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Review

Genotyping, evolution and epidemiological findings of *Rickettsia* species

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ABSTRACT

Rickettsiae are obligate intracellular bacteria that can cause mild to life-threatening diseases, including epidemic typhus, one of the oldest pernicious diseases of mankind. Clinical awareness of rickettsial diseases and molecular diagnosis have shown that rickettsioses should be viewed as new emerging and reemerging diseases. *Rickettsia* has been shown to be a large genus with a worldwide distribution, a very diverse host range, including hosts that have no relationship with vertebrate. Genomic studies have demonstrated genome reduction due to gene loss associated with increased pathogenicity and horizontal DNA acquisition according to a sympatric mode of evolution in hosts that contain several organisms. This article presents a review of genotyping techniques and examines the principle of genotype determination in terms of taxonomic strategies and detection methods. This article summarizes the epidemiological and pathological features of *Rickettsia* and discusses the genomic findings that help the understanding of the evolution of pathogenicity including the deleterious mutations of repair systems and the toxin–antitoxin systems.

Contents

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1. Introduction

Rickettsia species are obligate intracellular bacteria in the order *Rickettsiales*, α-Proteobacteria. *Rickettsia* are best known as vertebrate pathogens for which the vectors are blood-feeding arthropods (Raoult and Roux, 1997). The genus *Rickettsia* was traditionally classified based on their morphological, antigenic, and metabolic characteristics into the following groups: (i) the spotted fever group (SFG), which includes species transmitted by hard ticks such as *Rickettsia conorii*, the causative agent of Mediterranean spotted fever (Raoult et al., 1986) and *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever (Dumler and Walker, 2005); (ii) the typhus group (TG), which includes *Rickettsia typhi*, the flea-transmitted causative agent of murine typhus and *Rickettsia prowazekii*, the louse-transmitted agent of epidemic typhus (Bechah et al., 2008; Zinsser, 1935) and (iii) the group containing *Rickettsia tsutsugamushi*, the etiological agent of scrub typhus. Genotyping methods used for taxonomic purposes and the description of new species determined that the genus *Rickettsia* comprises 27 recognized species and several dozen as-yet uncharacterized strains (Fig. 1). *R. tsutsugamushi* has been shown to be distinct enough to warrant classification into a new genus, *Orientia* (Tamura et al., 1995). Species within the SFG have been subdivided into four subgroups in accordance with their phenotypic profiles; the subgroups are the *R. rickettsii*, *Rickettsia massiliae*, *Rickettsia helvetica*, and *Rickettsia akari* (Merhej and Raoult, 2011). The availability of complete genome sequences has allowed new approaches to phylogenetic inference and provided new perspectives on rickettsial evolution. Phylogenomic studies showed that the genus could be divided into four different phylogenetic groups: the well-known TG and SFG, the *Rickettsia belli* group and the *Rickettsia canadensis* group, which until recently comprised *R. canadensis* (Merhej et al., 2009a) (Fig. 1).

Clinical awareness of rickettsial diseases, extensive use of cell culture systems and the development of specific and sensitive molecular tools have allowed for the discovery of new rickettsioses and the identification of typical rickettsioses in places where no rickettsioses had been identified (Angelakis et al., 2012b; Raoult,

