AN OVERVIEW ON THE PHYLOGENETIC CLASSIFICATION OF *Brucella*

EVOLUCIÓN EN LA CLASIFICACIÓN FILOGENÉTICA DE *Brucella*

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SUMMARY

The genus *Brucella* is a globally distributed intracellular pathogen that affects animals and humans and presents low genetic variability, which is a challenge for its phylogenetic reconstruction. Genus differentiation originally occurred due to its preference for a certain host and analyses throughout the years to classify the genus *Brucella* and its strains, depended on the techniques used, the number of strains or the methods of phylogenetic reconstruction. Its history goes from recognizing the entire genus as unique *B. mellitensis* species with multiple varieties, to recognizing more than ten species. This review shows the journey through the techniques that have been used to differentiate the genus *Brucella*, which, although they have generated clarity in the grouping of some species, still leaves doubts in related to the oldest species, their divergence and the type of grouping of the new species discovered. The application of new technologies such as phylogenomics is contributing to solve these questions.

Key words: Monophyletic, Paraphyletic, Polyphyletic, Clade, Divergence.

RESUMEN

Las especies del género *Brucella* son patógenos de distribución mundial que afecta, tanto a los animales como al hombre, los cuales, presentan baja variabilidad genética, convirtiéndolo en un reto para su reconstrucción filogenética y diferenciación. La diferenciación originalmente se dio, debido a su preferencia por cierto hospedero. A través de los años, se han realizado diferentes análisis para clasificar el género *Brucella* y las variaciones que presenta, que dependen de las técnicas empleadas para su clasificación, las especies, el número de cepas o los métodos de reconstrucción filogenética. La historia pasa desde reconocer a todo el género como una única especie *B. mellitensis* con múltiples variedades, a reconocer más de diez especies. En esta revisión, se hace una travesía, a través de las diferentes técnicas que se han utilizado en el tiempo, para clasificar el género *Brucella* que, aunque han generado claridad en el agrupamiento de algunas especies, aun dejan dudas en relación a las especies más antiguas, su divergencia y el tipo de agrupación de las nuevas especies descubiertas. La aplicación de nuevas tecnologías, como la filogenómica, está contribuyendo a resolver estos interrogantes.

Palabras clave: Monofilético, Parafilético, Polifilético, Clado, Divergencia.

INTRODUCTION

Brucellosis is an infectious disease caused by a facultative intracellular pathogen that belongs to the genus *Brucella*; it is part of the 2α class of the Proteobacteria, whose members share the ability to interact intracellulary with the eukaryotic cell (Moreno *et al.* 1990; Paulsen *et al.* 2002; Chain *et al.* 2005; Ficht, 2010).

For many years, this genus comprised only six species, denoted the classical brucellae: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae* (Allardet-Servent *et al.* 1988; Vargas *et al.* 2011; Scholz & Vergnaud, 2013; Skendros & Boura, 2013). Mainly differentiated by their host preference and a set of antigenic and phenotypic characteristics. Since the 90's four *Brucella* species have been isolated from marine mammals, rodents and surprisingly from a breast

implant (Paulsen *et al.* 2002; O'Callaghan & Whatmore, 2011; Vargas *et al.* 2011; Ortega *et al.* 2013; Wattam *et al.* 2014). Other studies have reported the isolation of novel *Brucella* species denoted *Brucella vulpis* (Hofer *et al.* 2012; Scholz *et al*. 2016) *and Brucella papionis* (Whatmore *et al.* 2014) that belong to the atypical group of *Brucella* (Tiller *et al*. 2010), and other isolates from frogs, fish and additional rodents thus expanding the type of host of this bacterium (Eisenberg *et al.* 2012; Eisenberg *et al.* 2016; Scholz *et al.* 2016; Soler-Lloréns *et al.* 2016; Whatmore *et al.* 2016).

In relation with the genetics of this pathogen, in 1985 a high degree of identity (>90%) among *Brucella* species was found from studies made by DNA-DNA hybridization between the different species (Gee *et al.* 2004), which made them difficult to classify. Since then, a consensus has been reached about the grouping of the *Brucella* species by different authors, given that the results depend on the techniques, the number of strains used, and the inclusion of new species as well as the method of phylogenetic analysis used. The aim of this review was to compare from a methodological point of view, the *Brucella* species that are most widely studied and among which phylogenetically clear relations are presented.

