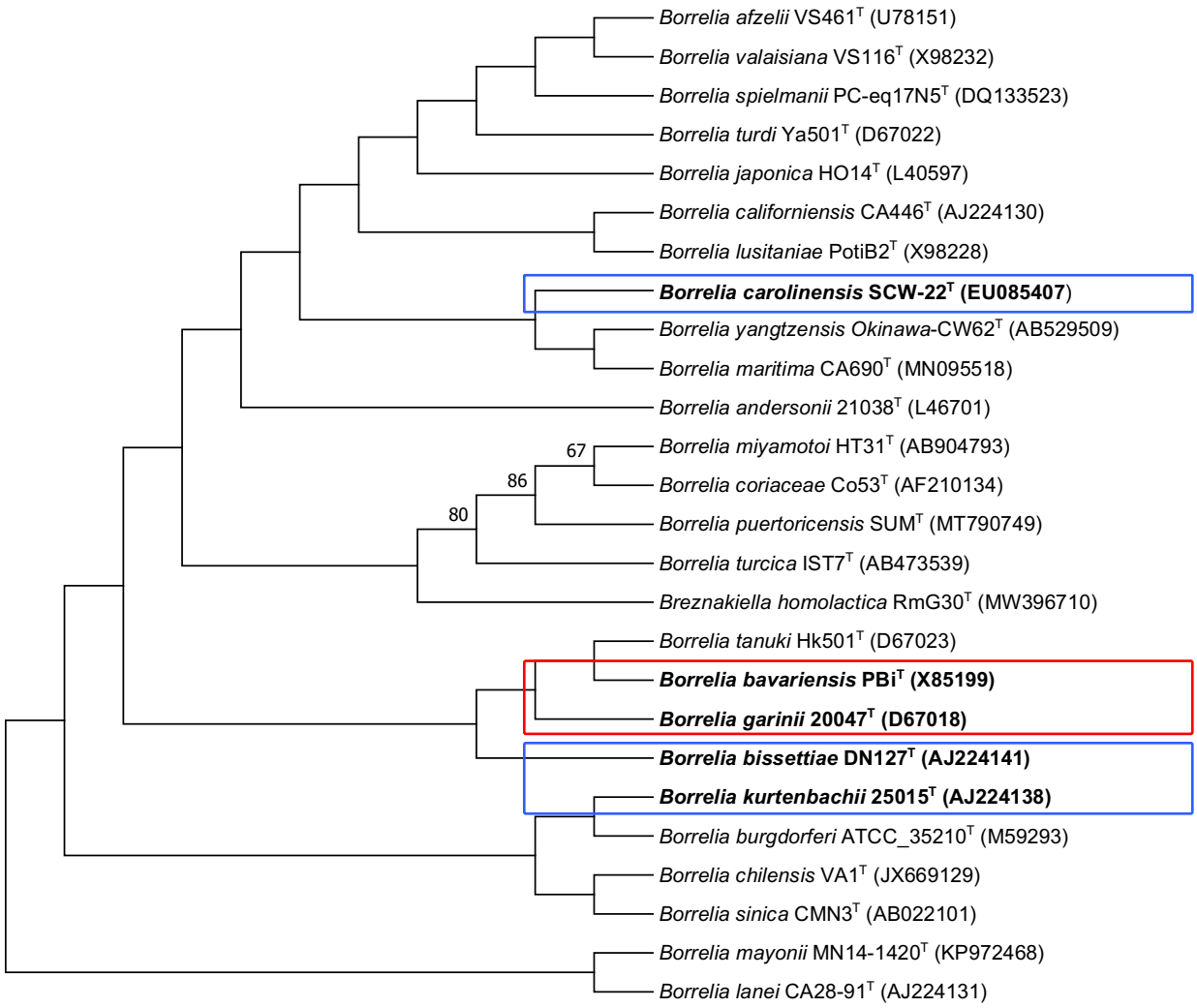


*Figure 37 :* ***The 16S rRNA Phylogenetic Tree based on the Maximum Parsimony Method.*** *The tree was inferred for the near-complete 16S rRNA gene sequences using the Maximum-Parsimony method (Nei & Kumar, 2000). The Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. This phylogenetic tree illustrates the close evolutionary relationship between Borreliella bavariensis PBi$^T$ and Borreliella garinii CIP 103362$^T$, plus Borreliella bissettiae DN127$^T$, Borreliella caroliniensis SCW 22$^T$, and Borreliella kurtenbachii 25015$^T$, and their taxonomic position among the other closely related type strains in the Borrelia Genus. Numbers at the nodes are bootstrap values, expressed as a percentage of 1000 resamplings (only values >50 % are shown) (Felsenstein, 1985). Breznakiella homolactica RmG30$^T$ was used as an outgroup. Bar 0.002 nucleotide substitution per site. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).*

## 3.2.2. Phylogenomic Delineation and Comparative Genomic Analysis

### 3.2.2.1. Pairwise Genomic Comparisons and dDDH Estimation

To further examine these the *Borrelia* taxonomic relationships, pairwise comparisons among the genomes were conducted using the GBDP approach via the Type (Strain) Genome Server (TYGS). Digital

DDH values and their confidence intervals were also calculated using the GGDC 4.0 (Meier-Kolthoff et al., 2013, 2022).

The obtained pairwise dDDH values (Table VIII) shows that the *Borrelia* analysed strains were generally above the 70 % DDH threshold commonly used for species delineation implying a significant genetic similarity among these species (Meier-Kolthoff et al., 2013; Moore et al., 1987).

The phylogenetic analysis conducted using the GBDP approach yielded a 16S rRNA-based phylogenetic tree **(Figure 38)** and a Whole genome based phylogenetic tree **(Figure 39)**. In both trees, the *Borrelia* strains of the two groups respectively display similar colours in the species cluster columns of the tree's metadata, indicating their grouping within the same genomic cluster. Additionally, these strains respectively cluster together in two separate monophyletic clades on both phylogenetic trees with exception to *B. carolinensis* in the 16S rRNA-based phylogenetic tree.

Therefore, the high dDDH values and the formation of a single monophyletic clade across both 16S rRNA and whole-genome phylogenies indicate that these strains share a close genomic relatedness. Furthermore, their uniform clustering in species columns of the tree's metadata (same colour) suggests that they constitute a single genomic group, rather than representing separate species.

