

Leptospira taxonomy: then and now*

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Summary

The genus *Leptospira*, isolated for the first time in 1915, is one of the most diverse genera of bacteria which includes species isolated worldwide. The first taxonomic classification was based on the serological similarities of the strains. The basic taxon in the serological classification is serovar. More than 300 serovars are currently known. Furthermore, for many years the genus was divided into two species, the saprophytic *Leptospira biflexa* and the pathogenic *Leptospira interrogans*. The introduction of genetic techniques has led to a new species classification. In this review, we described the changes that have taken place over more than a century in the classification system of these bacteria and present the current state of the taxonomy of the genus *Leptospira* based on genome analysis. A complete list of the currently recognize 68 species of the genus *Leptospira* is presented, together with their phylogenetic relationships reflecting virulence status.

Keywords: *Leptospira*, species, bacterial taxonomy, genomes

Leptospirosis is a spirochetal disease with a great impact on both human and veterinary public health, caused by members of the genus *Leptospira*. Presently, leptospirosis is considered an emergent or re-emergent disease, increasing the concerns about prevention measures for human illness, wastage in farm animals, and the role of wildlife in the whole epidemiological process (10, 20, 27, 32, 93-95). Leptospirosis is considered by the World Organization for Animal Health (OIE) as a disease of importance to international trade, and it is included in the list of "Multiple species diseases" (57). This zoonotic disease affects over 1 million humans, killing nearly 60 000 people annually (3). Although it has a greater incidence in tropical regions, it is considered to be one of the most geographically widespread zoonosis in the world. The spectrum of human diseases caused by these pathogens is wide, ranging from subclinical infections to a severe syndrome of multi-organs dysfunction, sometimes with a fatal conclusion (30, 42).

The most common sources of infection are water or soil contaminated with infected animal urine, the infected urine itself, or tissues from infected animals. Animals can be divided into maintenance hosts and accidental (incidental) hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts, but other animals (including humans) may become accidentally infected by contact with the infected urine (42).

Leptospira is a highly genetically and serologically heterogeneous organism and attempts to understand the taxonomy of these bacteria can be confusing for young researchers and practitioners, especially as the list of new serovars and species has grown considerably in recent years. The current version of the International Code of Nomenclature of Prokaryotes (ICNP) assigned *Leptospira* genus to the family *Leptospiraceae*, order *Leptospirales*, class *Spirochaetia*, Phylum: *Spirochaetota*, subkingdom: *Negibacteria*, kingdom: *Bacteria* (58, 61). This review concerns *Leptospira* taxonomy from an historical perspective to the modern-day and shows how recent advances in genome

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sequencing have provided a systematic classification of *Leptospira* species

Leptospira morphology

Leptospira cells are unusual among bacteria based on their helical cell morphology with hook-shaped ends and the existence of periplasmic flagella (PF) – the sole motility machinery with the ability of spontaneous super coiling (Fig. 1) (1, 42, 75, 88). Like all spirochetes, they have a double membrane structure consisting of a cytoplasmic membrane, a peptidoglycan layer in the periplasmic space, surrounded from the outside by an outer membrane (Fig. 1C) (29). The outer membrane is rich in proteins (outer membrane proteins, OMP) and lipopolysaccharide (LPS) – which constitute the main antigen for *Leptospira* (31). Leptospiral lipopolysaccharide has a composition similar to that of other Gram-negative bacteria but has lower endotoxic activity.

Either pathogenic or non-infectious environmental saprophytic *Leptospira* morphologically are indistinguishable, as we can observe in dark-field microscopy (Fig. 1A). Regardless of species, leptospires have a right-handed spiral cell body that is usually 6 mm by 20 mm in length, 0.1 µm spiral diameter, and a wavelength of about 0.5 µm. (Fig. 1B) (21). The cells have pointed ends with exhibit curvatures and a morphology that frequently changes between a spiral and a hook shape during movement (52). The pathogenicity of *Leptospira* is closely related to the cell motility and mobility decrease attenuate this spirochete's virulence (59, 89). Variability in bacterial motility associated with

pathogenicity does not translate into changes in morphology between *Leptospira* species. It leads to problems with identification which affect the complexity of the classification and diagnostic process.

