Rev. sci. tech. Off. int. Epiz., 2013, **32** (1), 149-162

Molecular characterisation of *Brucella* species

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Summary
The genus *Brucella* (Mayer and Shaw, 1920) currently consists of ten species with validly published names. Within most species further differentiation into biovars exists. Genetically, all *Brucella* species are highly related to each other, exhibiting sequence similarity values of 98% to 100% in aligned regions (core genome). The population structure is clonal. Despite this close genetic relatedness, the various species can be clearly distinguished from each other by application of high-resolution molecular typing tools, in addition to assessment of phenotype and host preference. Accurate species delineation can be achieved by conventional multiplex polymerase chain reaction (PCR), single nucleotide polymorphism (SNP) analysis and multilocus sequence typing (MLST) or multilocus sequence analysis (MLSA). The last is also suitable for phylogenetic reconstructions, owing to the highly clonal evolution of the different species. Highly discriminatory multilocus variable number of tandem repeats (VNTR) analysis (MLVA) allows both species delineation and differentiation of individual isolates and thus represents a perfect first-line tool for molecular epidemiological studies within outbreak investigations. More recently, whole genome sequencing (WGS) and the resulting global genome-wide SNP analysis have become available. These novel approaches should help in further understanding the evolution, host specificity and pathogenicity of the genus *Brucella*.

Keywords
Brucella spp. – Clonal population – High-resolution typing assay – Low genetic diversity – Monophyletic genus.

Introduction

The genus *Brucella*: historical background and current state of taxonomy and classification

To fully understand the current nomenclature, taxonomy, overall structure, and molecular diversity of the genus *Brucella*, knowledge about its creation and history is a prerequisite.

The genus *Brucella* (Class Alphaproteobacteria, Order Rhizobiales, Family Brucellaceae) was established by Meyer and Shaw in 1920, with *B. melitensis* (infecting humans and goats) as the type species and *B. abortus* (infecting cattle) as the second species (36). At that time, molecular techniques were not available and species delineation was based solely on phenotypic traits, such as host preference, differentiating biochemical reactions, growth behaviour on different culture media, and growth in the presence of different dyes.

In their study on 21 *B. melitensis* and 32 *B. abortus* strains, Meyer and Shaw (36) came to the following conclusion: 'The causative organism of undulant fever of man and of Malta fever of goats cannot be distinguished morphologically or biochemically from the organism responsible for infectious abortion in domesticated animals.'

Despite this statement, *B. melitensis* and *B. abortus* retained their names and status as separate species, most likely

because of practical reasons and their isolation from different hosts and geographical regions.

In the following years, *B. suis* (1929), isolated from pigs (28); *B. ovis* (1956), isolated from sheep (5); *B. neotomae* (1957), isolated from the desert woodrat (44); and *B. canis* (1968), isolated from dogs (6), were added to the genus as novel species. Like the first two species, they were exclusively classified and characterised on the basis of their phenotype and host preference.

Differentiation of the various species and biovars remained difficult, and it was (and still is) a fact that different results can be obtained by independent *Brucella* research groups when characterising representative *Brucella* strain collections by employing phenotypic assays. Differentiation and classification are made even more complicated by the existence of strains showing atypical biochemical reactions that cannot be attributed to existing *Brucella* species.

As early as 1968, the first studies at the molecular level, using the DNA-agar method and a filter method, were applied to members of the genus *Brucella* (25, 26). These studies supported *B. melitensis*, *B. abortus* and *B. suis* as members of a single genus. The studies further revealed that the 'beagle organism' (Carmichael) also belonged to the same genus. Soon afterwards, the 'beagle organism' became *B. canis* (6).

In 1985, using the modern DNA–DNA hybridisation technology (the gold standard for bacterial species delineation, with a threshold of 70% DNA hybridisation experimentally determined to fit with previous phenotypic classifications), Verger *et al.* confirmed the close genetic relatedness of the various species (50). Since all tested species and biovars had a relative DNA-binding ratio (genetic relatedness) of more than 80%, the authors came to the conclusion that only one species, *B. melitensis*, should be recognised in the genus *Brucella*. Two years later, in 1987, De Ley *et al.* came to the same conclusion (12).

Following the advent of the polymerase chain reaction (PCR) method and DNA sequencing technology, the close genetic relatedness among the various *Brucella* species became more and more evident. Today we know that all the above-mentioned species have identical 16S ribosomal (r)RNA- and *recA* gene sequences (21, 41) and are almost identical (only a few nucleotide substitutions) in the majority of housekeeping genes, supporting the conclusions of Verger *et al.* and De Ley *et al.*

In 2002, the first *Brucella* whole genome sequence (*B. melitensis* 16M) was published (13), followed shortly after by the genome sequence of *B. suis* strain 1330 (39). Comparative whole genome analysis confirmed the close genetic relatedness of these genomes (average nucleotide identity above 99%), in agreement with DNA–DNA hybridisation results. It became obvious that *Brucella*, and in particular *B. suis* strain 1330, shares extensive genetic similarity with some plant pathogens and microbes that live symbiotically with plants, such as *Agrobacterium* and *Rhizobium*, suggesting a soil-associated common ancestor (39).