***Table VIII :*** *Pairwise Digital DNA–DNA Hybridization values of the analysed Borrelia species.*

| | ***dDDH values*** | ***Confidence Intervals*** |
|---|---|---|
| ***B. garinii vs B. bavariensis*** | 77 % | 77.1 – 71.8 |
| ***B. bissettiae vs B. carolinensis*** | 80 % | 77.4 – 83.0 |
| ***B. bissettiae vs B. kurtenbachii*** | 63 % | 60.1 – 65.8 |
| ***B. kurtenbachii vs B. carolinensis*** | 72 % | 69.7 – 75.6 |

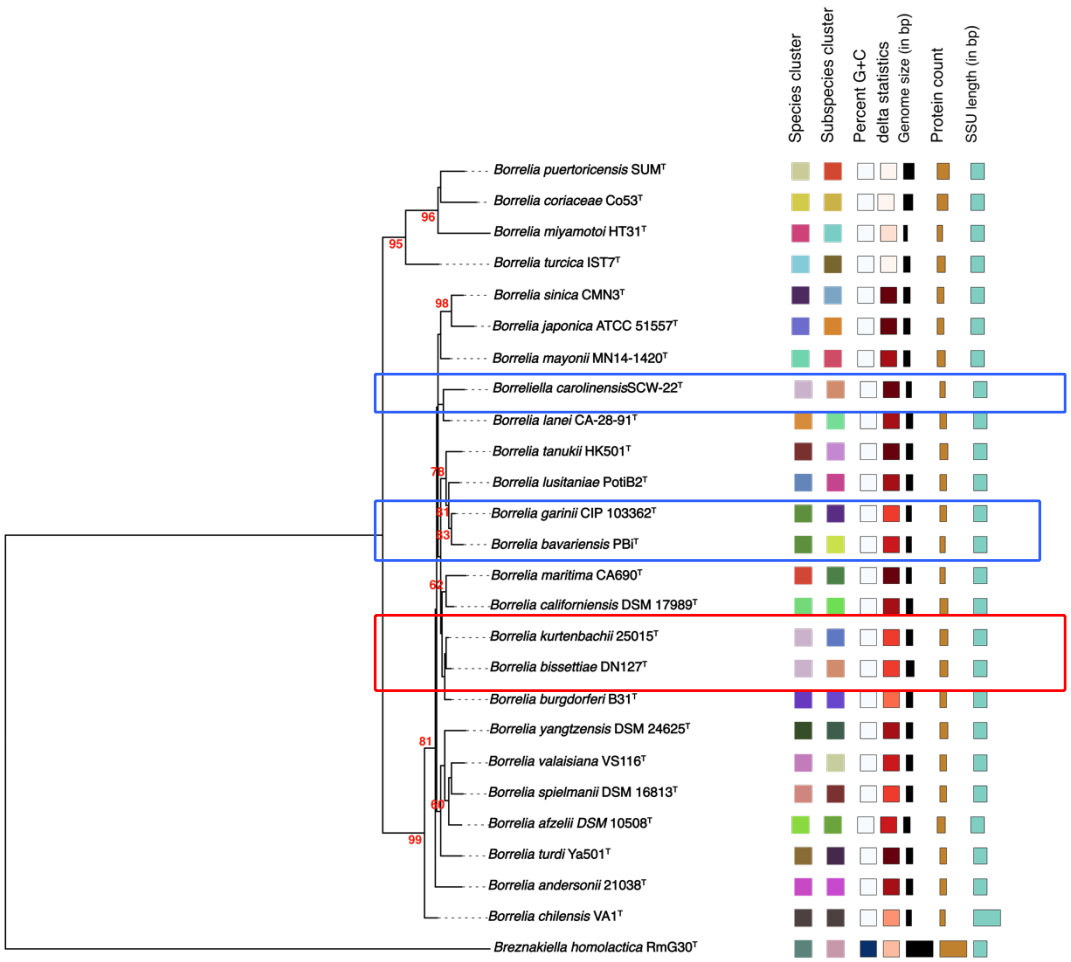


*Figure 38 :* ***The 16S rRNA Phylogenetic Tree based on the GBDP Approach.*** *This phylogeny was* inferred *with FastME 2.1.6.1 (Lefort et al., 2015) based on the GBDP approach (Meier-Kolthoff et al., 2013). It illustrates the evolutionary relationship among strains of taxonomically debated Borrelia species and their closest relatives with Breznakiella homolactica RmG30$^T$used as an outgroup. The numbers above the branches are GBDP pseudo-bootstrap support values from 100 replications (only values >50 % are shown). Color-coded metadata columns indicate key genomic features such as species/subspecies groupings, G+C content, genome size, and protein count. Clusters of the same species or subspecies are highlighted in matching colours.*

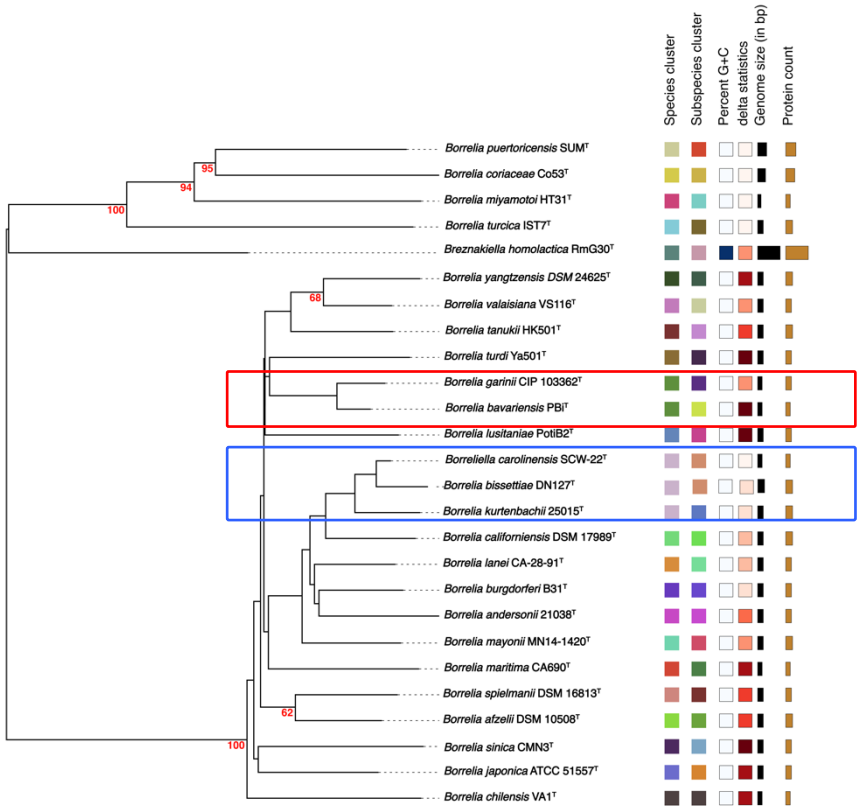


*Figure 39 :* ***Whole-genome Phylogenomic Tree.*** This phylogeny was *inferred with FastME 2.1.6.1 (Lefort et al., 2015) based on the GBDP approach (Meier-Kolthoff et al., 2013). It illustrates the evolutionary relationship among strains of taxonomically debated Borrelia species and their closest relatives with Breznakiella homolactica RmG30 $^T$ used as an outgroup. The numbers above the branches are GBDP pseudo-bootstrap support values from 100 replications (only values >50 % are shown). Color-coded metadata columns indicate key genomic features such as species/subspecies groupings, G+C content, genome size, and protein count. Clusters of the same species or subspecies are highlighted in matching colours.*