Historical overview of *Leptospira* and leptospirosis

The name “Spirochaete” for these bacteria was firstly used in 1838 by Ehrenberg to denote a long, motile, and flexible microorganism isolated from the water (84, 85). The first description of pathology was done in dogs as “canine typhoid fever” by Hofer in 1852. Nearly fifty years later without knowing of Hofer's observation, Klettin 1898 made a careful and extensive clinical study of illness that affected dogs during a canine exhibition in the city of Stuttgart and so he called it “Disease of Stuttgart” (82). Adolph Weil, a teacher of Medicine in Heidelberg (Germany),

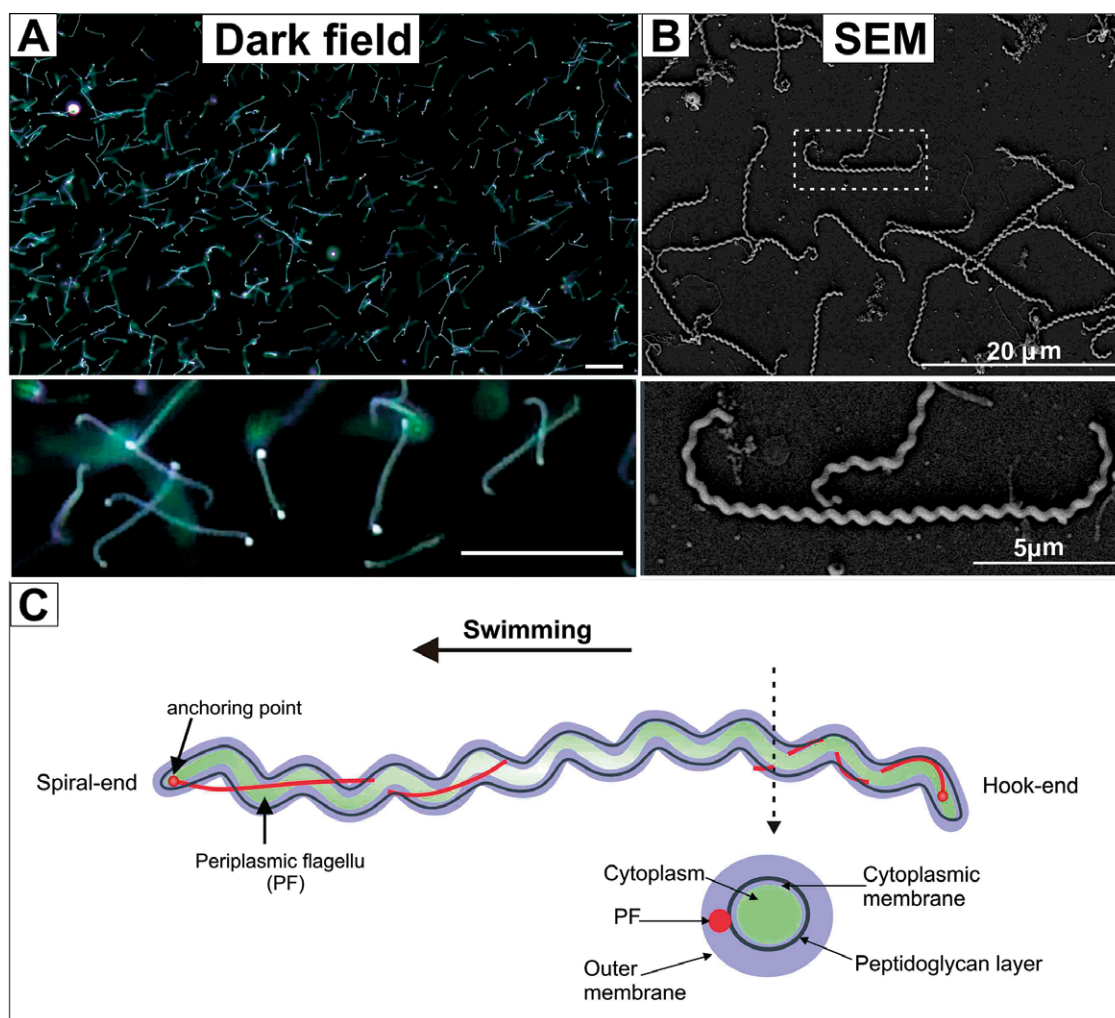


Fig. 1. Morphology of *Leptospira* cell (A-C). (A) Bacterial cultures were observed under a microscope with a dark-field examination [200 × magnification; bar = 10 mm]. (B) For Scanning Electron Microscope (SEM) analysis, *Leptospira* cells bound to glass cover slips were fixed in a mixture of 2.5% formaldehyde with 2.5% glutaraldehyde in a 0.05 M cacodylate buffer (Sigma-Aldrich; Saint Louis, MO, USA; pH 7.2). Next samples were postfixed with 1% osmium tetroxide, sputter-coated with gold and examined at an accelerating voltage of 20 kV or 10 kV using a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan). (C) Schematic graph of the *Leptospira* structure with a cross section of the cell body: outer membrane, periplasmic flagellum (PF), peptidoglycan layer, cytoplasmic membrane and cytoplasm (Photos: Arent Z., Pardyak L., Plachno B., Kotula-Balak M.)

carried out the first observations of clinical symptoms in humans between 1870-1882 and published them in 1886 (2). According to Weil, the disease developed in an acute way, characterized by high fever, splenic and liver enlargement, renal failure, and jaundice, and was subsequently named Weil's disease. The first demonstration of leptospires was done by Stimson in 1907 who examined them in the renal tubules belonging to a New Orleans man that died from a feverish jaundice disease, during a yellow fever epidemic (17, 78). Stimson named this microorganism *Spirochaeta interrogans*, indicating the specific observation that its shape resembled a question mark. Despite several bacteriologists, including Robert Koch, having tried without success to isolate the pathological agent of "Weil's disease", the etiology of leptospirosis was unknown until the beginning of the twentieth century. The isolation of the organisms was first reported from freshwater in 1914 by Wolbach and Binger, who called them *Spirochetabiflexa*, but as the agent of Weil's disease, the organism was isolated for the first time from the blood of miners by Inada and Ido in 1915 at the Kyushu University Medical School in Fukuoka (43). They succeeded in transmitting the infection to guinea pigs from the blood samples of the patient. Ido and colleagues suggested *Rattus norvegicus* as the natural reservoir of *Spirochaetaictero haemorrhagiae* (87). These findings were soon confirmed in Europe by German

