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## *Brucella* evolution and taxonomy

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### Abstract

The genus *Brucella* contains alpha-Proteobacteria adapted to intracellular life within cells of a variety of mammals. Controversy has arisen concerning *Brucella* internal taxonomy, and it has been proposed that the DNA–DNA hybridization-based genomospecies concept be applied to the genus. According to this view, only one species, *Brucella melitensis*, should be recognized, and the classical species should be considered as biovars (*B. melitensis* biovar *melitensis*; *B. melitensis* biovar *abortus*; etc.). However, a critical reappraisal of the species concept, a review of the population structure of bacteria and the analysis of *Brucella* genetic diversity by methods other than DNA–DNA hybridization show that there are no scientific grounds to apply the genomospecies concept to this genus. On the other hand, an enlarged biological species concept allows the definition of *Brucella* species that are consistent with molecular analyses and support the taxonomical standing of most classical species. Both the host range as a long-recognized biological criterion and the presence of species-specific markers in outer membrane protein genes and in other genes show that *B. melitensis*, *B. abortus*, *B. ovis*, *B. canis* and *B. neotomae* are not mere pathovars (or nomenspecies) but biologically meaningful species. The status of *B. suis* is, however, less clear. These approaches should be useful to define species for the marine mammal *Brucella* isolates, as illustrated by the grouping of the isolates from pinnipeds or from cetaceans by *omp2* gene analysis. It is shown that a correct *Brucella* species definition is important to understand the evolution of the genus.

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### 1. Introduction

Taxonomy seeks to discover the order hidden in the apparently bewildering diversity of living beings and, as a practical consequence, provides the framework to establish the

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identity of a given specimen. It is logically based on three interrelated areas: classification, nomenclature and identification. Classification is an information storage and retrieval system that uses hierarchical categories or ranks (Phylum, Class, Order, Family, Genus and Species) among which the basic one is the species (Dobzhansky et al., 1977; Brenner et al., 2001; Young, 2001). Although other criteria have been used in the past, hierarchical grouping of the taxons follows phylogenetic criteria in modern taxonomy (Moreno, 1997; Ludwig and Klenk, 2001; Young, 2001). In procaryotes, the overwhelming majority of the phylogenetic data are obtained from the analysis of “molecular chronometers” useful to draw molecular “phylogenetic trees” which constitute the backbone of the current classification for the higher taxons (Ludwig and Klenk, 2001). However, molecular chronometers do not provide enough resolution to delineate the terminal branches of the trees and are, therefore, of limited taxonomical value at species level (Fox et al., 1992; Stackebrandt and Goebel, 1994).

The importance of a well-constructed classification system cannot be overemphasized. It is clear that the way in which we perceive the taxonomic relationships among different bacteria influences our understanding of their basic biological and ecological features. The definition of species is of similar importance and not necessarily anthropocentric. Whereas other taxonomic categories are artificial concepts intended to introduce an order in the classification, the species is the only taxon that describes a biological entity that exists (or existed) in nature in a population sense (Goodfellow and O’Donnell, 1993). Critical biological events such as speciation and extinction also work at the species level. Also important are the practical implications of taxonomy. As it is stated in the introductory chapters of some Bergey’s editions, experience demonstrates that “a classification that is of little use to the microbiologist, no matter how fine a scheme or who devised it, will be ignored or significantly modified” (Staley and Krieg, 1984; Brenner et al., 2001).

The *Brucella* group provides one of the best examples of the challenges confronted during the construction of a coherent taxonomic system and also of its usefulness. For many years, the correct position of the group in the higher taxons was not known. The demonstration of the phylogenetic closeness between the *brucellae* and other bacteria associated with eucaryotic cells, no matter whether they are of animal or plant origin, (De Ley et al., 1987; Moreno et al., 1990; Moreno, 1992) has had a profound impact in the way we envisage the virulence mechanisms of these bacteria (Moreno and Moriyón, 2002). In addition, this perspective (Ugalde, 1999) has resulted in new research strategies and biological interpretations (Iñón de Iannino et al., 1998; Sola-Landa et al., 1998; O’Callaghan et al., 1999; Velasco et al., 2000; Briones et al., 2001). The definition of *Brucella* species also illustrates the importance of taxonomical issues (Verger et al., 1985; Anonymous, 1986; Verger et al., 1987; Meyer, 1990a; Anonymous, 1994; Moreno, 1997; Moreno and Moriyón, 2001). Through the years, *Brucella* taxonomists developed a classification system based on six species (*B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis* and *B. canis*) subdivided into biovars (or biotypes). This classification was challenged over a decade ago on the grounds that the high degree of DNA homology observed by DNA–DNA hybridization (Hoyer and McCullough, 1968a; Hoyer and McCullough, 1968b; Verger et al., 1985) demonstrates that *Brucella* is a monospecific genus (*B. melitensis*) of which the six classical species would not be but biovars (*B. melitensis* biovar *abortus*, *B. melitensis* biovar *suis*, etc.) (Verger et al., 1985; Verger et al.,