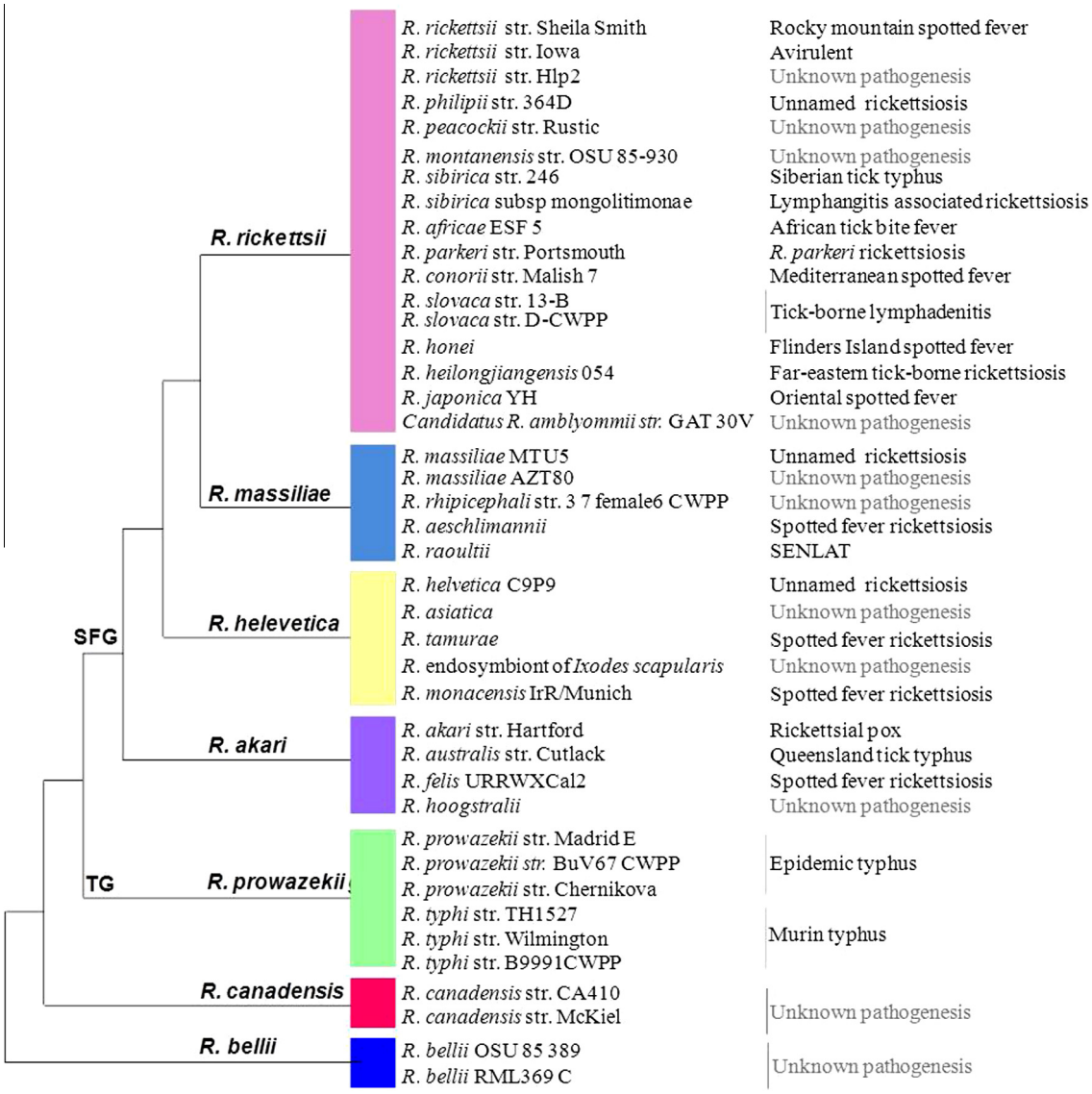

Fig. 1. Rickettsial phylogeny and pathogenic potential. Schematic cladogram of all the recognized *Rickettsia* spp. obtained from the majority consensus tree constructed on the basis of outer membrane proteins and citrate protein sequences. Sequences were aligned using CLUSTALW, five hundred bootstrap replicates were generated, and phylogenetic inferences using the Neighbor-joining method were obtained within PHYLIP package (Felsenstein, 1989; Larkin et al., 2007). TG, Typhus Group; SFG, Spotted Fever Group.