researchers who transmitted the organism to guinea pigs after isolating it from soldiers (43). Nevertheless, in 1917, Noguchi presented the results of a careful comparison between the strains of *Spirochaetaictero haemorrhagiae* and *Spirochaeta biflexa* isolated in different geographical areas. His observations led him to the conclusion that these organisms morphologically and biologically are the same and in 1918 the name *Spirochaetaictero haemorrhagiae* was changed to *Leptospira icterohaemorrhagiae* (84).

In subsequent years it was found that suspensions of living leptospires were agglutinated in the presence of sera from a patient with symptoms suspicious of "Weils Disease" and it was recognized that *Leptospira* strains could be distinguished by their antigens. As more different strains were isolated, serological relationships were demonstrated between some of them. The serological technique was adopted and improved as a test for the diagnosis of leptospirosis and it still is the basic method for leptospirosis diagnosis and is now called the microscopic agglutination test (MAT) (57, 79, 83).

Initially, antigenetically distinct strains were assigned the status of species. Namely, it was *L. ictero haemorrhagiae*, *L. hebdomadis*, *L. canicola* and *L. biflexa*. Concern about the classification system for new strains was recognized by Wolf and Broom (87). They proposed that serologically distinct strains should not be allocated in different species but serotypes. Furthermore, closely related serotypes could be clustered into serogroups. As discriminating methods, they proposed the cross-absorption test, which was able to differentiate two indistinct strains by MAT. This work became the basis for the development of the system based on antigenic classification and serotype was the basic taxon in the *Leptospira* classification system.

In addition to taxonomy based on similarity of surface-exposed epitopes subdivision in the "TSC meeting" in Montreal in 1962, the Subcommittee on the Taxonomy of *Leptospira* recommended the division of genus *Leptospira* into two species, namely *Leptospira interrogans* (representing the pathogenic strains) and *Leptospira biflexa* (saprophytic strains). The species were differentiated by phenotypic characterization (19, 22, 34, 35, 38, 77). Key events in the development of leptospiral taxonomy in relation to the general taxonomy of prokaryotic organisms are shown in Figure 2.