Despite the findings of Hoyer *et al.*, Verger *et al.*, De Ley *et al.* and the results of comparative genome analysis, the Subcommittee on the Taxonomy of *Brucella* agreed unanimously on a return to pre-1986 *Brucella* taxonomy and, as a consequence, to the re-approval of the six *Brucella* species with the recognised biovars (38). The reasons for this decision are essentially that isolates from the different species are consistently clustered in different groups showing host preference, suggesting that they do represent different ecotypes (8). In this view, they may qualify as different 'species' in spite of their very high sequence similarity. There is an ongoing discussion about *Brucella* classification, nomenclature and taxonomy, and the currently available minimal standards for the description of novel *Brucella* species from 1975 (9) are under review in order to take into account the advent of molecular methods and genetic population analysis.

Novel *Brucella* species in the genetic era

After the addition of *B. canis* in 1968, no new species were added to the genus until 2007, presumably because of the overall high relatedness among various *Brucella* species and the limited discriminatory power of phenotypic methods. In this context, the rare candidates that might represent new species within the genus could not be easily distinguished from atypical phenotypic variants within a known species.

Brucella pinnipedialis and *Brucella ceti*

In 1994 Ewalt *et al.* described an atypical novel *Brucella* strain isolated from a bottlenose dolphin (15). In 1997, following the isolation of a series of novel strains from sea mammals, Jahans *et al.* proposed that they comprised a new nomen species, which they called *B. maris* (30). The name *B. maris* was not officially recognised and after ten years of further research, the sea mammal isolates were subdivided into two novel *Brucella* species, according to specific phenotypic and molecular markers (7, 29). These were named *B. ceti* and *B. pinnipedialis*, with cetaceans and seals as their preferred hosts (17). More recent molecular analyses indicate that both *B. ceti* and *B. pinnipedialis* can further be subdivided into separate subclusters (2, 10, 33,

54) but with no indication in terms of ecotype that any of these sublineages would deserve species designation.

For the first time within the *Brucella* genus, modern molecular typing tools, in combination with classical phenotyping (i.e. a polyphasic approach), were used for species description (17).

Brucella microti and *Brucella inopinata*

A similar polyphasic approach, consisting of a battery of phenotypic and molecular analyses, was used to describe *B. microti*, originally isolated from the common vole *Microtus arvalis* as a novel species in 2008 (27, 42). This was followed by the description of *B. inopinata*, isolated from an endogenous infection of a breast implant in a 71-year-old woman in 2010 (11, 43).

Brucella microti and *B. inopinata* are phenotypically different from the classical *Brucella* species and, therefore, are often misidentified when only phenotypic methods are applied. Both *B. microti* and *B. inopinata* are characterised by rapid growth and high metabolic activity. Therefore, biochemical test systems such as the API® NE (non-enteric) and VITEK (both manufactured by Bio-Mérieux) misidentify both species as *Ochrobactrum*, which is the closest genetic neighbour to *Brucella* (42, 43).

Whereas *B. microti* is identical in its 16S rRNA and *recA* gene sequences to the corresponding consensus sequences of all other *Brucella* species, *B. inopinata* is characterised by unique 16S rRNA and *recA* gene sequences, differing in five and seven nucleotides, respectively (43). *Brucella inopinata* also exhibits a significantly lower level of sequence similarity in various housekeeping genes (11) when compared with other *Brucella* species. Consequently, it is the most divergent species within the genus *Brucella*. Although *B. inopinata* represents the most diverse species, it shares a DNA–DNA relatedness of > 80% with *B. melitensis* and therefore would still belong to the same species if the commonly accepted borderline for bacterial species delineation was applied.

With the addition of these novel species, the genus *Brucella* currently consists of ten species with validly published names (*B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. neotomae*, *B. canis*, *B. ceti*, *B. pinnipedialis*, *B. microti* and *B. inopinata*).

Emergence of potential novel *Brucella* species and *Brucella*-like organisms

The use of modern molecular methods facilitates the recognition of potential novel *Brucella* species. Application of these methods has revealed the existence of previously unknown *Brucella*-like organisms and potential novel *Brucella* species, isolated from humans and various animal sources including new hosts. The following paragraphs summarise what is currently known about the presence of potential novel *Brucella* species.

An unusual *Brucella* strain (BO2) was isolated from a lung biopsy specimen from a 52-year-old human patient in Australia who had chronic destructive pneumonia (47). Phenotypic and molecular analyses placed strain BO2 in close proximity to *B. inopinata*. Hence, the BO2 strain probably represents a novel lineage of *B. inopinata* (47). However, extended molecular analyses are necessary to confirm this proposition.

Seven strains, isolated in 1964 from three native rodent species in North Queensland, Australia, and previously reported as *B. suis* biovar 3, were reinvestigated to determine their exact classification (48). Molecular analysis indicated that the seven strains are unique and distinct from all known *Brucella* species and therefore may represent a novel species. Molecular data also indicate that the seven strains are related to *B. inopinata* and strain BO2 (48).

Atypical *Brucella* strains were recently isolated for the first time from non-human primates in association with two cases of stillbirth (40). Preliminary phenotypic and molecular analyses indicate that the two isolates are different from all known *Brucella* species (40).

Novel atypical *Brucella* strains have also been isolated from red foxes in Austria (33). These strains are slow growing and exhibit a large number of IS*711* elements, comparable to those of *B. ceti* and *B. pinnipedialis* (24). They show very low metabolic activity using the Taxa Profile™ assay to measure substrate utilisation and do not cluster with any known *Brucella* species (1). Deeper molecular analysis by multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) confirmed their separate position within the genus *Brucella* (H.C. Scholz, A.M. Whatmore & G. Vergnaud, unpublished).