2004). The aphorism, "the Rickettsioses follow rickettsiologists" (the more one searches, the more one finds), formulated years ago remains true (Raoult, 2009). The majority of the recently discovered pathogenic rickettsial agents was initially identified in arthropods and much later were identified in human specimens. In 1937, *Rickettsia parkeri* was identified in the Gulf Coast tick, *Amblyomma maculatum*, and it was not until 60 years later that the first confirmed human *R. parkeri* infection was described (Paddock et al., 2004). *Rickettsia slovaca* was first isolated in 1968 from *Dermacentor marginatus* ticks in Czechoslovakia, and the first documented case of a human *R. slovaca* infection was reported several years after (Raoult et al., 1997; Rehacek, 1984). Some *Rickettsia* spp. have been systematically associated with pathogenicity, and other *Rickettsia* spp. have remained without known pathogenic effects. The rickettsiae designated as human pathogens must be isolated or molecularly detected in the blood or tissues from patients with illnesses that are clinically compatible with rickettsioses and for whom seroconversion was indicated with reference laboratory methods. Given the difficulty in predicting the pathogenicity of new rickettsial species isolated from arthropods, these species should be considered potentially pathogenic to humans. Rickettsioses could be viewed as emerging and re-emerging diseases.

Molecular studies have revolutionized *Rickettsia* systematic studies and unveiled the great diversity of *Rickettsia* spp. with respect to host ranges, effects on hosts and geographical distribution. Many *Rickettsia* spp. have been discovered in diverse invertebrates and arthropods that do not feed on blood, including herbivorous arthropods, protozoans, helminthes, and leeches (Caspi-Fluger et al., 2012; Perlman et al., 2006). Transitions seem to have occurred between blood-feeding and non-blood-feeding hosts (Perlman et al., 2006). These findings revealed the complex evolutionary history of rickettsiae and suggested that *Rickettsia* spp. should be considered symbionts, i.e. living in intimate association with invertebrates, that can cause pathogenicity when transmitted to vertebrates through blood-feeding vectors (Perlman et al., 2006). The nature of the symbiotic relationship is a very subtle concept that should be treated with great caution. Some *Rickettsia* spp. have been described as arthropod reproductive manipulators (Renvoise et al., 2011). Further genetic manipulations might reveal the genetic basis of the host-association relationships and facilitate an understanding of the pathogenic mechanisms (Qin et al., 2004). The comparative analyses of pathogenic and less pathogenic *Rickettsia* and *Rickettsia* without known pathogenic effect showed parallel trends in genomic evolution that entailed gene losses related to the isolated intracellular lifestyle and horizontal DNA transfers related to the sympatric mode of evolution due to co-infection in the same host (Georgiades et al., 2011; Merhej et al., 2011). The present review summarizes the molecular approaches for rickettsial genotyping and the recent knowledge of the geographical distribution of rickettsiosis. It describes the large contribution of full genome sequencing as a means of illuminating the evolution of *Rickettsiae* and understanding the mechanisms that underlie the emergence of pathogenicity.

2. Genotyping of *Rickettsia* species

Bacteria have traditionally been classified according to the phenotypic criteria concerning the growth requirements and the microscopic morphology of the colony. These approaches are limited by the fastidious characteristics of the *Rickettsia* spp. Further differentiation among *Rickettsiae* has been obtained by using serotyping and genotyping methods. Different serotypes and genotypes have been identified, allowing for the evaluation of the geographical distribution and the diagnosis of rickettsial pathology.