### 3.2.2.2. Overall Genome Relatedness Indices (OGRIs)

#### 3.2.2.2.1 Average Nucleotide Identity (ANI)

To further assess the evolutionary relatedness among these *Borrelia* species, this analysis was performed.

Within the resulting ANI matrix (**Figure 40**), *B. garinii* CIP 103362$^T$ and *B. bavariensis* PBi$^T$ cluster closely together with an ANI value of **97 %**. Similarly, *B. bissettiae* DN127$^T$, *B. carolinensis* SCW-22$^T$, *and B. kurtenbachii* 25015$^T$ also cluster closely together with ANI values ranging from **96 %** to **98 %.** All these values are well above the 95 % ANI threshold value used for species delineation.

These observations further underscore the tight genetic similarity among the species within the two groups of Borrelia strains that were analysed and further suggests that organisms are so genetically similar that they should be considered part of the same species, rather than being separate species.

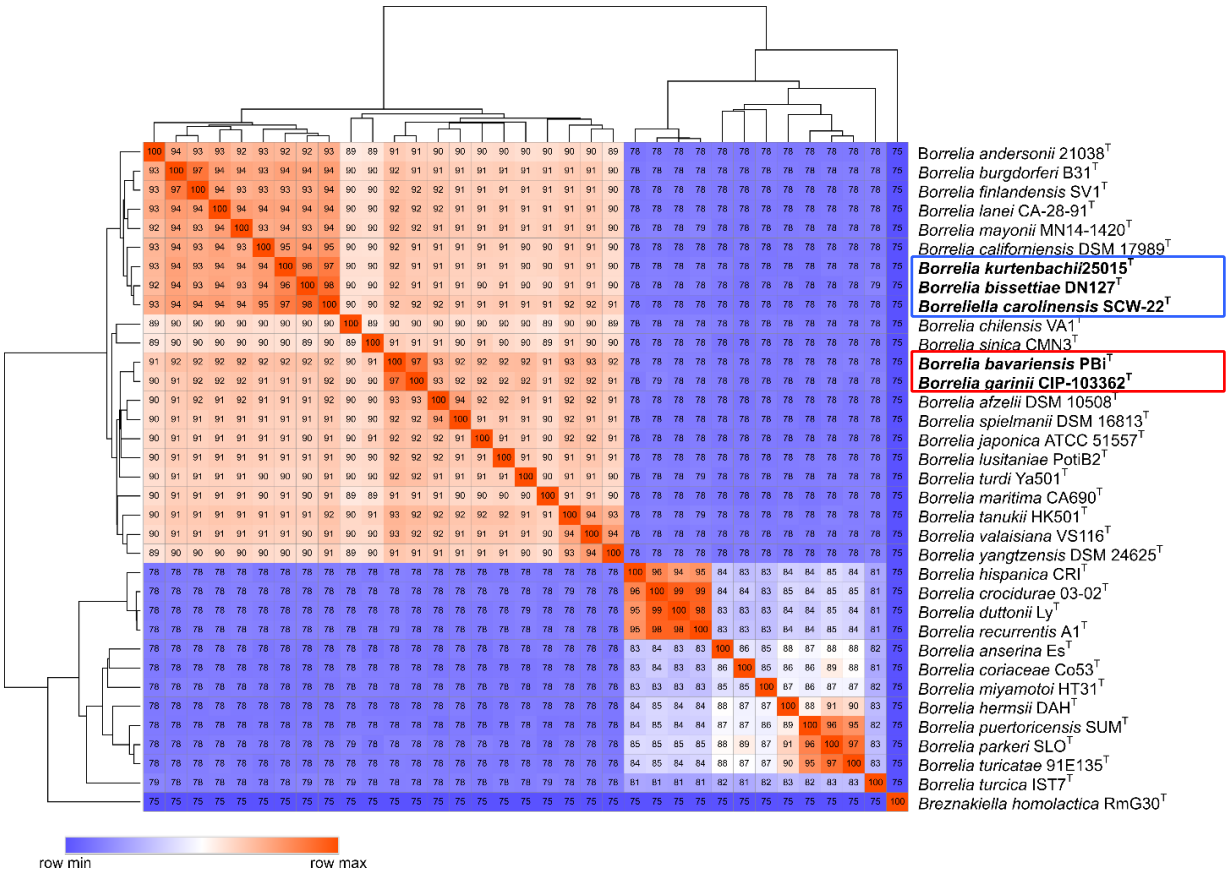


*Figure 40:* ***Heatmap of Average Nucleotide Identity (ANI) values.*** *This illustrates genomic relatedness among strains of Borrelia species and their closest relatives with Breznakiella homolactica RmG30$^T$ used as an outgroup. ANI values were calculated using FastANI v1.3, with the heatmap generated via hierarchical clustering based on the Euclidean distance metric (Danielsson, 1980) using Morpheus software (Broad Institute). Color gradients reflect ANI percentages, ranging from the lowest* ***75 %*** *(blue) to the highest* ***100 %*** *(red). Borreliella bavariensis PBi$^T$ and Borreliella garinii CIP 103362$^T$, plus Borreliella bissettiae DN127$^T$, Borreliella caroliniensis SCW 22$^T$, and Borreliella kurtenbachii 25015$^T$, display high ANI values indicating their close genetic relationship.*

### 3.2.2.3. Ortho Venn Gene Cluster Analysis

This analysis provided insights on orthologous gene clusters shared among the Borrelia species within the two analysed groups.