A potential novel *Brucella* species has been isolated from African bullfrogs (14). In contrast to all other *Brucella* species, the isolates are motile and equipped with a single laterally attached flagellum, as has been described for *O. anthropi*. Genome sequencing confirmed that it belongs to the genus *Brucella*. However, various additional genes related to genes of soil bacteria, e.g. *Agrobacterium*, could be identified, of which most encode ATP-binding cassette (ABC) transporters (H.C. Scholz, unpublished).

From these data it is obvious that we are only beginning to understand the full ecological spectrum of the genus *Brucella*. It is also obvious that a broad range of animal

species are carriers of a series of novel, as yet undescribed, *Brucella* species and *Brucella*-like organisms. At the present time the pathogenic potential of these novel *Brucella* species for humans is still unknown and the natural cycle of transmission and maintenance is not understood.

Methods for molecular characterisation of *Brucella* species

The availability of whole genome sequence data opened the way for in-depth molecular analyses and subsequent development of novel molecular typing tools, such as MLST, single nucleotide polymorphism (SNP) analysis and multilocus variable number of tandem repeats (VNTR) analysis (MLVA). These allow identification and differentiation of *Brucella* at the species, biovar and even at the individual strain level. These methods can be used to study genetic diversity among various *Brucella* species in an evolutionary context, or in outbreak scenarios.

Given that we have now entered a new genomic era of next generation sequencing, comparative whole genome sequence analysis of potential novel *Brucella* species and *Brucella*-like organisms will provide a better understanding of the evolution, host specificity and pathogenicity of this medically important genus.

In the following sections the most relevant methods for *Brucella* species identification, differentiation and further molecular in-depth characterisation will be presented.

PCR-based methods

One of the first PCR assays to differentiate among *Brucella* species was the so-called *Abortus–Melitensis–Ovis–Suis* (AMOS) PCR, developed by Bricker and Halling in 1994 (4). This PCR uses a single reverse primer, targeting the *Brucella*-specific insertion element IS*711*, and four different forward primers, each specific for a given species as estimated by testing representative isolates. Species are differentiated on the basis of different PCR fragment sizes. For many years, this assay was used by diagnostic laboratories to confirm pure cultures as either *B. melitensis*, *B. abortus*, *B. ovis* or *B. suis*. The disadvantage of this PCR was that not all species could be identified (i.e. *B. canis* and *B. neotomae*) and that some biovars within a given species gave negative results.

In 2006, a new conventional multiplex PCR (Bruce-ladder), using eight primer pairs in a single reaction, was developed by García-Yoldi and colleagues (20). Because this PCR covers all species and biovars it rapidly replaced the AMOS-PCR as a diagnostic tool and is still used in many diagnostic laboratories. Later, this PCR was enhanced, to cover novel species such as *B. microti* and *B. inopinata* (34). Although the enhanced Bruce-ladder allows accurate species delineation of all existing species, differentiation at the biovar level, or below, is not possible (Fig. 1).

Consequently, PCR assays that allow discrimination among biovars within a given species were developed. The most recent multiplex PCR assay to differentiate among *B. suis* biovars 1 to 5 (Suis-ladder) was developed by López-Goñi *et al.* (32).

To date, at least 400 reports have been published dealing with various PCR-based methods for *Brucella* detection and differentiation. For details on PCR-based assays see Yu and Nielsen (53).

Non-specific high-resolution typing assays

In the 1990s, the first PCR-based genotyping tools were used to study genetic diversity among *Brucella* species, biovars and individual strains. These techniques included enterobacterial repetitive intergenic consensus sequence

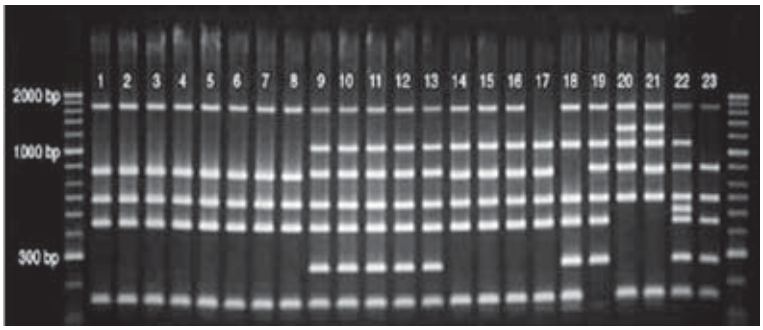

Fig. 1
Identification and differentiation of all known *Brucella* species and biotypes by modified multiplex polymerase chain reaction assay
Lanes 1–8: *B. abortus* biotypes 1–7 and biotype 9; lanes 9–13: *B. suis* biotypes 1–5; lanes 14–16: *B. melitensis* biotypes 1–3; lane 17: *B. ovis*; lane 18: *B. canis*; lane 19: *B. neotomae*; lane 20: *B. pinnipedialis*; lane 21: *B. ceti*; lane 22: *B. microti*; lane 23: *B. inopinata*
Source: Mayer-Scholl *et al.*, 2010 (34).