2.1. Serotyping

Rickettsia spp. are distributed throughout the world in diverse types of ecology (Figs. 1 and 2). Therefore, *Rickettsia* spp. present a high degree of antigenic heterogeneity (Kelly and Mason, 1990) susceptible to inducing a specific immune response. The serologic response is distinct enough to allow species definition. Rickettsial species from SFG were first characterized by the presence of a soluble antigen that fixes complement in the presence of serum from guinea pigs affected by Rocky Mountain spotted fever (Plotz et al., 1944). Other serological tests using the immune response include the toxin neutralization tests in mice (Bell and Stoenner, 1960) and cross-immunization in guinea pigs (Bozeman et al., 1960). The serological approach was shown to be sufficiently sensitive for detecting rickettsiae, and the microimmunofluorescence assay is the current reference method for the diagnosis of rickettsiosis (Philip et al., 1978). Cross-reactions undermine the efficiency of serological methods and the determination of *Rickettsia* species. Monoclonal antibodies have replaced polyclonal antibodies for the identification of rickettsial isolates, and other more sophisticated methods, such as cross-absorption techniques and Western blotting, have been developed (La Scola and Raoult, 1997). Protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), which targets the outer membrane proteins of interest, allow for the identification of SFG rickettsial species and the determination of isolates within the same species (Beati et al., 1992). Immunoblotting using monoclonal antibodies against the lipopolysaccharide-like (LPS-L) antigen and a 120-kDa protein allow for the specific detection of the typhus group (Eremeeva et al., 1994) (Fang et al., 2002). Cross-inhibition fluorescent-antibody tests demonstrated serological differences between the different strains of *R. conorii* (Goldwasser et al., 1974; Vitale et al., 1989). Serological assays that evaluate the antibody production allow for the detection of rickettsial infection for diagnostic purposes and further determination of the rickettsiae at the strain level.

2.2. Rickettsiae phylogeny

Because of the few distinctive phenotypic traits and a subsequent lack of specificity and interlaboratory reproducibility of serotyping methods, the precise identification and classification of *Rickettsiae* have become dependent on the study of genetic sequences. The genus *Rickettsia* has been shown to belong to group 1 of a subclass of *Proteobacteria* based on its 16S rRNA (*rrs*) gene sequences (Weisburg et al., 1989). The high level of conservation in the sequence of this gene in the members of the genus *Rickettsia* (the similarity level between two species exceeds 97.2%) constitutes an impediment for significant inferences about intragenus phylogeny (Roux et al., 1997). The genes encoding for citrate synthase (*gltA*) and for the outer surface proteins and surface cell antigens (*ompA*, *ompB*, *sca1*, *sca2*, and *sca4* genes) have been considered sufficiently variable to infer reliable phylogenetic relationships among *Rickettsiae* (Fournier et al., 1998; Ngwamidiba et al., 2005, 2006; Roux et al., 1997; Roux and Raoult, 2000; Sekeyova et al., 2001). The phylogenetic trees inferred from these datasets have divided the *Rickettsia* genus into six groups represented by the species: *R. rickettsii*, *R. massiliae*, *R. helvetica*, *R. akari*, *Rickettsia prowazekii* and *R. canadensis*. A multigenic approach combining several genes scattered across the rickettsial genomes confirmed the non-monophyletic characteristic of the SFG and distinguished a new subgroup represented by *Rickettsia montanensis* (Vitorino et al., 2007). The availability of complete genome sequences has enabled phylogenomic approaches that have helped to clarify the phylogenetic relationships among *Rickettsiae* (Merhej et al., 2009a). The different subgroups of SFG were defined with high