For the first group (**Figure 41**), a total of **934** gene clusters in ***B. garinii*** and 889 in ***B. bavariensis***. While 45 gene clusters were specific to *B. garinii*, no singletons were found in *B. bavariensis* genome.

For the second group (

*Figure 42*) a total of **1073** gene clusters in ***B. bissettiiae, 1069*** in ***B. kurtenbachii*** and **896** in ***B. carolinensis*** were identified. Among these, **889** orthologous gene clusters were shared across all three species, representing **82.85 %** of the gene clusters in *B. bissettiiae,* **83.16 %** in *B. kurtenbachii* and **99.21 %** in *B. carolinensis* (**Figure 33**).

Notably, *B. bissettii* and *B. kurtenbachii* share **161 clusters** not found in *B. carolinensis*. Also, Minimal overlap between *B. bissettiiae* & *B. carolinensis* (4 clusters), and *B. kurtenbachii* & *B. carolinensis* (3 clusters). While *B. bissettii* and *B. kurtenbachii* each possess **19** and **16 unique gene clusters** respectively, B. carolinensis does not have any species-specific (singleton) clusters.

The significant overlap in orthologous genes strongly points to a shared ancestral genetic origin, emphasizing the close evolutionary ties between these species. Furthermore, the lack of species-specific genes (singletons) in *B. bavariensis* and *B. carolinensis* within their respective groups further suggests a high degree of genomic similarity within these groups. These observations support the idea of their genetic proximity and provide additional evidence for reclassifying the Borrelia species as strains of the same species rather than separate species.

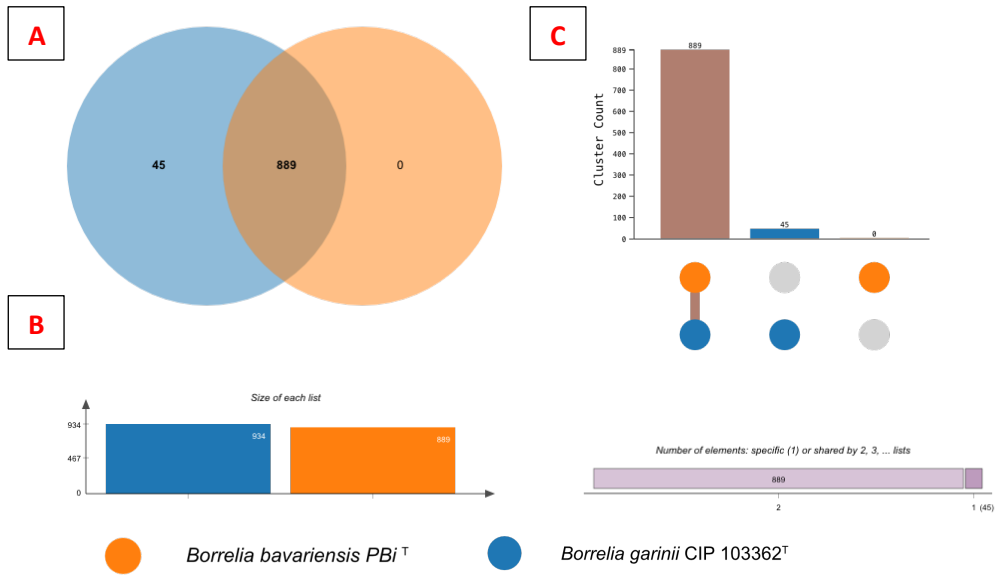


*Figure 41:* ***Distribution of Orthologous Gene Clusters among B. garinii and B. bavariensis****. (A) The Venn diagram displays orthologous gene clusters shared among the two strains and the strain-specific singletons. (B) Total number of clusters counts in each species (C) Bar graphs showing the distribution of shared cluster counts among these two strains.*

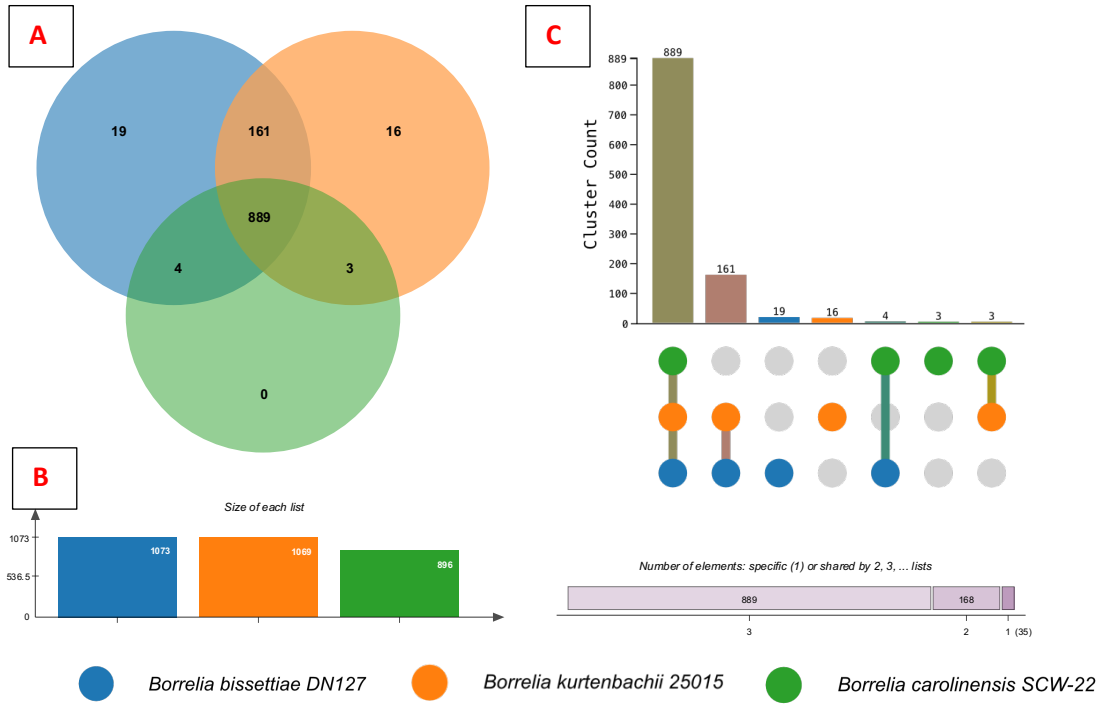


*Figure 42:* ***Distribution of Orthologous Gene Clusters among B. bissettiiae, B. kurtenbachii and B. carolinensis****. (A) The Venn diagram displays orthologous gene clusters shared among the three strains and the strain-specific singletons. (B) Total number of clusters counts in each species (C) Bar graphs showing the distribution of shared cluster counts among these three strains.*

### 3.2.2.4. Subsystem-Based Functional Gene Annotation Analysis

This analysis identified the functional gene clusters within each of the analysed *Borrelia* species.