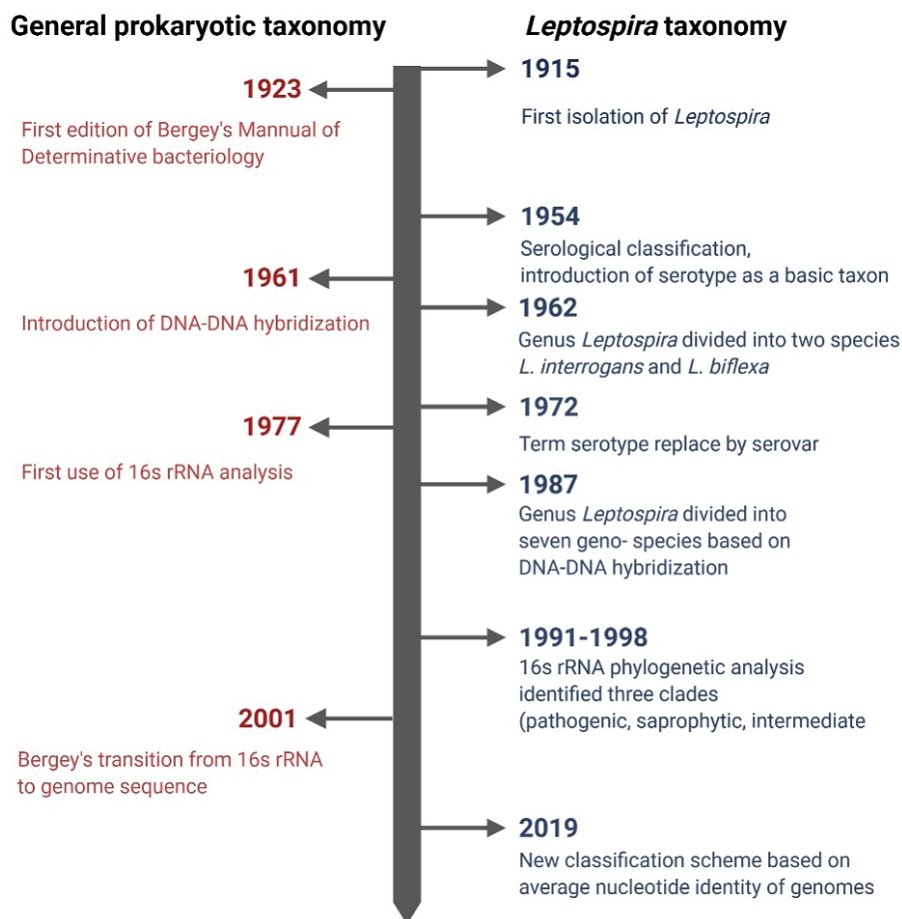


Fig. 2. Key events in the evolution of prokaryotic and *Leptospira* taxonomy

Serological classification

According to the classical serological classification introduced by Wolf and Broom in 1954 (87), both pathogenic and saprophytic *Leptospira* were divided into numerous serotypes defined by agglutination after cross-absorption with homologous antigen – the cross-agglutinin absorption test (CAAT). In 1973 the term “serotype” was replaced by the designation “serovar” (serologic variety) (35). According to the actual definition of the taxonomy of *Leptospira* two strains are considered to belong to different serovar if, after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in at least one of the two antisera in repeated tests (74). Serovars that are antigenically related have traditionally been, by convenience, grouped in serogroups, which have no taxonomic standing but are useful for epidemiological and diagnostic purposes (11, 42).

The Subcommittee on the „TSC meeting” of 1986 in Manchester approved the serovar list of pathogenic *L. interrogans* sensu lato complex consisting of 202 serovars divided by 23 serogroups and *L. biflexa* sensu lato complex composed by 63 serovars contained in 38 serogroups (36, 37). The serovars list is still revised and updated and actually above 300 serovars have been identified (43, 45, 60).

And although the modern bacterial taxonomy is based mainly on genetic diversity, identification of the serological status of *Leptospira* strains is still of great practical importance for two reasons. First, it helps to understand the mechanisms of pathogenesis and immunological response; second, it is important with regard to diagnosis and specific vaccine immunization. Antigenic diversity observed among *Leptospira* serovars, as mentioned above, is determined by carbohydrate side chains of lipopolysaccharide. LPS has been identified as an immuno dominant antigen, both in response to infection and to vaccination (18, 26). It is also considered to be the only protective antigen identified (49). Finally, *Leptospira* strains at the serovar level adapt to an animal species which acts as maintenance hosts, like Icterohaemorrhagiae in rats, Pomona in swine, Bratislava in swine and horses, and Canicola in dogs or Hardjo in cattle and sheep (4-8).