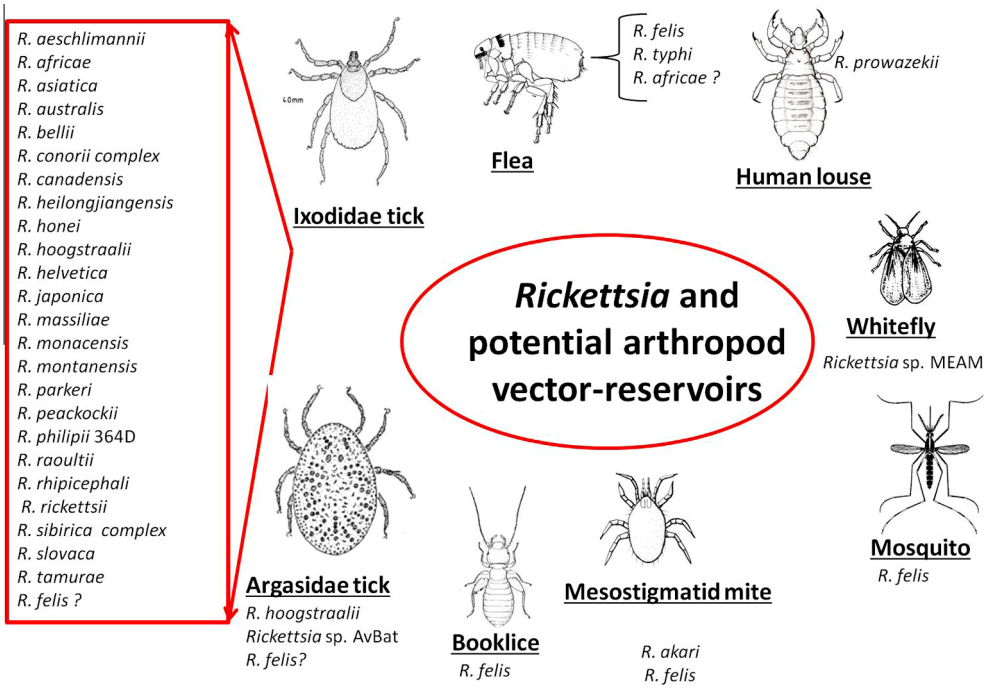

Fig. 2. Rickettsia and potential arthropod vectors and reservoirs.

phylogenetic resolution (Merhej et al., 2009a). *R. bellii*, which was first considered a SFG *Rickettsia*, based on its association with ixodid ticks, was found to be phylogenetically distant from all the other members of *Rickettsiae*. This finding is in agreement with the distinct phenotypic characteristics of *R. bellii*, including that it has the largest host range among the known rickettsiae (Raoult and Roux, 1997), and provided evidence to justify the creation of a new group formed by *R. bellii* (Merhej et al., 2009a; Merhej and Raoult, 2011). Genomic sequencing has provided a large amount of data that are useful for testing the reliability of recognized groups. Phylogenomic studies would be reinforced by the sequencing of more rickettsial species.

2.3. Genotyping methods and taxonomy

DNA taxonomy has been useful for the identification of rickettsial isolates at the genus, group, species and strains levels. Fournier et al. proposed multigene sequence-based guidelines for rickettsial species definition and classification using the 16S rRNA (*rrs*) gene, gene D, and four protein-coding genes, the *gltA*, *ompA*, and *ompB* genes (Fournier et al., 2003). To be classified as a member of the genus *Rickettsia*, an isolate should exhibit more than 98.1% and 86.5% similarity with the *rrs* and *gltA* sequences of the previously validated *Rickettsia* species, respectively. Fournier et al. determined thresholds of pairwise nucleotide sequence similarities that allow for the classification of an isolate into the typhus group or the SFG (Fournier et al., 2003). A multilocus sequence typing (MLST) scheme was used to evaluate the taxonomic relationship of rickettsial isolates by comparing the sequences of a few important genes, such as *ompB*, *ompA*, *gltA*, *sca4* and 16S rRNA (Fournier et al., 2003). MLST allowed for the detection of single nucleotide polymorphisms (SNP) that distinguish *Rickettsia* species (Ge et al., 2007). These findings highlight the lack of discriminatory power of coding DNA for the identification of *Rickettsiae* at the species levels and directed research for the development of new genotyping tools.