METHODS

The study includes a comprehensive search in electronic databases. A literature review was conducted with the following key words: *Brucella,* evolution of *Brucella,* phylogenetic of *Brucella,* classification of the genus *Brucella,* methods for classification of *Brucella,* species in *Brucella* and the combination of these terms. Records ranged from 40-50 articles, and articles published in English and Spanish were identified, classified and analyzed. Articles that provided relevant information on the basis and subject of the review were selected, the period considered in the research was from 1985 (first publication about *Brucella* genus) to 2016. Items whose thematic is not related to evolution or typification techniques were excluded

RESULTS AND DISCUSSION

Regarding the phylogenetic relationship of *Brucella* species, a consensus about the grouping of the different *Brucella* species is shown:

***Brucella abortus* and *Brucella melintesis*:** The phylogeny for these two species depends on factors such as type and number of species included in the analysis, as well as the technique and methodology used. In this classification, many authors have disagreed on through the years, the current consensus from the comparative study of the *Brucella* species through variations in the Omp2 gene sequences up to the last analyses realized from SNPs (single nucleotide polymorphisms) (Vignal *et al.* 2002; Ohishi *et al.* 2008; Wattiau *et al.* 2011) and of orthologous proteins, considers *Brucella melitensis* and *Brucella abortus* as part of a monophyletic group (Figure 1) (Mirnejad *et al.* 2012; Wattam *et al.* 2014). When we carried out phylogenetic analyzes, through the analysis of housekeeping genes including *O. anthropi as an external group,* showed *B. abortus* and *B. melitensis* as sister species, each of them monophyletic (Arboleda *et al.* 2017)

Complete genome sequencing is ideal; they have the advantage that mutational changes are totally reflected in the sequence, an event that does not occur in proteins, due to the degenerate character of the genetic code; clarifying in this way the main evolutionary mechanims involved in the different processes of speciation, a example of this type of study was made in 2015 in where the authors sequenced the complete genome of several *B. melitensis* strains, achieving not only discrimination between vaccine and endemic species, but also performing a phylogenetic reconstruction of the history of the species and proposing a possible global distribution of the bacterium (Whatmore *et al.* 2005; Tan *et al.* 2015).

Brucella suis* and *Brucella canis: In relation to these two species, it has been particularly difficult to distinguish among them because of the few genetic polymorphisms allowing to discriminate them at the molecular level (López-Goñi *et al.* 2011). Ficht *et al.* (1996) identified variations in the sequences of the gene that codes for the Omp2 protein, this membrane protein linked to the formation of porin, could be used for the differentiation of species given the high degree of sequence conservation (Ficht *et al.* 1990). Phylogenetic analyses postulated a recent divergence and a close relationship between these two species, proposing them as sister taxa with a common ancestor that is not shared with other species of *Brucella* (Whatmore *et al.* 2005).

With the use of techniques such as AFLP (amplified fragment length polymorphism) (Meudt & Clarke, 2007), MLEE (Multilocus enzyme electrophoresis) (Selander *et al.* 1986) and orthologous proteins, it is not possible to differentiate these two species and hence their limited use as tools (Gándara *et al.* 2001; Whatmore *et al.* 2005; Wattam *et al.* 2009) (Figure 2).

Techniques such as MLVA (Multilocus variable number of tandem repeats analysis), VNTR (variable number of tandem repeats) (Al Dahouk *et al.* 2007) and MLST (Multilocus sequence typing) (Maiden *et al.* 2013) show a clear dispersion between different strains of the same *B. suis* species (Figure 3), where the greatest distance is found between biovar 5 and the other biovars. Additionally, it exposes the separation of *B. suis* into two subclades, one formed by the biovar 2 and