1987). This proposal is now adopted in some prestigious data banks and culture collections (for example, the GenBank and the United Kingdom National Culture Collection) but not in many others. Part of the confusion was created by the acceptance by the Subcommittee on the Taxonomy of *Brucella* in 1986 that the monospecific classification system is the only taxonomically valid and that the “nomen species” (i.e. the classical species) should be retained just “to avoid confusion” (Anonymous, 1986). More recently, several *Brucella* strains that do not fit into the classical species have been isolated from marine mammals (Ewalt et al., 1994; Foster et al., 1996; Jahans et al., 1997; Clavareau et al., 1998; Miller et al., 1999; Forbes et al., 2000) and controversial names that do not follow the monospecific classification system have been proposed for them (Jahans et al., 1997; Cloeckert et al., 2001). These problems cannot be dismissed as a mere matter of semantics, since the acceptance of a single or several species (with a correct place for the isolates from marine mammals) substantially affects which biological traits we identify as relevant to understand both the biology and the evolution of the brucellae. Moreover, there are significant practical consequences with regard to the way in which we handle a set of bacteria which differ in epidemiological importance and impact in public health. In this article, we attempt to establish which concept of species should be used in the *Brucella* group and how it could be applied to new isolates. An account of how we envisage the evolution that has first led to the genus and then to *Brucella* speciation is also presented.

## 2. The brucellae and the concept of species

The concept of species was first used by scientists working with plants and animals, where pooling the information in the complete genome of two individuals by sexual mechanisms is (with exceptions) a necessary step for multiplication. For these organisms, the concept of “biological species” was defined (Table 1). It has to be emphasized that, defined in this way, the species are truly independent and internally coherent evolutionary units (Dobzhansky et al., 1977; White, 1978a) and this shows that, beyond its genetic wording, the concept uses a relevant biological criterion. An apparently alternative concept such as that of the “phylogenetic species” (Table 1) is not substantially different but for the fact that it could cover the exceptions (asexual reproduction and others) (White, 1978b) not included in the biological species definition.

Historically (like the genus–species binomial nomenclature) the use of the “species” in procaryotic taxonomy was adopted by bacteriologists when the wide genetical differences between procaryotes and the higher eucaryotes had not come into light. It is obvious that the fertility-based definition of “biological species” cannot be applied to procaryotes and, although the same terms are sometimes used in bacterial taxonomy discussions (Dykhuizen and Green, 1991; Lan and Reeves, 2001; Sneath, 2001), they are applied to groups of bacteria where exchange of genetic material can occur, a situation only superficially similar to that of sexual reproduction and for which the less equivocal term of “genospecies” (Table 1) was coined by Ravin in 1963 (Ravin, 1963). Likewise, the “phylogenetic species” concept is also of little use in bacterial taxonomy. Indeed, it overlaps with that of clone (Dijkshoorn et al., 2000; Young, 2001), does not provide criteria (see below) to delineate the group of bacteria that belong to the same species (Moreno,

Table 1  
Some species concepts

Concept	Definition	Comments	References
Biological species	Group of individuals that share the same genetic pool and that are isolated from other similar groups by reproductive barriers	Fertility is linked to pooling the genes of two individuals. The concept uses a biological criterion useful for over 99% of animals and plants Sometimes applied to procaryotes instead of the more precise “genospecies” concept (see below)	(Mayr, 1963; Dobzhansky et al., 1977; White, 1978a)
Phylogenetic species	Group composed of the smallest diagnosticable cluster of individuals within which there is a parental pattern of ancestry and descent	Describes situations in the higher eucaryotes not covered by the biological species concept	(Cracraft, 1983)
Genospecies	Groups of bacteria where exchange of genetic material can occur in nature	Overlaps with the concept of clone in procaryotes Too wide to be useful in procaryotes as there is increasing evidence for the widespread exchange of genes or group of genes among bacteria that are not phylogenetically related	(Ravin, 1963)
Taxospecies	Cluster of strains that have a high mutual phenetic similarity	Not necessarily objective or consistent with phylogenetic analyses Does not resolve bacteria which are different on the basis of molecular analysis but for which there are no clear phenotypic differences	(Ravin, 1963)
Nomenspecies or nominal species	Cluster of strains to which it is convenient to give a species name on basis other than taxonomical	Criteria used to give species names are practical but the group may be artificial.	(Ravin, 1963)
Genomospecies	Bacteria showing 70% or greater DNA–DNA hybridization and with 5 °C or less difference in $\Delta T_m$	Makes no provision for bacterial groups related at different DNA–DNA pairing values  It is based on a methodology that only superficially assesses genome similarities	(Wayne et al., 1987)

1997) and, as pointed out above, molecular clocks do not have enough resolution at this level (Fox et al., 1992; Stackebrandt and Goebel, 1994). Ravin (Ravin, 1963) also proposed the terms “taxospecies” and “nomenspecies” (Table 1) and, although these are nowadays considered a source of confusion (Dijkshoorn et al., 2000), the definitions had the merit of illuminating the provisional or subjective status of some bacterial species.

A consensus definition of bacterial species found in recent reviews is that of a group of strains of common origin (or clonal populations) which are more similar to each other than they are to any other strain (Goodfellow and O’Donnell, 1993; Dijkshoorn et al., 2000; Brenner et al., 2001; Young, 2001). This is a working definition that does not solve the problem of choosing the criteria to be used in delineating the species (Dijkshoorn et al., 2000), and it is this choice what has been and still is a matter of debate (Goodfellow and O’Donnell, 1993; Dijkshoorn et al., 2000; Brenner et al., 2001; Gillis et al., 2001; Sneath, 2001; Young, 2001). For years, the only possible criteria were those of conventional phenetic classification (hence the concept of taxospecies (Table 1)) but as molecular biology progressed more attention was given to the direct comparison of genomes, and the criterion that has dominated for the last 25 years has been DNA–DNA hybridization (Goodfellow and O’Donnell, 1993). On this basis, the concept of “genomospecies” (Table 1) was proposed (Brenner, 1973; Wayne et al., 1987; Brenner et al., 2001). “Genomospecies” are sometimes called “genomic species” (Goodfellow and O’Donnell, 1993) but this term is confusing because it is also used for the fertility-based concept of biological species of the sexual eucaryotes (see, for example, Palleroni (1993)). It is important to understand that the genomospecies definition is based on studies with enteric bacteria and related taxa that showed DNA–DNA hybridization discontinuities within these groups, with values in the 50–70% range being rare and with most species (taxospecies) previously established by conventional phenetic classification falling within the 70% DNA–DNA, 5 °C,  $\Delta T_m$  range (Table 1) (Brenner, 1973; Goodfellow and O’Donnell, 1993; Brenner et al., 2001). Thus, at least to some extent, it was the conventional phenetic approach that sustained the value of the genomospecies concept.