Classification in the genetic era (pre-genomic)

Initially, the taxonomy of bacteria, not only of the genus *Leptospira*, was largely intuitive and not fully defined. Undoubtedly, a major revolution in the classification of bacteria was introduced by techniques for the measurement of evolutionary divergence in the structure of nucleic acids. DNA-DNA hybridization studies have undoubtedly given rise to a new genetic era (68). Despite DNA-DNA hybridization, many other DNA-based classification methods have been

developed like DNA G+C content or pulse-field electrophoresis (48, 70). This has forced researchers to re-examine the nomenclatural system in the light of more recent taxonomic knowledge. During the Workshop on Reconciliation of Approaches to Bacterial Systematics of the International Committee for Systematic Bacteriology that took place in the Institute Pasteur in Paris, from 14 to 16 May 1987, it was agreed that the complete deoxyribonucleic acid sequence would be the reference standard to determine phylogeny and that phylogeny should determine taxonomy and nomenclature should agree with (and reflect) genomic information (81). Phylogenetic studies are directed at a basic understanding of pathways through which taxa have evolved from primordial and recent ancestors, calculated from analyses of evolutionary distances between selected semantides. From that, the species was the only taxonomic unit that could be defined in phylogenetic terms and the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness. The studies of molecular taxonomy, using the DNA-DNA hybridization, also made a significant contribution to an understanding of the genetic diversity within the genus *Leptospira* (41). Genetic typing has resulted in a novel classification scheme and seven new *Leptospira* genospecies were proposed (91). In addition to the two predefined species *L. interrogans* and *L. biflexa*, the following were also distinguished: *L. borgpetersenii*, *L. inadai*, *L. meyeri*, *L. noguchii*, *L. santarosai*, *L. weilii* and *L. wolbachii* (Tab. 1). Further genetic analysis of an additional seven serovars, not included in the previous studies, identified another new species *L. kirschneri* (65) but a newly isolated from a pig in Australia strain formed another species of *L. fainei* (62). Brenner et al. (13), in further determination of DNA relatedness, proposed four new *Leptospira* genospecies 1, 2, 3, 4, 5. For genospecies 2 the authors proposed the name *Leptospira alexanderi* to honor Aaron D. Alexander. In the TSC meeting of 2007 (Quito, Ecuador), the genomo species 1, 3, 4 and 5 proposed by Brenner were renamed because the subcommittee considered that the genomospecies had become a source of confusion in the literature. The subcommittee proposed that genomospecies 1 should be named *Leptospira alstonii*, genomospecies 3 should be named *Leptospira vanthielii*, genomospecies 4 should be named *Leptospira terpstrae* and genomospecies 5 should be named *Leptospira yanagawae* (46, 72). The next new *Leptospira* species recognized using genetic methods were *L. broomi* for two strains isolated from the blood of human patients with acute leptospirosis in Denmark and France (44), *Leptospira kmetyi* isolated from an environmental source in Malaysia (71), *Leptospira wolffii* recovered from a dog in Iran (92), *L. licerasiae* isolated from rats and humans in Peru (46, 51), nonpathogenic *Leptospira idonii* isolated

Tab. 1. List of 68 *Leptospira* species belonging to four main subclades: pathogenic (P1, P2) and saprophytic (S1, S2)