PCR (ERIC-PCR); repetitive intergenic palindromic sequence PCR (REP-PCR) (35, 45); randomly amplified polymorphic DNA PCR (RAPD-PCR) or arbitrarily primed PCR (AP-PCR) (16, 46); and restriction fragment length polymorphism PCR (RFLP-PCR) of the *omp*2 locus (7). Apart from the RFLP-PCR assay targeting the *omp*2 locus, these assays were based on more or less random amplification of genomic DNA and subsequent gel electrophoresis, resulting in very complex banding patterns. Because of this complexity, reproducibility between different laboratories was low, and therefore these approaches are not commonly used.

Specific high-resolution assays

Multilocus variable number of tandem repeats analysis

The MLVA assays take advantage of array-length variations in tandem repeats. Owing to the availability of whole genome sequences, tandem repeats can be readily identified, and tested for polymorphism. Most tandem repeats have been evaluated, essentially by three groups (Bricker, Vergnaud, Whatmore, and colleagues), and tens of polymorphic loci have been identified. Different selections of such loci, used in MLVA assays, have been proposed to suit different purposes.

HOOF-Prints and subsequent selections of loci for multilocus variable number of tandem repeats analysis

The first MLVA assay, named 'HOOF-Prints' (hypervariable octameric oligonucleotide fingerprints), was developed by Bricker *et al.* in 2003 (3). The *Brucella* genome contains a family of tandem repeats sharing the repeat unit 'AGGGCAGT'. Eight highly variable such loci, present in most *Brucella* species, were selected for use in the HOOF-Print assay. Variations of the repeat numbers at each locus can easily be investigated by amplifying the corresponding regions and subsequent gel electrophoresis or, preferably, capillary electrophoresis, given the short repeat unit size. This selection of tandem repeats has a very high discriminatory power and can be useful for local outbreak investigations. However, it cannot provide a species assignment owing to the high level of homoplasy (see below) at these loci.

With the HOOF-Print assay a reliable tool to study the relationship of human cases and outbreak dynamics became available for the first time (49). Indeed, high-resolution markers allow the discrimination of individual strains and therefore can be used for trace-back analyses and epidemiological studies in outbreak scenarios. A high discriminatory power is desired when investigating an outbreak with very limited geographical and temporal distribution, and highly variable loci will then be preferred. However, rapidly evolving VNTR markers often suffer from homoplasy, i.e. the appearance of the same genetic alteration in two or more branches of a phylogenetic tree. These phenomena can disrupt and confound the accurate phylogenetic placement of some isolates within an MLVA cluster and prevent accurate species-level designation.

A stronger phylogenetic signal and different selections of loci are needed when looking for species identification. Practical considerations (cost) also need to be taken into account. Although tens of VNTRs have been described in *Brucella* and are potentially useful, the number of loci in an MLVA assay should be kept to a minimum. Because the HOOF-Print MLVA assay could not be used for identification purposes at the species level, additional selections of tandem repeats were subsequently proposed (Table I).

The $MLVA15_{Orsay}$ and $MLVA16_{Orsay}$ selection (31) and/or their subpanels are currently the most commonly used, according to the literature, probably because of the existence of an accessible internet database which contains MLVA16 data from 1,492 isolates (see: http://mlva.u-psud.fr/brucella/ [accessed on 10 December 2012]).

The $MLVA16_{Orsay}$ assay is divided into two different panels: one with a low discriminatory index that allows quick allocation to a major branch and species, and a second panel with VNTR markers of high discriminatory power, suitable for outbreak investigations, some of which are used in the HOOF-Print assay (Table I). A minimum spanning tree based on $MLVA16_{Orsay}$ data from 1,925 isolates is shown in Figure 2.

Whereas MLVA is a highly efficient tool for clustering strains, it is not a phylogenetic tool, because rapidly evolving VNTR markers often suffer from homoplasy. However, clusters can be easily connected upon a phylogenetic backbone, as soon as a subset of representative strains has been analysed by both MLST (see below) and MLVA.

Given the clonal evolution of the main *Brucella* species, phylogeny makes sense, and phylogenetic investigations can conveniently be used to define lineages. In fact, MLST is probably the tool most commonly used in current phylogenetic investigations of *Brucella*.

Multilocus sequence typing of single-nucleotide polymorphisms

The conventional MLST approach uses sequence divergence in housekeeping genes. About seven to nine housekeeping genes are commonly analysed in order to obtain a reasonable balance between the acceptable identification power, time and cost for the strain typing. From each housekeeping gene, approximately 450 to

Table I

List of variable number of tandem repeats (VNTR) markers used in different assays

HOOF designations are from Bricker *et al.*, 2003 (3); 'Bruce' are from Le Flèche *et al.*, 2006 (31); 'VNTR' are from Whatmore *et al.,* 2006 (52); 'BruceVNTR' are from Tiller *et al.,* 2010 (47)