The genomospecies concept has unquestionable merits as it has helped to clarify the internal taxonomy of some important groups of procaryotes. Moreover, it has the appeal of the simplicity inherent to classification systems that use a single (monothetic) criterion,<sup>1</sup> and this contributes to its apparent objectiveness. Also, it provides an apparently scientific basis for the principle of balance<sup>2</sup> to be fully applied at species level for the first time in bacterial taxonomy. These three circumstances have favored the reductionistic view that the only valid scientific concept of species in procaryotes is that of the genomospecies (Brenner et al., 2001) and, therefore, that the classical *Brucella* species are mere “nomenspecies” (Table 1) (Anonymous, 1986; Brenner et al., 2001) with no true taxonomical standing (Vergler et al., 1985; Anonymous, 1986). Nevertheless, the genomospecies concept is not as universally accepted as it is claimed: it has been criticized

<sup>1</sup> Monothetic classification systems are those based on the assumption that a taxon is strictly defined by a feature invariably present in all individuals. Historically, this notion was found to be unsatisfactory for phenetic bacterial taxonomy and was substituted by a polythetic approach according to which members of a group have a maximum of features in common (Goodfellow and O’Donnell, 1993).

<sup>2</sup> This principle states that “retrieval of information is greatly facilitated if the taxa at a given categorical rank are, as far as possible, of equal size and degree of diversity” (Mayr, 1998).

because of the technical problems involved and, more important, also on scientific grounds.

A weakness of the genomospecies definition is that DNA–DNA hybridization only superficially assesses genome similarities or differences (van Belkum et al., 2001). In the case of *Brucella*, this is a significant criticism (Meyer, 1990b) since other methods allow a refined analysis of the genus (see below). However, the most serious and general problem of the genomospecies concept is that it makes no provision for bacterial groups related at different DNA–DNA pairing values (Sneath, 1989; Moreno, 1997; Dijkshoorn et al., 2000). That this should occur stems from the fact that the enormous diversity of procaryotes (Service, 1997) and the variety of niches they colonize result in different population structures and, indeed, it is at the level of populations where the species concept should be biologically meaningful (Goodfellow and O'Donnell, 1993). From what is known about the mechanisms of gene exchange in procaryotes, two extreme situations can be envisaged (Maynard Smith et al., 1993; Moreno, 1997). The first one is that of bacterial populations living in environments where biological competition is intense and that can exchange portions of the genome by means of conjugation, transformation or transduction. A related genetic trait of these bacteria is that they very often carry plasmids of several kinds that confer adaptive plasticity. The effects of genetic exchange are counterbalanced by mechanisms such as the repair systems that recognize heteroduplex DNA with extensive mismatches and, although possibly to much less extent, by restriction enzymes (Maynard Smith et al., 1991). Both sorts of phenomena should result in reticulate populations (Fig. 1) in which genomic differences assessed by the DNA–DNA hybridization method are “relaxed.” Examples would be some bacteria present in soil and rhizosphere or in animal-associated

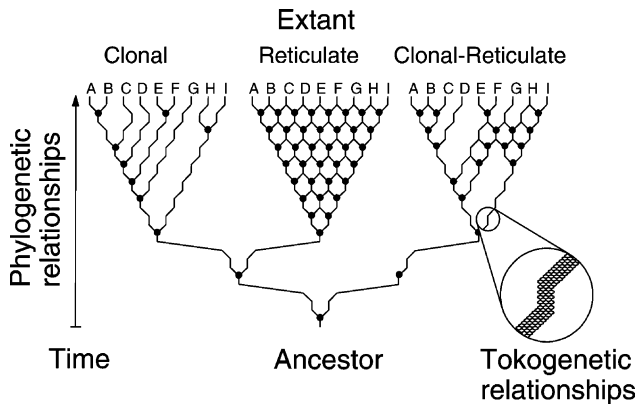


Fig. 1. Hypothetical phylogenetic trees and tokogenetic relationships among bacteria. Three different alternatives for generating bacterial species or strains (A–I) during evolution are presented. Speciation will depend on the probabilistic transmission and expansion of exogenous and endogenous genetic events as well as natural selection. Endogenous events such as mutation, internal recombination of genes and duplication or deletion of sequences are vertically transmitted and clonally expanded by the ancestor (●). Exogenous events include the horizontal acquisition or recombination of foreign bacteria sequences, plasmids and lysogenic phages (∧). Examples of clonal species are the *Brucella* spp., *Bartonella* spp., *Anaplasma* spp. and *Rickettsiae* spp.; examples of reticulate or clonal-reticulate bacteria are members of the *Rhizobiaceae* and *Enterobacteriaceae* families. Modified from Moreno (1997), with permission.

environments such as the gut and some mucosal surfaces. The analysis of multilocus enzyme electrophoresis data provides experimental support for the existence of this kind of population structure in *Rhizobium meliloti* divisions A and B, *Neisseria gonorrhoeae*, *N. meningitidis*, and some *Salmonella* serotypes (Maynard Smith et al., 1993). Moreover whole genome analysis of several strains also supports this pattern for *E. coli* (Ochman and Jones, 2000; Ohnishi et al., 2001).