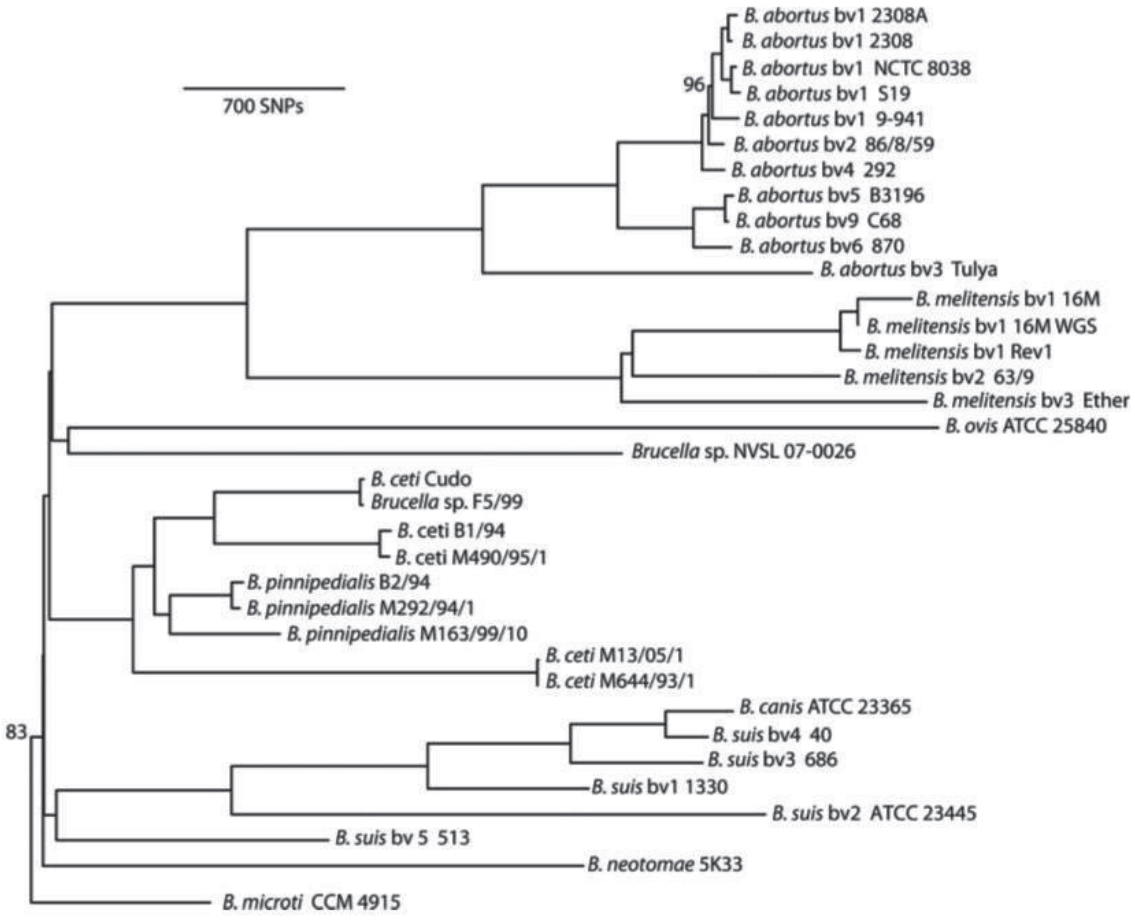

Figure 1. Phylogenetic analysis based on maximum parsimony from SNPS (Wattam *et al*. 2014). All branches have 100% support unless otherwise noted, which permits restricted use. License number 3798391155478.

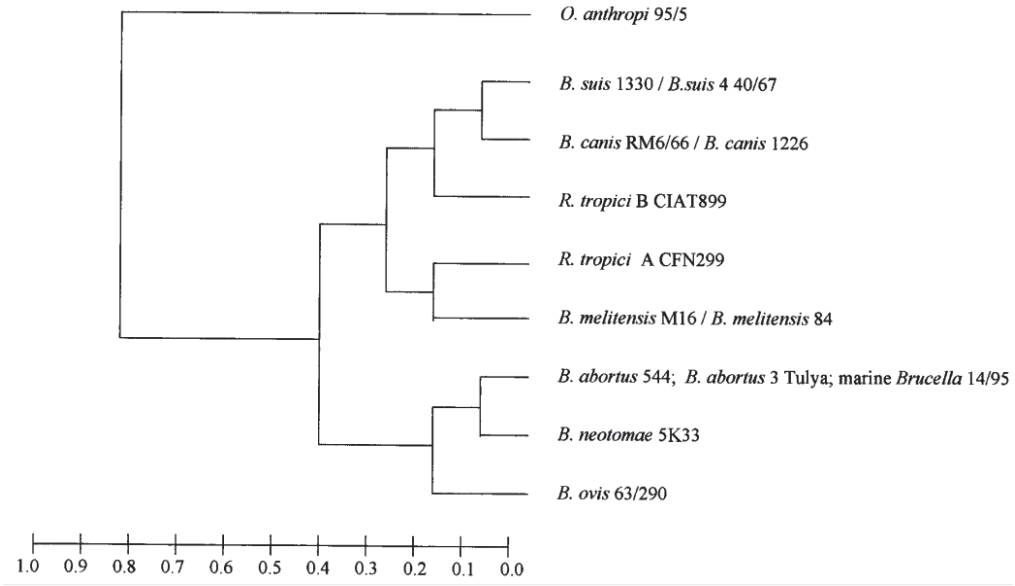

Figure 2. Dendogram from multilocus enzyme electrophoresis (Gándara *et al*. 2001), which permits restricted use. License number 3798390614711.

one that contains biovars 1, 3 and 4, making this species a paraphyletic group (Wang *et al.* 2016), converting it into the most diverse strain of this bacterial genus (Le Flèche *et al.* 2006; Sankarasubramanian *et al.* 2016). This research also shows the direct emergence of *B. canis* from a *B. suis* ancestor (Figure 2) (Le Flèche *et al.* 2006).