Locus_repeat unit size	Aliases	Chr.	Published HGDI values		HOOF-Prints	$MLVA8_{Orsay}$	$MLVA10_{Orsay}$	$MLVA11_{Orsay}$	$MLVA15_{Orsay}$	$MLVA16_{Orsay}$	$MLVA21_{Weybridge}$	$MLVA15_{Flagstaff}$	$MLVA10_{Ames}$
BRU28_13bp	Bruce54 VNTR21	1	0.26	0.39							x		x
BRU211_63bp	Bruce11	1	0.84			x	x	x	x	x			
BRU217_15bp	Bruce46 BruceVNTR25	1	0.5									x	
BRU233_18bp	Bruce45 VNTR7	1	0.65	0.54		x	x	x	x	x	x		
BRU379_12bp	Bruce43	1	0.55			x	x	x	x	x			
BRU424_125bp	Bruce42	1	0.75			x	x	x	x	x			
BRU588_8bp	Bruce09 HOOF8 BruceVNTR30	1	0.72	0.80	x				x	x	x	x	x
BRU1134_18bp	Bruce08 VNTR26	1	0.53	0.44		x	x	x	x	x	x		
BRU1234_15bp	Bruce36 VNTR27	1	0.38	0.41							x		
BRU1250a_8bp	Bruce07 VNTR5A BruceVNTR3*	1	0.78	0.78					x	x	x	x*	x
BRU1250b_8bp	VNTR5B BruceVNTR3*	1		0.88							x	x*	x
BRU1322_134bp	Bruce06	1	0.73			x	x	x	x	x			
BRU1505_8bp	Bruce30 HOOF2	1	0.7	0.77	x				x	x	x		
BRU1543_8bp	Bruce04 HOOF6 BruceVNTR28	1	0.87	0.86	x				x	x	x	x	
BRU1915_8bp	Bruce25 HOOF3 Bruce VNTR29	1	0.9	0.74	x						x	x	x
BRU1938_8bp	Bruce01 HOOF7 BruceVNTR1	1	0.95	0.90	x						x	x	
BRU1940_8bp	Bruce24 HOOF5 BruceVNTR2	1	0.88	0.89	x						x	x	
BRU2066_40bp	Bruce55 VNTR24	1	0.69	0.75		x	x	x	x	x	x		

Table I
List of variable number of tandem repeats (VNTR) markers used in different assays (cont.)

Locus_repeat unit size	Aliases	Chr.	Published HGDI values		HOOF-Prints	$MLVA8_{Orsay}$	$MLVA10_{Orsay}$	$MLVA11_{Orsay}$	$MLVA15_{Orsay}$	$MLVA16_{Orsay}$	$MLVA21_{Weybridge}$	$MLVA15_{Flagstaff}$	$MLVA10_{Ames}$
BRU18_8bp	Bruce14 VNTR12B BruceVNTR33	2	0.93	0.91							x	x	
BRU19_8bp	Bruce13 VNTR12A	2	0.85	0.85							x		
BRU73_15bp	Bruce12	2	0.82			x	x	x	x	x			
BRU275_8bp	Bruce74 BruceVNTR7	2	0.63									x	
BRU285_28bp	Bruce73	2	0.51										
BRU322_8bp	Bruce22 HOOF1	2	0.9	0.92	x						x		x
BRU324_3bp	Bruce19**	2	0.79					x		x			
BRU329a_8bp	Bruce20 HOOF4	2		0.92	x						x		x
BRU329b_8bp	Bruce21	2	0.57				x	x	x	x			
BRU339_8bp	Bruce18 VNTR17 BruceVNTR16	2	0.82	0.71			x	x	x	x	x	x	x
BRU344_5bp	Bruce17 VNTR16 BruceVNTR31	2	0.79	0.82							x	x	x
BRU396_9bp	BruceVNTR14	2		0.00								x	
BRU542_12bp	Bruce80 BruceVNTR20	2	0.18									x	
BRU548_8bp	Bruce16 VNTR2 BruceVNTR27	2	0.85	0.75					x	x	x	x	x
BRU564_18bp	Bruce68	2	0.47										
BRU574_6bp	BruceVNTR21	2		0.06								x	
BRU609_31bp	Bruce67	2	0.52										
BRU652_17bp	Bruce66 VNTR14	2	0.32	0.31							x		

Chr.: chromosome
HOOF-Prints: hypervariable octameric oligonucleotide fingerprints
HGDI (Hunter–Gaston diversity index): discriminatory power
MLVA: multilocus VNTR analysis
* The primers for BruceVNTR3 amplify simultaneously VNTR5A and VNTR5B

** Bruce19 was initially published by Le Flèche *et al.*, 2006 (31) as a 6 base pair (bp) repeat unit VNTR. However, owing to a secondary internal deletion in the array, present in some lineages, it is considered as a 3 bp repeat unit for the purposes of allele-calling consistency