Species	References	Subclade	Species	References	Subclade
<i>L. adleri</i>	76	P1	<i>L. saintgironisae</i>	76	P2
<i>L. ainazensis</i>	40	P1	<i>L. sarikeiensis</i>	80	P2
<i>L. ainlahdjerensis</i>	40	P1	<i>L. selangorensis</i>	80	P2
<i>L. alexanderi</i>	13	P1	<i>L. semungkikensis</i>	80	P2
<i>L. alstonii</i>	72	P1	<i>L. venezuelensis</i>	64	P2
<i>L. barantonii</i>	76	P1	<i>L. wolffii</i>	71	P2
<i>L. borgpetersenii</i>	91	P1	<i>L. abararensis</i>	40	S1
<i>L. ellisii</i>	76	P1	<i>L. bandrabouensis</i>	80	S1
<i>L. gomenensis</i>	80	P1	<i>L. biflexas.s.</i>	22	S1
<i>L. interrogans.s.</i>	22	P1	<i>L. bourretii</i>	80	S1
<i>L. kirschneri</i>	65	P1	<i>L. bouyouniensis</i>	80	S1
<i>L. kmetyi</i>	71	P1	<i>L. brenneri</i>	76	S1
<i>L. mayottensis</i>	12	P1	<i>L. chreensis</i>	40	S1
<i>L. noguchii</i>	91	P1	<i>L. congkakensis</i>	80	S1
<i>L. santarosai</i>	91	P1	<i>L. ellinghauseni</i>	50, 80	S1
<i>L. stimsonii</i>	15, 16, 80	P1	<i>L. harrisiae</i>	76	S1
<i>L. tipperaryensis</i>	53, 80	P1	<i>L. jelokensis</i>	80	S1
<i>L. weilii</i>	91	P1	<i>L. kanakyensis</i>	80	S1
<i>L. yasudae</i>	15, 16, 80	P1	<i>L. kemamanensis</i>	80	S1
<i>L. andrefontaineae</i>	80	P2	<i>L. levettii</i>	76	S1
<i>L. broomii</i>	44	P2	<i>L. meyeri</i>	91	S1
<i>L. dzoumogneensis</i>	80	P2	<i>L. montravelensis</i>	80	S1
<i>L. fainei</i>	62	P2	<i>L. mtsangambouensis</i>	80	S1
<i>L. fletcheri</i>	80	P2	<i>L. noumeaensis</i>	80	S1
<i>L. fluminis</i>	80	P2	<i>L. perdikensis</i>	80	S1
<i>L. haakeii</i>	76	P2	<i>L. terpstrae</i>	72	S1
<i>L. hartskeerlii</i>	76	P2	<i>L. vanthielii</i>	72	S1
<i>L. inadai</i>	91	P2	<i>L. wolbachii</i>	91	S1
<i>L. johnsonii</i>	50, 80	P2	<i>L. yanagawae</i>	72	S1
<i>L. koniamboensis</i>	80	P2	<i>L. idonii</i>	67	S2
<i>L. langatensis</i>	80	P2	<i>L. ilyithenensis</i>	80	S2
<i>L. licerasiae</i>	51	P2	<i>L. kobayashii</i>	80	S2
<i>L. neocaledonica</i>	76	P2	<i>L. ognonensis</i>	80	S2
<i>L. perolatii</i>	76	P2	<i>L. ryugenii</i>	50, 80	S2

from environmental water beside a swimming pool in Japan (67) and *Leptospira mayottensis* isolated from humans (12).

As described above, DNA-DNA hybridization has been the “gold standard” for species delineation not only in the *Leptospira* genus (Fig. 2). The next milestone was undoubtedly the studies initiated by Woese in 1977 on small subunit ribosomal RNA (86). It was shown that rRNA can provide an objective evolutionary framework across the tree of life. The introduction of rapid analysis of the 16S rRNA sequence very quickly redefined prokaryotic taxonomy. The transition from a traditional phenotype-based classification

to a 16S rRNA-based phylogenetic framework was presented in the second edition of Bergey’s Manual of Systematic Bacteriology (25, 33, 86). The primary structure of 16S rRNA is far easier and more accessible for many laboratories to determine than hybridization between DNA strands (9, 73). Phylogenetic studies on 16S rRNA *Leptospira* strains identified three clades that reflected species designation based on the pathogenicity status, namely pathogenic, saprophytic and intermediate test rains. The last group: intermediate, contains strains of unclear pathogenicity, which usually have been recovered from patients but do not cause death, or clinical symptoms, or do not establish renal infection using animal models (17, 62, 69). It has been suggested that pathogens might have evolved from an environmental ancestor by the acquisition of new functions through lateral gene transfer associated with the adaptation to new hosts (23, 90).