For the first group (Figure 43), the results indicate that ***B. garinii*** and ***B. bavariensis*** exhibit a significantly high functional similarity as evidenced by the equal number of subsystems and genes across all the analysed functional subsystems with a very subtle difference in the number of genes responsible for stress response, defence, virulence and RNA processing.

For the second group (Figure 44), the results indicate that ***B. bissettiiae, B. kurtenbachii*** and ***B. carolinensis*** also exhibit a significantly high functional similarity as evidenced by the equal number of subsystems and genes across all the analysed functional subsystems with a very subtle difference in the number of genes responsible form metabolism, energy and stress response, defence, virulence processes.

These results demonstrate significant functional similarity both within the *B. garinii* and *B. bavariensis* group, as well as within the *B. bissettiiae*, *B. kurtenbachii*, and *B. carolinensis* group. Overall, this provides further evidence supporting the idea of grouping these species more closely, potentially as strains of the same species.

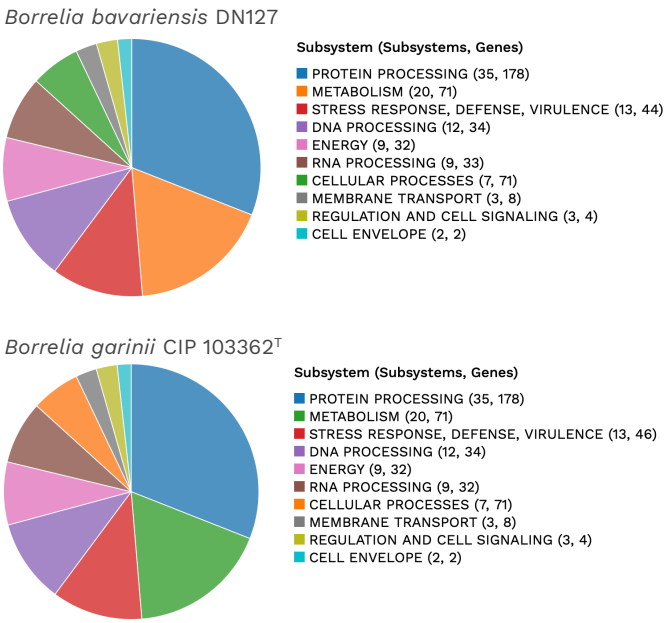


*Figure 43:* ***Functional Categorization of Annotated Genes within the B. garinii and B. bavariensis by the RAST toolkit.***

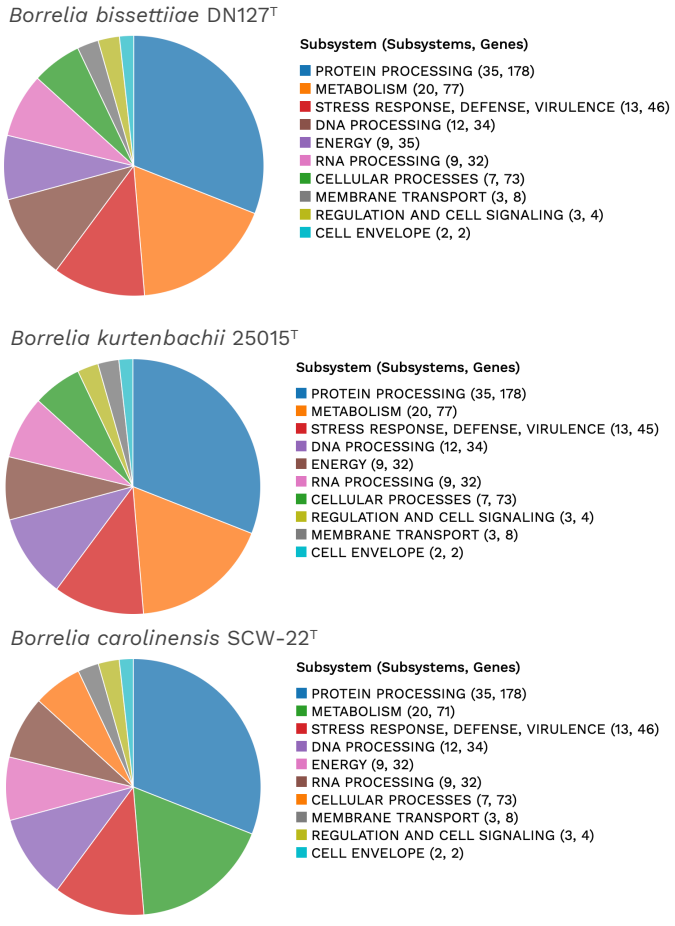


*Figure 44:* ***Functional Categorization of Annotated Genes within the B. bissettiiae, B. kurtenbachii and B. carolinensis by the RAST toolkit.***

# CONCLUSION

# TAXONOMIC CONCLUSION

## a. Taxonomic Conclusion on *Bordetella Species*

This comprehensive phylogenomic analysis of *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* revealed a very close taxonomic relationship among these species.

Phylogenetic analyses based on 16S rRNA gene sequences, whole genomes, and core genomes consistently grouped them into a single, well-supported monophyletic clade across all phylogenetic trees, indicating strong evolutionary relatedness.

Furthermore, the OGRIs calculations including digital DNA-DNA hybridization (87–91 %), Average Nucleotide Identity (98–99 %), and Average Amino acid Identity (98 %), consistently exceed species-level thresholds (dDDH ≥70 %, ANI/AAI ≥95–96 %). This indicated a shared evolutionary origin and a high degree of gene conservation among the analysed strains.

Then, Complementary insights from the OrthoVenn and Subsystem-based functional analyses further reinforce the hypothesis that these species evolved from a common *B. bronchiseptica*-like ancestor, undergoing genome reduction along a pathoadaptive trajectory associated with host specialization.

So, despite their known differences in host specificity and pathogenicity, our collective findings support the unification of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* as host-adapted variants of a single species.

Therefore, in accordance with the International Code of Nomenclature of Prokaryotes (ICNP), we propose a reclassification ***B. bronchiseptica*** (Ferry 1912) Moreno-López 1952 (Approved Lists 1980) and ***B. parapertussis*** (Eldering and Kendrick 1938) Moreno-López 1952 (Approved Lists 1980) as later heterotypic synonyms of ***B. pertussis*** (Bergey et al. 1923) Moreno-López 1952 (Approved Lists 1980).