Genetic methods based on the comparison of intergenic spacers have proven useful for genotyping *Rickettsia* strains. The gene sequences were shown to have limited interstrain variability (Fournier et al., 2004). Ge et al. found only 3% genetic variation between the strains Madrid E and Breinl of *R. prowazekii* using DNA microarray hybridization (Ge et al., 2004). Because intergenic spacers are less subject to evolutionary pressures than coding sequences, they exhibit nucleotide polymorphisms among *Rickettsiae* that make them more appropriate for typing bacteria at the strain level than genes. Multi-spacer typing (MST) based on the sequencing of variable intergenic spacers, was useful for genotyping *Rickettsiae* at the strain level (Fournier et al., 2004; Ge et al., 2007). MST using 25 intergenic spacers showed that several *R. prowazekii* strains circulated in human body lice during an outbreak of epidemic typhus in Burundi (Zhu et al., 2005b). The analysis of sequences of intergenic regions evaluated the genetic variability among rickettsial isolates from different geographic locations and revealed the existence of different genotypes (Wenjun et al., 2009; Parola et al., 2008; Fournier and Raoult, 2007; Karpathy et al., 2007). Three variable intergenic spacers were shown to be sufficient for the identification of rickettsial isolates at the species level and for further classification into subspecies taxonomic ranks (Fournier and Raoult, 2007). The MST method helped an international committee of rickettsiology experts to propose a guideline for the classification of rickettsial isolates at various taxonomic levels and to clarify the nomenclature within the genus *Rickettsia* (Raoult et al., 2005). The combination of MST and MLST helped to identify four subspecies in the *R. conorii* complex, *R. conorii* subsp. *conorii* subsp. nov. (type strain = Malish, ATCC VR-613), *R. conorii* subsp. *indica* subsp. nov. (type strain = ATCC VR-597), *R. conorii* subsp. *caspia* subsp. nov. (type strain = A-167), and *R. conorii* subsp. *israelensis* subsp. nov. (type strain = ISTT CDC1) (Zhu et al., 2005a) and two subspecies in the *R. sibirica* complex, *R. sibirica mongolitimonae* and *R. sibirica* (Fournier et al., 2006).

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) typing was developed to characterize genetic diversity within different rickettsial strains (Vitorino et al., 2005). VNTRs comprise multimeric repeats that vary in copy number, yielding inter-individual length polymorphisms that could be detected by

a PCR assay, because the regions that flank the repeats are generally well conserved. Tandem repeat motifs could be used to genotype *Rickettsiae* and provide an accurate discrimination of *R. conorii* complex strains (Vitorino et al., 2005) and *R. rickettsii* strains (Eremeeva and Dasch, 2009). Four sites of *R. rickettsii* were shown to contain variable numbers of VNTR sites, and an analysis of their concatenated sequences clustered all the studied isolates into 6 groups (18 genotypes). This technique demonstrated the unique lineage of the *R. rickettsii* isolates associated with *Rh. sanguineus* in Arizona (Eremeeva et al., 2006) and the unique genotype of *R. rickettsii* found in *Rh. sanguineus* in southern California (Wikswo et al., 2007) and demonstrated that circulating *R. rickettsii* in Panama shared a *rickA* VNTR genotype with isolates found in Central and Southern America that were previously associated with *Amblyomma cajennense* (Estripeaut et al., 2007). Molecular approaches have clarified rickettsial taxonomy and allowed for the identification of new species and strains and the reorganization of *Rickettsia* genus. Genotyping methods helped to assess the genetic variability and to study the geographical distribution and pathogenic potential of rickettsiae.

3. Epidemiology

3.1. Emergence of rickettsioses

The newly discovered species of *Rickettsiae* have changed our understanding of the clinical features and epidemiology of historically recognized rickettsioses. Beyond the classic symptom triad, i.e. fever, rash and headache, that were considered as the major clinical signs for rickettsioses diagnosis, each rickettsiosis was shown to present with specific characteristics including the severity and rate of the inoculation eschar (Bechah et al., 2008; Parola et al., 2013; Socolovschi et al., 2013; Walter et al., 2012). Unlike the infections caused by *R. conorii conorii*, patients infected with African tick bite fever tipically present with multiple eschars associated with a few maculopapular or vesicular lesions (Parola et al., 2013; Raoult et al., 2001). The SENLAT (Scalp Eschar and Neck LymphAdenopaThy) syndrome is the presence of an inoculation eschar on the scalp that is associated with cervical lymphadenopathy after a tick bite (Angelakis et al., 2010b; Parola et al., 2009, 2013). The characteristic rash in Rocky Mountain Spotted Fever occurs as generalized petechial lesions on the body, including the palms and soles. In cases of lymphangitis-associated rickettsiosis, the patients present with an expansion of the inoculation eschar to the draining node and/or painful regional adenopathy (Edouard et al., 2013; Fournier et al., 2005). Relapses have been described for *R. prowazekii* (Brill-Zinsser disease) and *R. felis* (Socolovschi et al., 2010).