An almost opposite situation can be envisaged for bacteria that are ecologically confined in environments where they reside as pure cultures. These bacteria have little chances of taking up heterologous DNA, and also lack plasmids because the phenotypic plasticity linked to these structures is useful only in open environments (Moreno, 1998). Moreover, the chances for genetic drifts are also reduced because of the stable selective pressure exerted by the environment. These circumstances result in a reduced genetic diversity with more “tight” limits for the biological group. Perhaps the best examples are those intracellular parasites of animals that are transferred from host to host and have a limited or no ability to survive in an open environment. In some cases, adaptation can be so extreme as to be manifested as a narrow host range, viscerotropism or preference for some cell types, thus resulting in biological populations that are evolutionarily committed in the sense that they are unlikely to revert or be converted to a different host or cell range. It seems obvious that the brucellae fall within this second group: their ecological isolation is clear, plasmids have never been detected and there is no firm evidence for lysogenic phages (Anonymous, 1986; Rigby, 1990; Meyer, 1990a; Meyer, 1990b; Moreno and Moriyón, 2001). Certainly, genetic uniformity (as tested by DNA–DNA hybridization) is very high in the brucellae (Hoyer and McCullough, 1968a; Hoyer and McCullough, 1968b; Verger et al., 1985; Verger et al., 2000). However, this is not a rigorous argument favoring the existence of a single biological group, but rather the manifestation of a population structure reflecting an adaptation which does not exclude biological diversity, and then the existence of several species.

The preceding considerations show that, although useful, the genomospecies concept should be restricted to certain bacterial groups and that its application to the brucellae and similar groups cannot be done without neglecting basic aspects of their biology. The inadequacy of this concept does not clarify which *Brucella* species are valid or how new situations should be handled. In this regard, it is important to retrieve the basic notion that species are natural realities active at population level, as it is the case of the biological species in plant and animal kingdoms. Accordingly, the definition of the bacterial species should be based, rather than on reductionistic operational criteria, on a thorough understanding of their biology, including the population structure. This is also a “biological species” concept (Moreno, 1997) which, since it acknowledges biological diversity, encompasses other definitions when these are restricted to groups where the delineating criterion chosen is biologically relevant (for example, the fertility-based definition of biological species in most of the higher eucaryotes, and the genomospecies concept for bacteria susceptible to fluid genetic exchange). In bacteria, this enlarged “biological species” concept may lack the simplicity of the genomospecies definition and disregards the principle of balance. However, this principle is anthropocentric, and the biological concept is totally scientific since it aims to describe what exists in nature. In addition, the biological concept is consistent with the polyphasic approach that postulates that phenetic,

genetic, phylogenetic, and ecological data must be integrated in modern bacterial taxonomy (Gillis et al., 2001).

### 3. Practical considerations: biosafety and *Brucella* species

All brucellae are currently classified as biosafety group three microorganisms but, despite this apparent uniformity, every worker in the area knows that the virulence for humans and the ease of transmission is not the same for all classical species. Two examples illustrate this point. Epidemiological evidence shows that *B. suis* biovar 2 and *B. ovis* are not pathogenic for humans, and that *B. abortus* biovar 5, *B. neotomae* and *B. canis* pose little danger, while they are pathogenic for animals (Meyer, 1990b). Also, veterinarian services do not consider in the same category of risk *B. melitensis*, *B. suis*, *B. canis* or *B. ovis*. Thus, regardless of the outcome of our scientific discussions, the monospecific classification system has little or no chance of being universally adopted since it would be foolish for Animal or Human Health Authorities to abandon the system that, in practice, has proved useful and safe for their purposes. A related problem is illustrated by the fact that, in those culture collections that have adopted the monospecific system, it is not presently possible to identify the classical species unless the correspondence between the reference number of the particular strain and the species name is retrieved from specialized publications.<sup>3</sup> The listing of all brucellae under the same species name is confusing and clearly conveys a danger to persons with little or no experience on *Brucella* and brucellosis research. It is to avoid situations like this that the *nomen periculosum* “dangerous name” rule was created (Judicial Commission, 1985). According to this rule, biosafety must prevail over other considerations in the taxonomical nomenclature and, therefore, it is clear that the monospecific classification system should not be used in databanks or culture collections. These issues are also connected to the need to modify the biosafety regulations concerning those brucellae that pose no risk to human health.