This taxonomic revision better reflects their genomic coherence and evolutionary history which provides a unified framework that can aid in diagnostics, vaccine design, and epidemiological surveillance.

## b. Taxonomic Conclusion on *Borrelia Species*

Similarly, the comprehensive phylogenomic analysis of the *B. garinii* and *B. bavariensis* group, as well as the *B. bissettiae*, *B. kurtenbachii*, and *B. carolinensis* group, revealed a very close taxonomic relationship among these species.

Phylogenetic analyses based on their 16S rRNA gene sequences and whole genomes consistently clustered each group into a single monophyletic clade across all phylogenetic trees, indicating strong evolutionary relatedness.

Additionally, their digital DNA-DNA hybridization and Average Nucleotide Identity values consistently exceeded their respective species-level thresholds (dDDH ≥70 %, ANI ≥95–96 %). This clearly suggests a common evolutionary origin and a strong level of gene conservation among the strains analysed. The OrthoVenn and Subsystem-based functional analyses also pointed out a high degree of genomic similarity among the species within those *Borrelia* groups.

The results collectively underscore the close evolutionary and taxonomic relatedness of the analysed *Borrelia* species in the two groups challenging their current classification as separate species.

Therefore, in accordance with the International Code of Nomenclature of Prokaryotes (ICNP), we propose a reclassification of ***Borrelia bavariensis*** (Margos *et al.* 2013) as a later heterotypic synonym of ***Borrelia garinii*** (Baranton *et al.* 1992). Similarly, we propose a reclassification of ***Borrelia carolinensis*** (Rudenko *et al.* 2011) and ***Borrelia kurtenbachii*** (Margos *et al.* 2014) as a later heterotypic synonym of ***Borrelia bissettiae*** (Margos *et al.* 2016).

## c. Limitations of Our Study.

Several challenges were encountered during the *Borrelia* analysis that significantly limited the overall effectiveness of this study.

First, we were unable to perform some key analyses i.e. the Average Amino acid Identity and Core genome analysis. The efforts to reach the EDGAR platform support team in order to perform the Average Amino Acid Identity (AAI) analysis were unsuccessful. Its alternative, the Kostas Lab platform, remained persistently under maintenance throughout the study period. These missing analyses are essential components of a robust and comprehensive taxonomic assessment.

Given that taxonomic resolution often relies on integrating results from multiple complementary methods, each compensating for the limitations of others, the absence of these analyses limited our ability to draw a definitive conclusion regarding the taxonomic ambiguities within the *Borrelia* group.

Additionally, our *Borrelia* dataset itself may have been of sub-optimal quality. This was reflected in the very weak bootstrap values observed across all the generated 16S rRNA phylogenetic trees, which may have negatively impacted the resolution and reliability of the phylogenetic inference.

Due to the time constraint of this dissertation, we were unable to address these issues within the *Borrelia* section. This therefore underscores the need for further in-depth investigations to clarify the taxonomic positions of these Borrelia species. We hope that the findings presented here will provide a valuable starting point for such future efforts.

## d. Future Perspectives.

The development of multiple platforms and software tools capable of performing key analyses in taxonomic research would help prevent delays in future studies caused by overreliance on a limited

number of existing servers. This would ensure greater continuity and efficiency in research, particularly in situations where widely used platforms are unavailable due to downtime or maintenance.

Greater efforts should be made to ensure that all genomic data available in public databases is consistently of high quality. This is essential to minimize inaccuracies in the results of taxonomic studies that rely on such data and allow better reproducibility of results within the scientific community.

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# APPENDICES

## Appendix A

Characteristics of the Genome Sequences of the *Bordetella* strains and outgroups used in our study i.e. *Bacillus subtilis* ATCC 6051$^T$ & *Achromobacter xylosoxidans* NCTC 10809$^T$.