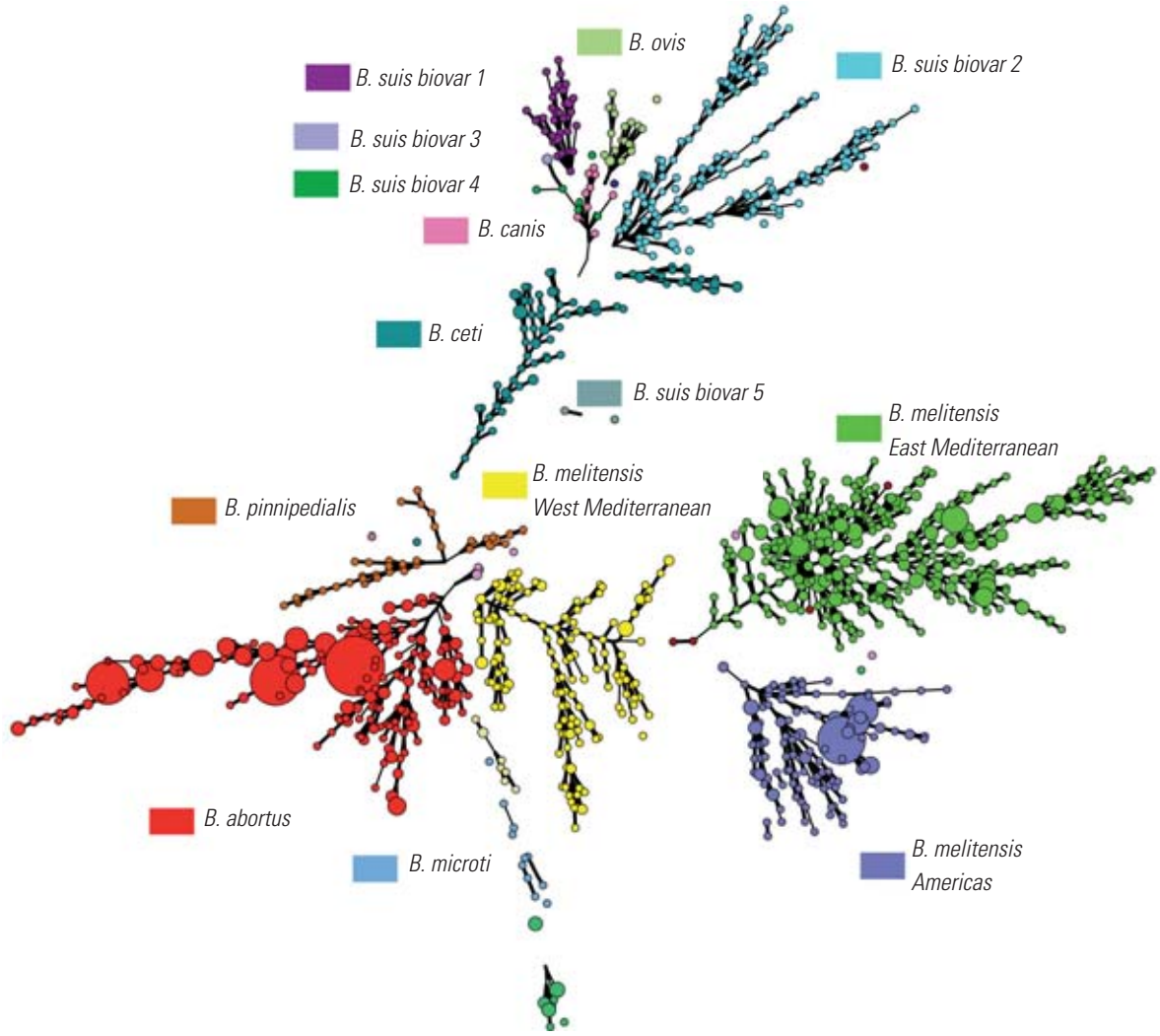

Fig. 2

Minimum spanning tree based on multilocus variable number of tandem repeats analysis (MLVA15$_{Orsay}$) data from 1,925 isolates

The majority were compiled from the literature. Branch lengths up to three are shown. The figure illustrates how MLVA can be used to draw the image of a population at low cost, and then pick the rare isolates (shown in different colours) with long branches for whole genome draft sequencing.

500 base pairs (bp) are amplified by PCR, followed by DNA sequencing and subsequent comparative sequence analysis. Each unique sequence is assigned a specific allele number and alleles are combined into an allelic profile and further assigned to a specific sequence type (ST). New alleles result in a new combination and therefore in a novel sequence type.

Because accumulated changes occur slowly and are regarded as selectively neutral, the MLST approach is a reliable tool for the overall characterisation of microbial populations and the investigation of phylogenetic relationships. However, the slow molecular clock rate and the limited number of genes do not allow in-depth phylogenetic reconstructions and analysis of local epidemiological studies. Thus, unlike MLVA, MLST is of little value for outbreak investigations.

In 2007 Whatmore *et al.* developed an MLST assay based on nine different loci (51). Of the nine loci, seven correspond to classical housekeeping genes. The remaining two loci target the *omp25* gene and an intergenic region, respectively, resulting in more discriminatory power. Nine loci are sequenced, totalling almost 4,400 bp, or 0.1% of the genome. Owing to clonal evolution and congruence of the phylogeny of individual loci, these data are usually analysed as MLSA in which the sequence from all nine loci is concatenated. Such a tree is shown in Figure 3.