### 4. The definition of *Brucella* species

There is no consensus on molecular criteria to define the bacterial genus and its definition is based mostly on phenotypic circumscriptions (Young, 2001). In nature, the brucellae are pathogens that do not multiply in open environments, characteristically cycle from host to host and, as far as it is known, are always found intracellularly but in the moments that precede dissemination. Thus, the definition of the genus is mostly based on these biological characteristics, and this was satisfactorily settled in the twenties long before the phylogeny and the true taxonomic position of the group was known (Meyer, 1990b). In this same line of thought, a very significant characteristic of many brucellae is that they exhibit a marked although not absolutely strict host range. This is shown not only

<sup>3</sup>For example, in the National Culture Collection, *B. melitensis* 10512 is actually the classical reference strain of *B. ovis*. The equivalence between the NCTC number and the reference strain of *B. ovis* can be found in Alton et al. (Alton et al., 1988) but not in the webpage of the National Culture Collection. Here, it is merely said that NTCC 10512 is a type strain (of what?) and that was isolated from ram semen.



as a preference for a given animal species but also by the observation that, when cross-infections between different animal species occur, the bacterium is seldom perpetuated in non-reservoir, non-preferential hosts once the primary host is removed (Alton, 1990; Blasco, 1990; Carmichael, 1990; Crawford et al., 1990; Meyer, 1990b). Also significant is that no naturally occurring genetic exchange (or laboratory manipulation) has been described that converts one of the classical species into another. This genetic stability marks a clear difference with bacteria like pathogenic *E. coli* in which both the host range and the type of damage induced depends on the acquisition and type of relatively few adhesin, invasin and toxin genes. Thus the classical *Brucella* species that display a marked host range (*B. melitensis* for sheep and goats; *B. abortus* for cattle, *B. canis* for dogs, and as far as it is known *B. neotomae* for desert wood rats), or this feature plus significant differences in pathology (*B. ovis*), are not mere pathovars. On the contrary, these bacteria constitute genetically committed groups evolutionarily linked to their preferred hosts, and for which biologically meaningful species can be defined. A conspicuous exception to this is *B. suis* since host range seems to be a characteristic of the biovars (1 and 2 for pigs; 2 for pigs and hares; 4 for reindeer; 5 for wild rodents) rather than of the species (Alton et al., 1988). Whether this reflects in all cases a true host range or the geographical isolation of some biovars remains controversial (Meyer, 1990b).

The insight of the early *Brucella* researchers that the host range is a key biological characteristic in the definition of *Brucella* species is also consistent with the results of molecular analyses. Of these, the first work that supported the division of the genus into the classical species was the numerical analysis of the cellular fatty acids performed by Tanaka et al. (Tanaka et al., 1977). It has to be noted that, whereas this study included a significant number of *B. melitensis*, *B. abortus* and *B. canis* isolates (12 strains and all biovars, 50 strains and all biovars and 12 strains, respectively), fewer *B. suis*, *B. ovis* and *B. neotomae* isolates (6 (biovars 1, 2 and 3), 1 and 1, respectively) were examined and, therefore, no conclusions could be drawn on the internal diversity of these three species. Moreover, consistent with the fact that DNA–DNA hybridization only superficially assesses genome similarities or differences (van Belkum et al., 2001), markers that overlap with most of the classical *Brucella* species have been found. Studies with a limited number of strains, including in some cases the reference strains of the classical species and biovars, show that they can be discriminated by restriction fragment length polymorphism (RFLP) analyses of whole DNA (Allardet-Servent et al., 1988; Jensen et al., 1999), the Bru-RS1 and Bru-RS2 elements (Halling and Bricker, 1994), the insertion sites of IS711 (Bricker and Halling, 1994; Ouahrani et al., 1996; Clavareau et al., 1998; Bricker, 1999), the REP and ERIC sequences (Mercier et al., 1996; Tcherneva et al., 2000) and other DNA sites (Cloeckert et al., 1996), with total or partial success depending on the particular method, DNA region, and the level of discrimination (i.e. a single species, several or all species, or even most but not all biovars using a single method). From a taxonomical standpoint, the species-specific localization of IS711 is particularly meaningful since these elements are in a relatively high number in *Brucella* genomes and represent a possible source of internal diversity for a group where, as discussed above, there are little chances for genetic exchange. Moreover, molecular markers for *B. melitensis*, *B. abortus*, *B. ovis*, *B. canis* and *B. neotomae* (all of which display characteristic host ranges) have been identified in *omp25* (*omp31a*), *omp31*, *omp2a* and *omp2b* (Table 2) in studies that followed earlier work that suggested that the

Table 2  
Species and biovar specific molecular markers in *Brucella* outer membrane protein genes

Species	Biovar	Target genes (restriction site) for differentiation at the level of		Reference
		Species	Biovar	
Classical				
<i>B. melitensis</i>	Biovar 1, 2, 3	<i>omp25</i> (EcoRV)	n.d. <sup>a</sup>	(Cloeckert et al., 1995)
<i>B. abortus</i>	Biovar 1, 2, 4	$\Delta omp31$	<i>omp2a</i> ( $\Delta 120$ bp)	(Cloeckert et al., 1995; Vizcaíno et al., 1997; Vizcaíno et al., 1999; Vizcaíno et al., 2001)
	Biovar 3, 5, 6, 9		n.d. <sup>a</sup>	(Vizcaíno et al., 1997; Vizcaíno et al., 1999; Vizcaíno et al., 2001)
<i>B. suis</i>	Biovar 1, 3, 4, 5	n.d. <sup>a</sup>	<i>omp2aomp2b</i> <sup>b</sup>	(Cloeckert et al., 1995)
	Biovar 2		<i>omp31</i> (AvaII)	(Vizcaíno et al., 1997)
<i>B. ovis</i>		<i>omp25</i> ( $\Delta 36$ bp); <i>omp31</i> (Sau3AI); <i>omp2b</i> (EcoRI or PvuII)	n.a. <sup>c</sup>	(Cloeckert et al., 1995; Vizcaíno et al., 1997)
<i>B. canis</i>		<i>omp31</i> (AvaII)	n.a. <sup>c</sup>	(Vizcaíno et al., 1997)
<i>B. neotomae</i>		<i>omp2a</i> (AluI); <i>omp2b</i> (HaeIII)	n.a. <sup>c</sup>	(Cloeckert et al., 1995)
Proposed for marine mammals <sup>d</sup>				
<i>B. pinnipediae</i>		<i>omp2b</i> (StyI)	?	(Cloeckert et al., 2001)
<i>B. cetaceae</i>		<i>omp2a</i> (AluI or BglIII or ClaI or EcoRI)	?	(Cloeckert et al., 2001)

<sup>a</sup> n.d., not described.