| ORGANISM SCIENTIFIC NAME | ASSEMBLY ACCESSION | TAXONOMY ID | ASSEMBLY NAME | SOURCE | ANNOTATION | LEVEL | CONTIG N50 | SIZE | GENE COUNT | BIOPROJECT | BIOSAMPLE | GC % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Bordetella petrii DSM 12804*** | GCA_000067205.1 | 94624 | ASM6720v1 | GENBANK | Annotation submitted by Bielefeld Univ | Complete Genome | 5287950 | 5287950 | 5095 | PRJNA28135 | SAMEA3138272 | 65.5 |
| ***Bordetella pseudohinzii 8-296-03*** | GCA_000657795.2 | 1331258 | gbh03v02 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Contig | 256610 | 4538520 | 4247 | PRJNA202161 | SAMN02263900 | 66.5 |
| ***Bordetella bronchiseptica NBRC 13691*** | GCA_001598655.1 | 1216982 | ASM159865v1 | | | Contig | 62488 | 5115418 | | PRJDB244 | SAMD00046893 | 68.5 |
| ***Bordetella bronchialis AU3182*** | GCA_001676705.1 | 463025 | ASM167670v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 5878756 | 5878756 | 5179 | PRJNA318508 | SAMN05257177 | 67.5 |
| ***Bordetella flabilis AU10664*** | GCA_001676725.1 | 463014 | ASM167672v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 5835727 | 5954318 | 5322 | PRJNA318508 | SAMN05257178 | 66 |
| ***Bordetella parapertussis NCTC5952*** | GCA_900445785.1 | 519 | 52451_D01 | | Annotation submitted by SC | Contig | 4034469 | 4775492 | 4578 | PRJEB6403 | SAMEA24553918 | 68 |
| ***Bordetella holmesii NCTC12912*** | GCA_900445775.1 | 35814 | 51726_B01 | | Annotation submitted by SC | Contig | 3275926 | 3780341 | 3846 | PRJEB6403 | SAMEA4504060 | 62.5 |
| ***Bordetella avium HAMBI_2160*** | GCA_034424645.1 | 521 | ASM3442464v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 3721798 | 3721798 | 3449 | PRJNA1047486 | SAMN38562714 | 61.5 |
| ***Bordetella trematum NCTC12995*** | GCA_900445945.1 | 123899 | 52067_A01 | | Annotation submitted by SC | Contig | 4476238 | 4498245 | 4204 | PRJEB6403 | SAMEA4504055 | 65.5 |
| ***Bordetella hinzii NCTC13199*** | GCA_900637615.1 | 103855 | 52191_A01 | | Annotation submitted by SC | Complete Genome | 5033537 | 5033537 | 4794 | PRJEB6403 | SAMEA4530653 | 67 |
| ***Bordetella pertussis CIP63.1*** | GCA_965137715.1 | 520 | CIP63.1T | | Annotation submitted by Collection de l'Institut Pasteur | Scaffold | 18080 | 3852834 | 3892 | PRJEB85433 | SAMEA117660713 | 68 |
| ***Bordetella pertussis J448*** | GCA_001831455.1 | 520 | ASM183145v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 4386396 | 4386396 | 4192 | PRJNA279196 | SAMN05770316 | 68 |
| ***Bordetella pertussis B199*** | GCA_002892765.1 | 520 | ASM289276v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 4314502 | 4314502 | 4165 | PRJNA279196 | SAMN04388407 | 67.5 |
| ***Bordetella pertussis H640*** | GCA_004008975.1 | 520 | ASM400897v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 4088701 | 4088701 | 3962 | PRJNA279196 | SAMN08136977 | 67.5 |
| ***Bordetella bronchiseptica 59327*** | GCA_044619415.1 | 518 | ASM4461941v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Contig | 268279 | 5087024 | 4804 | PRJNA1079785 | SAMN40084193 | 68.5 |
| ***Bordetella bronchiseptica 59325*** | GCA_044619475.1 | 518 | ASM4461947v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Contig | 340604 | 5264860 | 5036 | PRJNA1079785 | SAMN40084191 | 68 |
| ***Bordetella bronchiseptica NCTC10543*** | GCA_900636925.1 | 518 | 45137_F01 | | Annotation submitted by SC | Complete Genome | 5199761 | 5199761 | 4847 | PRJEB6403 | SAMEA3893452 | 68.5 |
| ***Bordetella parapertussis 12822*** | GCA_000195695.1 | 257311 | ASM19569v1 | | Annotation submitted by Sanger Institute | Complete Genome | 4773551 | 4773551 | 4403 | PRJNA25 | SAMEA1705915 | 68 |
| ***Bordetella parapertussis KACC 11942*** | GCA_003428255.1 | 519 | ASM342825v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Contig | 188600 | 4727047 | 4505 | PRJNA484648 | SAMN09767464 | 68 |
| ***Bordetella parapertussis B160*** | GCA_004008215.1 | 519 | ASM400821v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 4775493 | 4775493 | 4494 | PRJNA287884 | SAMN08105890 | 68 |
| ***Achromobacter xylosoxidans NCTC10807*** | GCA_001457475.1 | 85698 | NCTC10807 | | Annotation submitted by SC | Complete Genome | 6813182 | 6813182 | 6192 | PRJEB6403 | SAMEA2517358 | 67.5 |
| ***Bacillus subtilis ATCC 6051*** | GCA_031316525.1 | 535026 | ASM3131652v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Contig | 2215957 | 4264639 | 4557 | PRJNA553198 | SAMN12236531 | |

## Appendix B

Characteristics of the Genome Sequences of the *Borrelia* strains and Outgroup used in our study i.e. *Breznakiella homolactica* RmG30$^{T}$.