Classification in the genomic era

DNA–DNA hybridization together with sequence comparison of select DNA markers (mainly RNA) has revolutionized our understanding of the microbial world and led to a rapid increase in the number of descriptions of novel taxa as shown above. The increasing number of the full genome sequence, in the last twenty years, gave undoubtedly a new insight into prokaryotic divergence and initiated the transition from gene phylogeny to genome phylogeny. Even before the introduction of genomics, it was clear that the complete deoxyribonucleic acid (DNA) sequence would be the reference standard for determining phylogeny and that phylogeny should determine taxonomy (81). Whole-genome sequencing (WGS) comparisons began to highlight the disadvantage of 16S rRNA sequence analysis, which is its limited taxonomic resolution. The 16S rRNA gene represents only 0.05% of an average 3-Mbp prokaryotic genome (33) and is often highly conserved and therefore often lacks sufficient variable characters to make a robust phylogenetic inference at the species level. The number of available sequences of the entire bacterial genome began to increase rapidly as researchers gained access

to new sequencing techniques such as next-generation sequencing (NGS) and also simple tools to measure similarity between two genomes (24). Konstantinidis and Tiedje (39) proposed similarity measures between pairs of genomes either at the level of nucleotides (average nucleotide identity – ANI) or encoded proteins (Average amino acid identity – AAI) of orthologous genes.

The first sequencing of the whole genome of *Leptospira* strains was published for three of the most common species, namely *L. interrogans*, *L. borgpetersenii*, and the saprophyte *L. biflexa* between 2003-2008. *Leptospira* genomes are 3.73-4.99 Mb in size and are located on two circulate chromosomes (14, 55, 56, 63, 66, 80). Since then, the number of available genomes has increased exponentially and a comparison of them showed that coupled *Leptospira* strains that have 100% identical 16S rRNA have ANI values corresponding to unknown, potentially novel species. This observation has forced a revisiting of the taxonomy of the genus. An example of such reclassification is a newly isolated strain in Europe GWTS#1 assigned previously to the species *L. alstonii*, based on the 16S rRNA and *secY* genes. However, the ANI value with the reference strain of *L. alstonii* was at a range of only 79.2%. Using the ANI cutoff of 95% as the metric to delineate bacterial species, its affiliation has been revised and it creates a new species *L. tipperaryensis* (53, 54, 80).

Furthermore, the increase in the number of successful *Leptospira* isolations in recent years, especially from environmental samples, has also contributed to the knowledge of their diversity. These bacteria are fastidious and difficult organisms to isolate, but the modification of isolation media, especially by using a new combination of antimicrobials to prevent contamination, has allowed many of them to be isolated from the environment (76). This has contributed to a significant increase in the number of genomes in accessible databases. A comparison of ANI values of genomes of the new isolates and representative genomes of known species revealed 45 new *Leptospira* species: 24 of pathogenic status and 21 saprophytic. Currently, 68 species of *Leptospira* have been identified (15, 16, 40, 50, 64, 76) (Tab. 1).

Phylogenetic analysis of described species has identified two major clades and four subclades. The first major clade is the „Pathogens”, containing the species responsible for animal and human infections. This clade is subdivided into two subclades: P1 – grouping species formerly referred to as pathogens and P2 – formerly referred to the as intermediate group. The second clade contains “Saprophytes” – species isolated from the environment, which is also divided into two subclades: S1 and S2 (80) (Tab. 1).

WGS studies of bacteria are currently in a golden age. By applying genomic sequencing to a large collection, the need for systematic, standardized de-

scriptions of bacterial genotypic variation remains a priority. Sequence variation in the genome represents an important starting point if different organisms are to be compared, but all types of variation must be cataloged effectively to make comprehensive associations between phenotype and genotype. It requires the construction of sustainable resources: both for relating WGS data to typing and taxonomy) and to use interoperable data sets that are archived in accessible databases (47). Recently, to meet these needs, a new core genome multilocus sequence typing (cgMLST) was proposed, which extends the classical MLST to 545 core genes representing the entire phylogenetic diversity of the genus. The proposed cgMLST strategy allows high-resolution genotyping of *Leptospira* isolates across the phylogenetic breadth of the genus. The unified genomic taxonomy of *Leptospira* strains, available publicly at <http://bigsd.b.pasteur.fr/leptospira>, will facilitate the global harmonization of *Leptospira* genotyping (28).

In conclusion, the review unveils the serological and genomic diversity of the *Leptospira* genus. In practice, an ideal taxonomy would involve one system. Classical serotyping, based on the similarity of surface-exposed epitopes determined by lipopolysaccharides anchored to the outer membrane of the bacterial cell, is still useful for identifying reservoirs and generating a control strategy but does not give insight into genetic diversity. Understanding how genetic speciation occurs in the *Leptospira* population could help to explain bacterial evolution and its role in the acquisition of virulence factors. Therefore, both classification systems, based on serology and genetic differences, are used by researchers, although they are not always compatible.

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