In a comprehensive study, Whatmore and colleagues investigated 160 *Brucella* isolates belonging to all known species and biovars (51). The assay grouped *B. abortus*, *B. melitensis*, *B. ovis* and *B. neotomae* as well-separated clusters according to their species affiliation. *Brucella suis* biovars 1 to 4 also clustered together but demonstrated higher intra-species heterogeneity when compared with the other species. *Brucella suis* biovar 5 formed a separated lineage. The ten *B. ovis* isolates, although from different

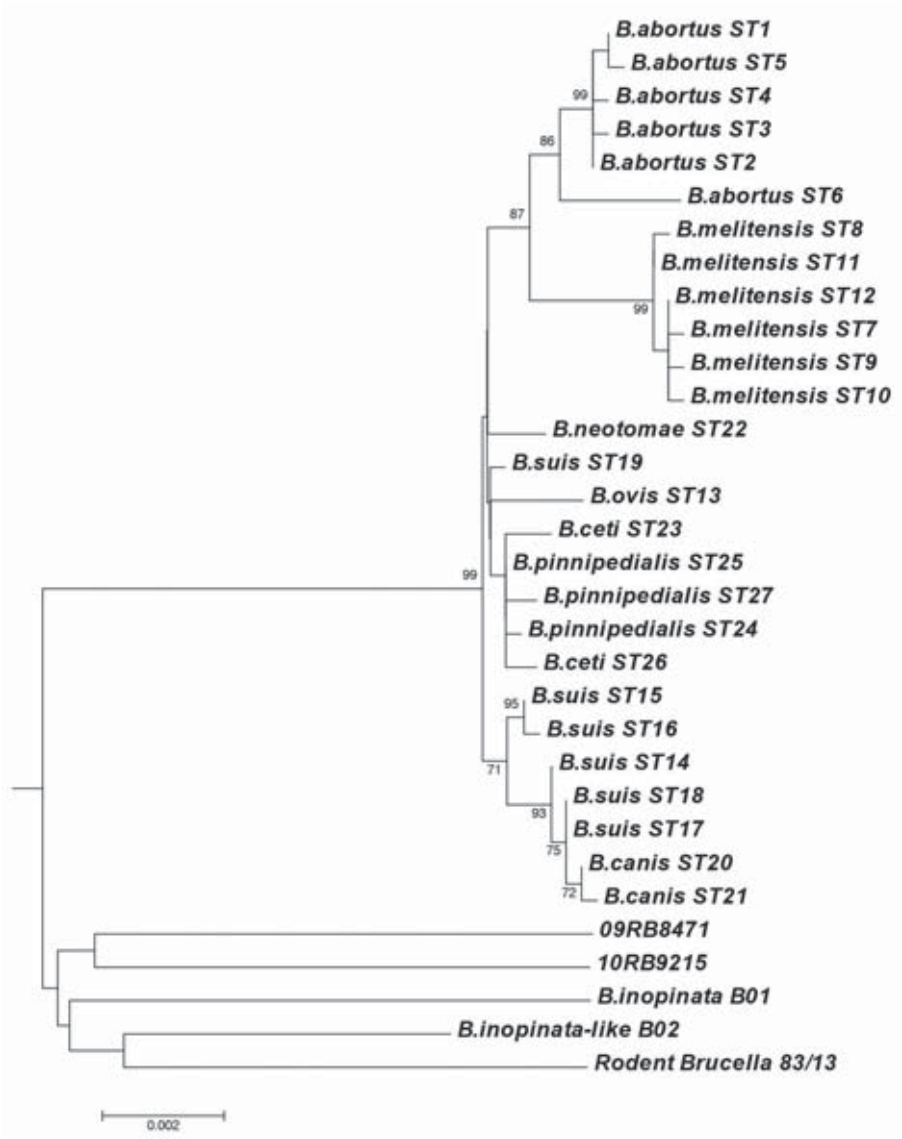

Fig. 3

Relationships among the *Brucella* complex based on eight-locus multilocus sequence analysis

The extended *Brucella* group, including *B. inopinata*, the rodent isolate 83/13, strain BO2 and the two isolates 09RB8471 and 09RB9215 from African bullfrogs cluster separately from the core *Brucella*. Sequences were concatenated and the phylogenetic tree constructed using the neighbour-joining approach. Numbers at nodes correspond to proportions of 100 resamplings that support the topology shown, with only values greater than 70% indicated. Bar: number of substitutions per nucleotide position

geographical regions, were represented by a single sequence type. The *Brucella canis* strains grouped in proximity to *B. suis* biovars 3 and 4 but were distinguishable by a different sequence type. *Brucella* isolates from marine mammals also grouped as a separated cluster.

The results of that study confirmed the close proximity of *B. suis* biovars 3 and 4 with *B. canis* and the separate position of *B. suis* biovar 5, already suggested by phenotypic assays and molecular methods. On the basis of these results the authors concluded that this assay can be used to provide a strong phylogenetic backbone for the *Brucella* genus.

Real-time polymerase chain reaction assays of single-nucleotide polymorphisms

The MLST technique requires amplification of the corresponding target genes by PCR, followed by double-strand DNA sequencing of the products and subsequent sequence analysis. The overall procedure is expensive and time consuming. Consequently, a new platform for the rapid detection of SNPs was developed.

Based on the results from MLST, Gopaul *et al.* developed a rapid assay to distinguish the major

Brucella clades using the Minor-Groove-Binder (MGB) real-time PCR technology (22). For each allele a highly specific 5′ labelled, 3′ MGB Taqman probe was designed. The assay was evaluated by testing over 300 isolates of *Brucella*. Almost at the same time, a similar approach to distinguish among the various *Brucella* species was developed by Foster *et al.* (19). However, it is important to keep in mind that assays based on typing selected SNPs are highly biased. They collapse tree branches along linear trees.