| ORGANISM SCIENTIFIC NAME | ASSEMBLY ACCESSION | TAXONOMY ID | ASSEMBLY NAME | SOURCE | ANNOTATION | LEVEL | CONTIG N50 | SIZE | GENE COUNT | BIOPROJECT | BIOSAMPLE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Borrelia turicatae* 91E135** | GCA_000012085.2 | 314724 | ASM1208v2 | | Annotation submitted by Lab of Human Bacterial Pathenogenesis, RML, NIAID, NIH | Complete Genome | 917330 | 1165365 | 1092 | PRJNA13597 | SAMN02603508 |
| ***Borrelia duttonii* Ly** | GCA_000019685.1 | 412419 | ASM1968v1 | | Annotation submitted by Marseille-Nice Genopole | Complete Genome | 931674 | 1574881 | 1469 | PRJNA18231 | SAMN02603585 |
| ***Borrelia recurrentis* A1** | GCA_000019705.1 | 412418 | ASM1970v1 | | Annotation submitted by Marseille-Nice Genopole | Complete Genome | 930981 | 1242163 | 1140 | PRJNA18233 | SAMN02603586 |
| ***Borreliella valaisiana* VS116** | GCA_000170955.2 | 445987 | ASM17095v2 | | Annotation submitted by J. Craig Venter Institute | Complete Genome | 913294 | 1258864 | 1395 | PRJNA19843 | SAMN02436326 |
| ***Borreliella finlandensis* SV1** | GCA_000181875.2 | 498741 | ASM18187v2 | | Annotation submitted by J. Craig Venter Institute | Scaffold | 179974 | 1281782 | 1384 | PRJNA28631 | SAMN02436286 |
| ***Borreliella bavariensis* PBi** | GCA_000196215.1 | 290434 | ASM19621v1 | | Annotation submitted by Fritz Lipmann Institute (former Institute of Molecular Biotechnology, IMB) | Complete Genome | 904246 | 986914 | 969 | PRJNA12554 | SAMN02603240 |
| ***Borreliella bissettiae* DN127** | GCA_000222305.1 | 521010 | ASM22230v1 | | Annotation submitted by University of Maryland School of Medicine - Institute for Genome Sciences | Complete Genome | 900755 | 1403443 | 1518 | PRJNA29363 | SAMN02604204 |
| ***Borrelia hispanica* CRI** | GCA_000500065.1 | 1417229 | AB2 | | | Contig | 216648 | 1783846 | | PRJNA226260 | SAMN02471307 |
| ***Borrelia parkeri* SLO** | GCA_000568735.2 | 1313294 | ASM56873v2 | G | Annotation submitted by Integrated Genomics | Chromosome | 917680 | 1053291 | 1121 | PRJNA195597 | SAMN03081465 |
| ***Borreliella chilensis* VA1** | GCA_000808095.1 | 1245910 | ASM80809v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 900694 | 982238 | 940 | PRJNA266551 | SAMN03166165 |
| ***Borrelia crocidurae* 03-02** | GCA_000825665.2 | 29520 | Borrelia crocidurae str. 03-02 | | | Scaffold | 13697 | 920157 | | PRJEB7269 | SAMEA2768622 |
| ***Borreliella garinii* CIP 103362** | GCA_001922545.1 | 29519 | ASM192254v1 | E | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 905638 | 1156110 | 1149 | PRJNA350560 | SAMN05941958 |
| ***Borrelia anserina* Es** | GCA_001936255.1 | 1365188 | ASM193625v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 906833 | 1042690 | 963 | PRJNA212123 | SAMN04359737 |
| ***Borreliella mayonii* MN14-1420** | GCA_001945665.1 | 1674146 | ASM194566v1 | N | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 904387 | 1311545 | 1270 | PRJNA321302 | SAMN04979181 |
| ***Borrelia turcica* IST7** | GCA_003606285.1 | 1104446 | ASM360628v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 957653 | 1283624 | 1273 | PRJNA449848 | SAMN08918700 |
| ***Borrelia maritima* CA690** | GCA_008931845.1 | 2761123 | ASM893184v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 902176 | 1109596 | 1069 | PRJNA450015 | SAMN08925038 |
| ***Borreliella spielmanii* DSM 16813** | GCA_014201705.1 | 88916 | ASM1420170v1 | B | Annotation submitted by DOE Joint Genome Institute | Scaffold | 222578 | 1165303 | 1161 | PRJNA632298 | SAMN14908353 |
| ***Borreliella yangtzensis* DSM 24625** | GCA_014201775.1 | 683292 | ASM1420177v1 | | Annotation submitted by DOE Joint Genome Institute | Scaffold | 212868 | 1350499 | 1409 | PRJNA632131 | SAMN14908401 |
| ***Borreliella afzelii* DSM 10508** | GCA_014202295.1 | 29518 | ASM1420229v1 | A | Annotation submitted by DOE Joint Genome Institute | Scaffold | 222940 | 1331350 | 1358 | PRJNA632126 | SAMN14908385 |
| ***Borreliella californiensis* DSM 17989** | GCA_014205885.1 | 373543 | ASM1420588v1 | | Annotation submitted by DOE Joint Genome Institute | Scaffold | 213364 | 1352451 | 1426 | PRJNA632130 | SAMN14908344 |
| ***Borrelia miyamotoi* HT31** | GCA_019668505.1 | 47466 | ASM1966850v1 | | Annotation submitted by Joint Faculty of Veterinary Medicine, Yamaguchi University | Complete Genome | 906165 | 906165 | 862 | PRJDB10961 | SAMD00264446 |
| ***Borrelia coriaceae* Co53** | GCA_023035295.1 | 144 | ASM2303529v1 | N | Annotation submitted by Baylor College of Medicine | Complete Genome | 920509 | 1787333 | 1750 | PRJNA637792 | SAMN18441555 |
| ***Borrelia hermsii* DAH** | GCA_023035675.1 | 314723 | ASM2303567v1 | | Annotation submitted by Baylor College of Medicine | Complete Genome | 927976 | 1498568 | 1493 | PRJNA637792 | SAMN18441553 |
| ***Borrelia puertoricensis* SUM** | GCA_023035875.1 | 2756107 | ASM2303587v1 | K | Annotation submitted by Baylor College of Medicine | Complete Genome | 109607 | 1868579 | 1979 | PRJNA637792 | SAMN19000909 |
| ***Borreliella tanukii* HK501** | GCA_030436345.2 | 56146 | ASM3043634v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 913560 | 1342466 | 1374 | PRJNA431102 | SAMN10141379 |
| ***Borreliella lanei* CA-28-91** | GCA_030437705.2 | 373540 | ASM3043770v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 907274 | 1202123 | 1204 | PRJNA431102 | SAMN10141376 |
| ***Borreliella kurtenbachii* 25015** | GCA_030437945.2 | 1196056 | ASM3043794v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 901026 | 1345655 | 1388 | PRJNA431102 | SAMN10141375 |
| ***Borreliella turdi* Ya501** | GCA_030439285.2 | 57863 | ASM3043928v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 908244 | 1270789 | 1244 | PRJNA431102 | SAMN10141384 |
| ***Borreliella lusitaniae* PotiB2** | GCA_030440365.2 | 100177 | ASM3044036v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 903614 | 1202374 | 1203 | PRJNA431102 | SAMN10141377 |
| ***Borreliella andersonii* 21038** | GCA_032595875.2 | 42109 | ASM3259587v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 902021 | 1228260 | 1232 | PRJNA431102 | SAMN34060371 |
| ***Borreliella sinica* CMN3** | GCA_033969665.2 | 87162 | ASM3396966v2 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 902863 | 1225378 | 1248 | PRJNA431102 | SAMN34060373 |
| ***Borreliella burgdorferi* B31** | GCA_040790805.1 | 139 | ASM4079080v1 | | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 907880 | 1216633 | 1221 | PRJNA1130942 | SAMN42233977 |
| ***Borreliella japonica* ATCC 51557** | GCA_900099615.1 | 34095 | IMG-taxon 2597490341 annotated assembly | | Annotation submitted by DOE - JOINT GENOME INSTITUTE | Scaffold | 221163 | 1184862 | 1169 | PRJEB15958 | SAMN02983004 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Borreliella carolinensis* SCW-22** | GCA_032595915.1 | 478174 | ASM3259591v1 | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 901551 | 985046 | 937 | PRJNA431102 | SAMN34060367 |
| ***Breznakiella homolactica* RmG30** | GCA_016616095.2 | 2798577 | ASM1661609v2 | NCBI Prokaryotic Genome Annotation Pipeline (PGAP) | Complete Genome | 4646109 | 4646109 | 4153 | PRJNA686720 | SAMN17121750 |

THE END

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**UNIVERSITY OF SAAD DAHLAB – BLIDA 1**

جامعة سعد دحلب البليدة 1

Faculty of Natural and Life Sciences (SNV)

Department of Biology

**FINAL PROJECT GRADUATION THESIS**

For purpose of obtaining a Master's degree in the field of SNV.

Sector: **BIOLOGICAL SCIENCES**

Speciality: **MICROBIOLOGY**

Theme :

# Comprehensive genome-based reclassification of several pathogenic species within the genera *Bordetella* and *Borrelia*.


**Presented by:**

- NABAGALA IMMACULATE
- BAGUMA AZIIZI
- MUKWAYA JOSEPH


**Presented on** : 01/07/2025

**Before the Jury:**

| | | | | |
|---|---|---|---|---|
| Dr. BOKRETA S. | : | MCA/USDB1 | : | **President** |
| Dr. EDDAIKRA A. | : | MCA/USDB1 | : | **Examiner** |
| Dr. BOUZNADA K. | : | MCA/ENS-KOUBA | : | **Supervisor** |
| Pr. MEKLAT A. | : | Pr. /ENS-KOUBA | : | **Guest of Honor** |

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