<sup>b</sup> Differentiation can be achieved by analysis of *omp2aomp2b* with a combination of restriction enzymes (Cloeckert et al., 1995).

<sup>c</sup> n.a., not applicable (there are no biovars in the species).

<sup>d</sup> The proposal follows both the host range criterion and the demonstration of species specific markers.

genes of the major outer membrane proteins contain taxonomically relevant information (Ficht et al., 1990; Ficht et al., 1996). A conspicuous characteristic of *B. abortus* is the existence of a large deletion (25 kb) that encompasses not only *omp31* (Table 2) but also a large number of flanking genes (Vizcaíno et al., 2001). Also, a marker for *B. ovis* exists in the *O*-polysaccharide biosynthesis *wbkA* gene (Cloeckeaert et al., 2000b). No markers have been found so far for the classical biovars of *B. melitensis* or for some *B. abortus* biovars (Table 2). Noteworthy, the present state of the studies suggest an almost opposite situation for *B. suis* where no species specific markers have been found in the *omp* genes (Table 2) or elsewhere. On the other hand, discrimination of some *B. suis* biovars can be achieved by means of specific markers (biovar 2) or by a combination of restriction enzymes (Table 2).

The above-described results support the validity of the host range as a taxonomical criterion for defining the *Brucella* species and, therefore, confirm the true taxonomical standing of *B. melitensis*, *B. abortus*, *B. neotomae*, *B. ovis* and *B. canis*. It seems also that the situation is not so clear with regard to *B. suis* and that either the consistency of this species should be reassessed or additional criteria adopted. Indeed, this may represent a less tight adaptation to a given host that would be in keeping with the closeness of *B. suis* to the ancestor of the genus (see below). A similar problem is posed by the marine mammal strains. As a group, the isolates obtained so far can be differentiated from the brucellae that parasitize the terrestrial species by the electrophoretic profiles of *Xba*I DNA digests (Jensen et al., 1999), IS711-based DNA fingerprinting (Bricker et al., 2000; Cloeckeaert et al., 2000a), ribotyping with *Hind*III (Verger et al., 2000), and by the presence of a group-specific marker in the *omp2b* gene (Cloeckeaert et al., 2001). However, the electrophoretic profiles of the DNA digests (Jensen et al., 1999), the IS711 fingerprints (Bricker et al., 2000) and the polymorphism at the *omp2* genes (Cloeckeaert et al., 2001) show that they are a more heterogeneous group than the “terrestrial” species. Therefore, consistent with the variety of sea mammals from which they have been isolated (four species of seals, four species of dolphins, harbor porpoises and minke whales) (Ewalt et al., 1994; Garner et al., 1997; Jahans et al., 1997; Clavareau et al., 1998), or where serological evidence for *Brucella* infection exists (Jepson et al., 1997; Tryland et al., 1999; Nielsen et al., 2001; van Bressema et al., 2001), the molecular analyses show that it is not appropriate to group them into a single species “*B. maris*” (Jahans et al., 1997). The strains isolated from dolphins, porpoises and minke whales on one hand, and the strains isolated from seals on the other, can be classified into two different groups by the polymorphism at the *omp2* locus (Cloeckeaert et al., 2001), suggesting a broad host range distribution corresponding to cetaceans and pinnipeds. On these bases, a proposal consistent with the molecular taxonomical criteria used in the classical species would be to accept two new species, *B. cetaceae* and *B. pinnipediae* (Cloeckeaert et al., 2001). The IS711-based fingerprints show patterns that correspond rather than to cetaceans and seals, to the particular host species, and at least the isolates from common, harbor, ringed and harp seals can be differentiated from each other (Bricker et al., 2000). This is also the case for the IS711-based fingerprints of the biovars of some classical species, and so it is not clear whether this molecular criterion is useful for species separation. Indeed, it may be that a narrower host range exists than that suggested by the *omp2* locus studies. It seems that this issues cannot be presently solved without a better knowledge of the biology of the marine mammal brucellae, and at least their epidemiology and mode of transmission are relevant to determine to what extent the

groups defined by the molecular analyses constitute genetically committed evolutive groups similar to those defined for the terrestrial mammal brucellae.

## 5. *Brucella* phylogeny and evolution

*Brucella* organisms are alpha-*Proteobacteria* phylogenetically related to plant pathogens and symbionts such as *Rhizobium* and *Agrobacterium*, intracellular animal parasites such as *Bartonella* and *Rickettsia* and to opportunistic and free living bacteria like *Ochrobactrum* and *Caulobacter* (De Ley et al., 1987; Moreno et al., 1990; Velasco et al., 1998). Complete chromosomal sequences of species belonging to these genera of alpha-*Proteobacteria* have been released recently ([www.tigr.org/tdb/mbd/](http://www.tigr.org/tdb/mbd/)), allowing genetic comparisons with *Brucella* (Moreno and Moriyón, 2002; DeVecchio et al., 2002). In general, these data greatly support and complement previous analyses on *Brucella* evolution which were based in limited genotypic and phenotypic characters as well as the life style of its bacterial relatives (Moreno, 1992; Moreno, 1998; Moreno and Moriyón, 2001).