In our 2016 research, in which *B. suis* is observed as the species with the greatest genetic diversity among the strains studied; and the proximity between *B. suis* and *B canis* is observed, forming part of the same clade, with low divergence (Arboleda *et al.* 2017).

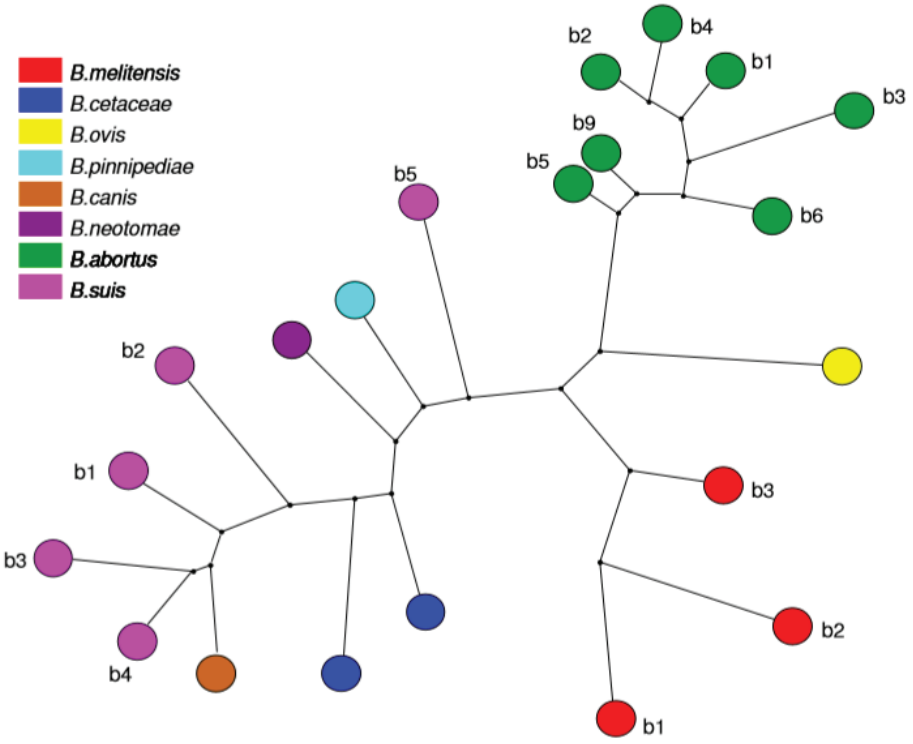

Figure 3. Phylogenetic analysis based on maximimum parsimony from MLVA (Le Fléche *et al*. 2006), which permits unrestricted use.

Brucella ceti* and *Brucella pinnipedialis: Referring to these two species, the first one was isolated from porpoises, whales and dolphins while the second one was isolated from common seals (Gándara *et al.* 2001; Foster *et al*. 2007). It is from these reports that the largest number of isolates have been reported and the host range has been significantly expanded (Verger *et al.* 1985). Since their discovery, the marine *Brucella* strains have been subjected to a range of tests such as DNA-DNA hybridization and ribotyping, in order to compare them with the terrestrial strains (Verger *et al*. 2000).

The first inclusion of marine species in the phylogeny of *Brucella* was made by Jensen *et al.* (1999), where an unusual grouping between these was demonstrated, similar to what was found between *Brucella canis* and *Brucella suis* serotype 1 (Michaux-Charachon *et al.* 1997). The ones isolated from the marine mammals presented different profiles from the other species of *Brucella,* placing them in a different clade or branch of the tree (Figure 1, Figure 4) (Whatmore *et al.* 2007).