Whole genome sequencing and global analysis of single nucleotide polymorphisms

Draft whole genome sequencing is increasingly being used in replacement of MLST and large-scale SNP typing because it is an unbiased approach and provides an incomparable wealth of data, at a cost which is now getting closer to that of the previously described assays. Hundreds of isolates are currently being sequenced and analysed at draft level for whole genome SNP discovery (37). This will provide much higher resolution than MLSA. From the resulting information, it will be possible to devise specific SNP assays that efficiently complement MLVA typing and clustering analysis when necessary (18).

Associated databases

The result of all sequence-based typing assays, MLST, SNPs, MLVA, and even whole genome SNP discovery, can be conveniently stored in flat text files, and/or be made accessible via the internet. Multiple sources of polymorphisms have been uncovered in *Brucella* by the use of whole genome sequencing technologies. Paradoxically, whereas for decades the challenge was to find genetic polymorphisms within these highly monomorphic pathogens, this is no longer the case. On the contrary, the current challenge is to provide the means to extract the relevant information and interpret it. The development of databases presenting typing data from selected polymorphisms has become the main issue. These databases need to be structured in such a way that different laboratories in the community will find an interest in providing them with their typing data. A balance probably needs to be found between a request for well-curated and 'certified' data, and the desire for comprehensive databases containing data from thousands of isolates of worldwide origin. These different models also have different associated running costs.

A prototype *Brucella* MLVA database was set up by the Paris Sud University in Orsay in 2006 (mlva.u-psud.fr) (23). This database has been regularly updated since that time, by compiling data from the literature. The current release (December 2012) contains data from 1,492 isolates (see Fig. 2) and the latest version of the software (version 4) can accommodate any numeric dataset, including MLST and SNP data.

Acknowledgements

The authors thank Adrian M. Whatmore for providing Figure 1.

Caractérisation moléculaire des espèces de *Brucella*

H.C. Scholz & G. Vergnaud

Résumé

Le genre *Brucella* (Mayer et Shaw, 1920) comprend dix espèces connues à ce jour, dont le nom a été validé et publié. La plupart de ces espèces se compose de plusieurs biovars. Au plan génétique, les espèces de *Brucella* sont étroitement apparentées, affichant des valeurs de similitude de séquences de 98 % à 100 % dans les régions d'alignement (dans le génome). La population a une structure clonale. En dépit de leur étroite parenté génétique, il est possible de différencier clairement ces espèces grâce à des outils de typage moléculaire de haute résolution utilisés en complément de l'analyse des phénotypes et des préférences d'hôtes. Une délimitation précise des espèces peut être réalisée au moyen de l'amplification en chaîne par polymérase multiplexe classique, par analyse du polymorphisme nucléotidique simple (SNP) et par typage par séquençage multiple de gènes (MLST) ou analyse multi-locus de séquences (MLSA). Cette dernière

méthode est également adaptée aux reconstructions phylogénétiques, du fait du caractère fortement clonal de l'évolution des différentes espèces. L'étude de minisatellites polymorphes (méthode MLVA, pour *multiple locus variable number tandem repeat analysis*, basée sur la détection de petites séquences répétées en tandem sur le génome, appelées VNTR) permet à la fois une délimitation des espèces et une différenciation des isolats et représente donc un outil de première ligne idéal pour les études moléculaires dans le cadre des enquêtes sur les foyers épidémiques. Sont également disponibles depuis peu le séquençage du génome entier et l'analyse qui en résulte des SNP à l'échelle du génome. Ces approches innovantes devraient contribuer à mieux comprendre l'évolution, la spécificité d'hôte et la pathogénicité du genre *Brucella.*

Mots-clés
Brucella spp. – Essai de typage de haute résolution – Faible diversité génétique – Genre monophylétique – Population de structure clonale.

Caracterización molecular de las especies de *Brucella*

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Resumen
El género *Brucella* (Mayer y Shaw, 1920) cuenta actualmente con diez especies cuyo nombre ha sido públicamente validado, la mayoría de las cuales presentan además distintos biovares. Genéticamente, todas las especies de *Brucella* están estrechamente emparentadas, con valores de similitud de secuencia que van del 98% al 100% en las regiones alineadas (genoma básico). La estructura de la población es clónica. Pese a tan estrecho parentesco genético, es posible distinguir claramente entre las diversas especies aplicando herramientas de tipificación molecular de alta resolución, además de observar el fenotipo y las preferencias por uno u otro hospedador. Es posible delimitar con exactitud las distintas especies aplicando técnicas de: reacción en cadena de la polimerasa (PCR) múltiple convencional; análisis de poliformismo de nucleótido único; y tipificación multilocus de secuencias (MLST) o análisis multilocus de secuencias (MLSA). Este último método también es útil para realizar reconstrucciones filogenéticas porque la evolución de las distintas especies tiene un carácter muy clónico. El análisis multilocus de un número variable de repeticiones consecutivas (*tandem repeats*) es muy discriminatorio y permite tanto delimitar las especies como diferenciar cultivos aislados, por lo que constituye una perfecta herramienta de primera línea para realizar estudios moleculares a la hora de investigar brotes. Últimamente se ha logrado una secuenciación genómica completa, y a partir de ahí se ha analizado el poliformismo de nucleótido único del genoma completo a escala mundial. Estos nuevos métodos deberían ser de ayuda para entender mejor la evolución, la especificidad respecto del hospedador y la patogenicidad del género *Brucella.*

Palabras clave
Brucella spp. – Ensayo de tipificación de alta resolución – Escasa diversidad genética – Género monofilético – Población clónica.

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