### 5.1. Before the *Brucella* genus

By observing the most parsimonious evolutionary trails, according to the ancestor descendent rules of characters and by estimating the connection between ortholog and paralog genes (Moreno and Moriyón, 2001; Moreno and Moriyón, 2002), it can be predicted that the ancestor of *Brucella* was an aerobic heterotroph free living motile bacteria inhabiting the rhizosphere of plants. With the exception of glycolysis, this bacterium was more likely endowed with all major biosynthetic pathways, including the hexose monophosphate shunt, citric acid cycle, urea cycle, the Entner–Doudoroff pathway and the erythritol pathway. In addition, it already harbored two chromosomes and plasmids with genes necessary to deal with local environmental conditions found in soil such as resistance to antibiotics and heavy metals. It also possessed an ancestral DNA replicative machinery conserved in alpha-*Proteobacteria*, from the strict parasitic *Rickettsia* which is the closest relative to mitochondria to the aquatic free living *Caulobacter* (Brassinga et al., 2001). The cell envelope of this hypothetical bacterium harbored an inner membrane with all the necessary components for classical oxidative phosphorylation based in cytochromes, ubiquinone-10 and proton ATPases, in addition to enzymes to adapt to low oxygen tension and nitrate respiration which probably allowed microaerophilic and anoxybiontic growth under some conditions. The periplasmic space also harbored osmoregulated cyclic glucans, Fe and Cu–Zn superoxide dismutases and catalase for dealing with peroxide and free oxygen radicals. Phosphatidylcholine, phosphatidylethanolamine, cardiolipin and phosphatidylglycerol substituted by fatty acids C<sub>16:0</sub>, C<sub>18:1</sub> and C<sub>19:0</sub> cyclopropane were already the major membrane phospholipids present in this precursor. In addition, the outer membrane was endowed with conserved family of outer membrane proteins such as RopA and porins as well as ornithine containing lipids and a unique lipopolysaccharide (LPS) molecule. This latter glycolipid was probably built of a phosphorylated diaminoglucose disaccharide substituted with two 3-deoxy-D-manno-2-otulosonic acid (Kdo) molecules, long chain fatty acids (some of them above 30 carbon

atoms), neutral sugars and uronic acids, but without the heptoses which characterize other LPS molecules. Linked to this, the outer membrane represented an effective barrier to environmental noxious substances in combination with the action of efflux pumps. Although flagella were conspicuous in this bacterium, it already lacked pili and fimbriae. Among some of the probable potential characteristics of this bacterium was its ability of being an opportunistic inhabitant of animal cells, establishing by this its future trail as intracellular parasite. Phenotypically, the closest extant species would be *Ochrobactrum intermedium* (Velasco et al., 1998; Velasco et al., 2000).

## 5.2. Rise of *Brucella* genus and *Brucella* speciation

One of the valuable things for establishing lines of ancestor descendent relationships is that they allow to distinguish between trails that have been maintained or modified to accomplish a different set of functions, from those that have been acquired or lost by idiosyncratic manner during evolution. If we accept that the *Brucella* ancestor was a bacterium that evolved into an animal intracellular parasite by losing structures, modifying preexisting ones and generating or acquiring new trails, these characteristics must be reflected in the genome as well as in the phenotype and life cycle of the extant *Brucella*. Commensurate with these features are the intermediate values regarding the genome size and the G + C content of *Brucella* in comparison with its free living/plant and the obligate intracellular alpha-*Proteobacteria* relatives. The presence of two chromosomes with the same G + C content and almost identical proportion of potential coding regions in relation to the chromosomal sizes as well as the equilibrated distribution of housekeeping genes, reveal that both replicons have had a long coexistence. Indeed, the closest *Brucella* relative, the free-living and opportunistic *Ochrobactrum* possesses two chromosomes (Jumas-Bilak et al., 1998), suggesting that the ancestor of these two genera already exhibited two megareplicons (Moreno, 1998). It is tempting to speculate that the smaller chromosome of the *Brucella/Ochrobactrum* ancestor evolved from a megaplasmid. Translocation of housekeeping genes to the ancestral megaplasmid, promoted by an extensive number of insertion elements and transposases, could have transformed this megareplicon into a chromosome. Indeed, certain clusters such as the arginine and ornithine cyclodeamidase genes and the *virB* operon, all located in chromosome II, are homologous to genes located in the same order in the Ti plasmid of *A. tumefaciens*. Concomitantly to the evolution of the chromosomes, plasmids and lysogenic phages probably were permanently excluded from the genome as consequence of the restricted life cycle of *Brucella* within host cells, as previously proposed (Moreno, 1998).