Based on studies of the Omp2 gene polymorphisms these authors proposed two subdivisions, recognizing two marine species, *Brucella pinnipediae* isolated from seals and *Brucella ceti* isolated from dolphins and porpoises (Cloeckaert *et al.* 2001), while other authors, through techniques such as VNTR, MLST, Fingerprint, demonstrated the separation of the marine species into three isolated groups (Le Flèche *et al.* 2006; Whatmore *et al.* 2006; Dawson *et al.* 2008): the first was obtained from species of dolphins, the second consisted in strains isolated from

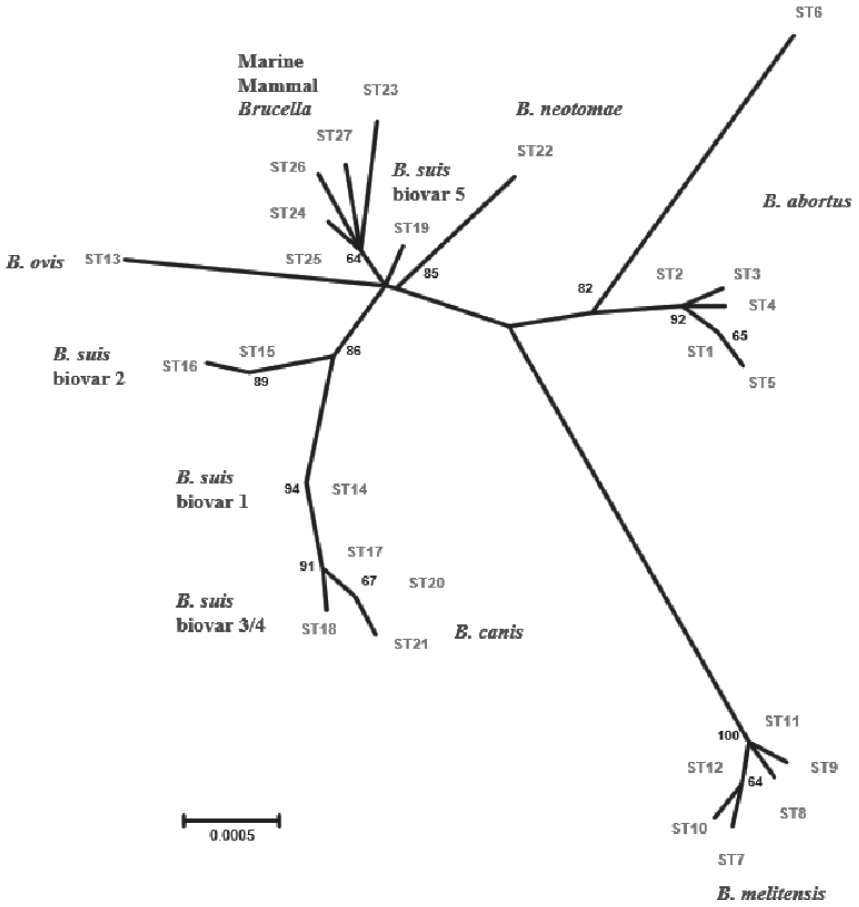

Figure 4. Phylogenetic analysis based on concatenated sequences through neighbor joining (Whatmore *et al.* 2007), which permits unrestricted use.

porpoises and the third contained isolates of different seal species (Groussaud *et al.* 2007; Dawson *et al.* 2008; Sidor *et al.* 2013). From these studies we retained the subdivisions within the *B. ceti* species because of their host preference between dolphins and porpoises. In a similar manner we speak of an ecological subdivision between the members of the pinnipediales species, demonstrated by a subgroup of hooded seals (Dawson *et al.* 2008).

Two groups have been agreed on based on more specific techniques: *B. ceti* and *B. pinnipediales,* with very small genetic distances, indicating a simultaneous divergence (Wattam *et al.* 2014); however it does not match the phylogeny of its main host. Guzmán-Verri *et al*. (2012) discusses the ability of the *Brucella* species to jump from one host to another and in some cases to generate new species depending on their preferred host, but they are in no case closely related with the divergence time of the host species, assuming a contamination of the marine species by the food chain (Dawson *et al.* 2008; Guzmán-Verri *et al.* 2012).

Brucella ovis* and *Brucella neotomae: Since the initial studies on the phylogeny of *Brucella, B. ovis* and *B. neotomae* species have been reported as the most divergent (Ficht *et al.* 1996; Michaux-Charachon *et al.* 1997; Jensen *et al.* 1999; Wattam *et al.* 2009), these data have differed depending on the techniques used and the inclusion of one or two species in the same analysis, placing these two species in the same clade is influenced by the used technique.

To define the basal or the most divergent species, different techniques have been proposed: the first one with MLVA, MLST and orthologous protein (protein in different species that connected through vertical evolutionary descent) (Fitch, 1970), where the authors confirm the previous divergence of *B. ovis* forming a separate clade from the other *Brucella* species (Le Flèche *et al.* 2006; Whatmore *et al.* 2007; Wattam *et al.* 2009) corroborated with the SNP orthologous study, suggesting that brucellosis in animals such as pigs, goats and cattle occurred from contact with infected sheep, approximately in the last 86.000 to 296.000 years (Foster *et*

al. 2009; Yang & Rannala, 2012). Our phylogenetic analysis, including *O. anthropic* as an external group yielded results according, placed *B. ovis* as the first lineage to be divided from the rest of the species of *Brucella* and recognize it as the basal species (Arboleda *et al*. 2017).

The second species, *B. neotomae* reported by Audic *et al.* (2011), from the analysis of the orthologous genes and the inclusion of a greater number of species, showed an initial divergence from *B. microti* and after this event the divergence of *B. suis* and *B. neotomae* demonstrated by the loss of a 2.6 kb fragment from these two species (Wattam *et al.* 2009; Audic *et al*. 2011).

***Brucella* of early divergence:** Throughout the years and with the advances in molecular analysis, new species of *Brucella* have been discovered, this causes variations in phylogenies (Audic *et al.* 2009; Whatmore *et al.* 2016) sequenced and examined the phylogeny of MLVA based on VNTRs from *B. microti*, initially isolated from voles, foxes and later from soils, being the only species with a reservoir outside a mammalian host (Rónai *et al*. 2015), contradicting the assumption that *B. ovis* is the most basal species and showing that *B. microti* is an even more basal species than *B. ovis*, also indicating that *B. inopinata* isolated from humans presents a previous divergence (Figure 5).

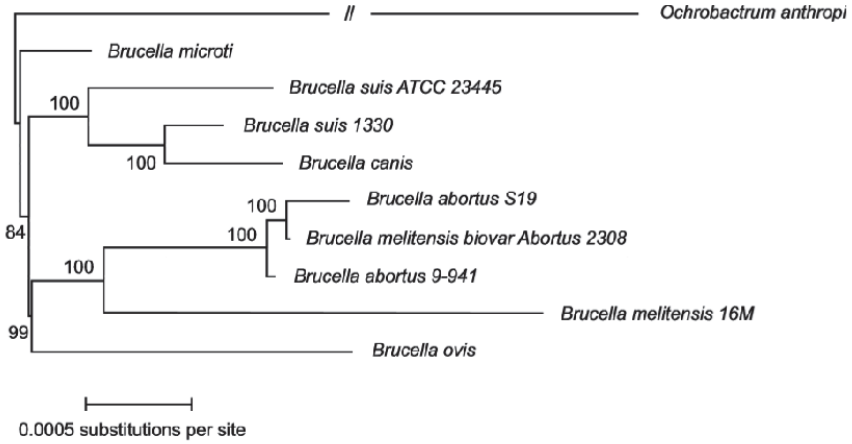

Figure 5. Phylogenetic reconstruction from 1,486 orthologous genes (Audic *et al*. 2009), which permits unrestricted use.

CONCLUSIONS

In conclusion there is a clear transition in the evolutionary tree of *Brucella* from its initial analysis 20 years ago, where a single species with multiple biodiversities was established (Foster *et al*. 2009). Phylogenetic studies have helped to see gene transfer events, thereby allowing not only the differentiation of the various *Brucella* species, but also the acquisition of virulence factors including the type IV secretion system, a perosamine based O antigen, and systems for the maintenance of metals and lineages involved in its host preference (Wattam *et al*. 2014; Tan *et al*. 2015).

The development of new studies to reach a better understanding of *Brucella* evolutionary history, host specificity and pathogenicity, is still needed to include a greater number of representatives genomes of each species, and to incorporate and to evaluate previous data (Moreno *et al*. 2002). Phylogenomics is the best alternative to ensure a better resolution to these questions, considering others speciation processes that can cause variations in the resulting trees (Eisen & Fraser, 2003) because it provides a direct way to estimate the evolution of the species from all the shared genes, and in which no duplication patterns or horizontal gene transfers are involved.

This review article demonstrates how molecular and bioinformatics advances have led to a better understanding of the genus *Brucella,* defining small but significant differences between species, which can be used to identify important interactions that contribute to the invasion, persistence, transmission and even virulence of each one of them.

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