Some of the orthologous characteristics identified as or predicted to be relevant for virulence did not probably undergo substantial modifications, as they are shared by the phylogenetic neighbors. Many are concentrated at the cell envelope, and include ornithine containing lipids, phosphatidylcholine, long chain fatty acids, periplasmic glucans, catalase and superoxide dismutase (Moreno and Moriyón, 2002). The absence of certain structures such as flagella, extracellular polysaccharides different from perosamine homopolymers, and of lysogenic phages, efflux pumps active on hydrophobic compounds, heavy-metal pumps and enterobactin iron chelator has been demonstrated in *Brucella* grown in vitro. Despite this, there are chromosomal sequences tentatively coding for all of

them, suggesting that either they are only expressed during intracellular parasitism or represent just a set of truncated DNA or “fossil” ancestral sequences remaining in the *Brucella* genome. At least in the case of genes coding for flagella and phages, the sequences seem to be truncated. Proteomic analysis of bacteria growing inside cells will contribute to discern between these alternatives. Other modifications that are in all likelihood the result of the adaptive evolution to pathogenicity include several outer membrane features. Whereas the structure of the LPS lipid A was not substantially modified with respect to that of the phylogenetic neighbors, it is postulated that the loss of the acidic sugars in the core oligosaccharide would simultaneously bring about a marked resistance to bactericidal peptides and an increased outer membrane permeability (Velasco et al., 2000) thus rendering the action of some efflux pumps inefficacious. Although these characteristics have not been examined in the marine mammal brucellae, at least resistance to bactericidal peptides can be predicted on the basis of the cultural characteristics (Foster et al., 1996). Thus, these changes in LPS structure must have occurred before speciation. Similarly, the high conservation of the two component BvrR/BvrS sensory regulatory system within the brucellae examined, and the striking difference in its periplasmic sensing region with those of the phylogenetic neighbors (López-Goñi et al., 2002) also support the idea that this adaptation has preceded speciation. In the same line of thinking, the type IV secretion system in plant endosymbionts functions in the establishment of the bacteroid condition and in *Agrobacterium* transports Ti DNA (Christie and Covacci, 2000), whereas in *Brucella* it may be devoted to the transference of molecules inside phagosomes for controlling intracellular trafficking. Further evolution of the LPS structure was achieved by horizontal acquisition of *O*-polysaccharide genes. After this, speciation events must have occurred including those leading to the different surface characteristics of the *Brucella* species that are attached to the LPS, and possibly some quantitative changes in Omps (Santos et al., 1984) and fatty acids (Tanaka et al., 1977). As cetaceans separated from the common branch earlier than other *Brucella* hosts about 55 millions of years ago and cross-infections with terrestrial mammals seem unlikely, it can be speculated that this would be the minimal time after which speciation occurred. This earlier divergence of cetaceans and the emergence of the pinnipeds only about 25 million years ago is consistent with the greater diversity observed for the marine mammal strains in molecular analyses.

By comparing dendrograms and phylogenies it seems feasible to hypothesize that *B. abortus* and *B. melitensis* shared the same ancestor (Moreno and Moriyón, 2001), and this is consistent with the time calculated for the divergence of their hosts (about 20 million years ago). Likewise, *B. canis* and *B. suis* shared a common ancestor. Indeed, the size of the two chromosomes of *B. canis* and biovars 2 and 4 of *B. suis* correspond; similarly, the size of the two chromosomes of *B. abortus* and *B. melitensis* also correspond, supporting the topologies of the trees. It has been hypothesized on the basis of the nutritional requirements that *B. suis* is the closest species to the *Brucella* ancestor (Plommet, 1991). This postulate is sustained by several observations. For instance, *B. suis* is the most diverse of the classical brucellae in genomic structure and host preferences (Moreno and Moriyón, 2001) and because this species has a wider range of biochemical alternatives, its metabolic capability comes closest to heterotrophy. Moreover, in trees constructed on the basis of biochemical characteristics or protein cross-reactivity and in which the heterotrophic *O. intermedium* is included as an outgroup, *B. suis* strains are the brucellae most closely

related to *Ochrobactrum* (Velasco et al., 1998). If we accept that speciation in some clonal bacterial populations, with no horizontal transfer of genes, may be gradual and commensurate with time, then *B. suis* is the most diverse among the *Brucella* species that parasitize terrestrial mammals, and the most closely related to the heterotrophic ancestor.

## 6. Conclusions: maintaining order in the taxonomy of the genus *Brucella*

In one of her 1990 reviews on *Brucella* Taxonomy and evolution, Margaret Meyer wrote a section entitled “Bringing Order into the Taxonomy of the genus *Brucella*” (Meyer, 1990b). Meyer did not agree with the idea that all brucellae should be grouped into a single species. She reasoned that, since “no one is certain about what constitutes a bacterial species” and the monospecific concept was in her opinion misleading with regard to “what we do know about the evolutionary paths in the genus,” more genetic and evolutionary information was necessary before adopting the proposed changes in *Brucella* nomenclature (Meyer, 1990b). In the opening chapters of the new edition of Bergey’s manual, it is stated that “. . . considering the perception of a bacterial species, taxonomists either sustain a coherent species definition without questioning if this corresponds to a biological reality, or they try to visualize bacterial species as condensed nodes in a cloudy and confluent taxonomic space” (Gillis et al., 2001). In this article, we have argued in favor of the second line of thought for the definition of *Brucella* species, and this addresses the first point made by Meyer with the result that there are no scientific grounds for accepting the idea that *Brucella* is a monospecific genus. Also, although Meyer’s views on the evolution of *Brucella* species (Meyer, 1990b) have been proved not to be correct, her insight that this aspect of *Brucella* biology would support the multiple species system seems basically right. Indeed, there are issues that remain to be resolved, and we have pointed out above the ones which are, in our view, the most important ones. The last proposal for minimal standards for new *Brucella* species was made in 1975 (Corbel and Morgan, 1975), and it seems timely to review them. The classical host range criterion is supported as a valid one by the molecular and evolutionary findings, and it seems likely that these tools and concepts will also be useful to define species and to understand the biology of the brucellae isolated from marine mammals.

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