Globalization and warming trends are two supplementary factors that might influence rickettsial transmission by arthropods (Parola et al., 2008). High temperatures increase the aggressiveness and propensity of *Rh. sanguineus*, the brown dog tick, leading to increased human attacks (Hemmersbach-Miller et al., 2004). A recent unusual cluster of spotted fever cases, including Mediterranean spotted fever (MSF) and *R. massiliae* infections, was reported during an atypical period of the year with summer-like temperatures (Parola et al., 2008). The transmission of *R. rickettsii* by the brown dog tick in Arizona, and the increased MSF incidence in the Mediterranean region were credited to the influences of warmer weather and the climate (Demma et al., 2005; Mouffok et al., 2009; Vescio et al., 2008). In recent years, with the increase in international travel to increasingly distant locations and with recent measures to protect wild fauna in combination with land rehabilitation and management practices, particularly forestry, the potential exposure to arthropods and the consequent risk of rickettsial transmission have increased. Among travelers returning from Sub-Saharan Africa, rickettsial infections, primarily tick-borne spotted fever, occurred more frequently than typhoid or dengue (Freedman et al., 2006).

3.2. Arthropods as vectors and reservoirs

To date, ticks, lice, and fleas in the orders *Ixodidea*, *Phtiraptera*, and *Siphonaptera*, respectively, are known to be competent vectors of rickettsial agents (Fig. 2). Mosquitoes were recently suggested as potential vectors for *R. felis* (Socolovschi et al., 2012c). Arthropods can acquire bacteria by vertical transmission or by co-feeding, which occurs when several arthropods feed next to each other on the same host (Socolovschi et al., 2009). When rickettsiae are transmitted efficiently, both transstadially (from larva to nymph and/or from nymph to adult) and transovarially (from females to their offspring via eggs), in an arthropod vector, this arthropod can serve as a bacterial reservoir, and the distribution of rickettsiosis will be identical to that of its arthropod hosts (Parola and Raoult, 2001).

3.2.1. Tick-borne rickettsiae

Ticks are obligate parasites of vertebrates and there are nearly 900 currently recognized species divided among three families: *Ixodidae* (hard ticks), *Argasidae* (soft ticks) and *Nuttalliellidae* (one species in South Africa) (Estrada-Pena et al., 2012; Parola and Raoult, 2001). These are the most important vectors and reservoirs of rickettsiae in nearly every region of the world. The use of multiple hosts and different stages (larva, nymphs, adults), the long life span (sometimes longer than their vertebrate host), and the use of intracellular digestion facilitating the penetration of rickettsia to tick tissues enhance the vectorial capacity of ticks. *Ixodidea* ticks are confirmed vectors and reservoirs of most disease-causing *Rickettsia* including *R. rickettsii*, *Rickettsia africae*, *R. conorii conorii*, *R. honei*, *R. sibirica*, *R. slovaca*, *Rickettsia raoultii*, *R. parkeri*, *R. massiliae*, *R. aeschlimannii*, *R. helvetica* (Fig. 2) (Parola et al., 2013; Socolovschi et al., 2013). Ticks are vectors for *R. australis*, *R. heilongjiangensis*, *R. japonica*, *R. sibirica mongolitimonae*, *R. monacensis*, and *R. philipii*, but their roles as reservoirs have not been elucidated for these species. Tick-borne rickettsiae that are possibly associated with human illnesses include *R. bellii*, *R. canadensis*, *Rickettsia asiatica*, *Rickettsia hoogstraalii*, *Rickettsia montanensis*, *Rickettsia rhipicephali* and *Rickettsia tamurae*. Numerous new *Rickettsia* species have been detected by molecular tools have been isolated in ticks (Parola et al., 2013; Socolovschi et al., 2012a), but their potential for pathogenicity remains unknown. *Rickettsia peacockii* might not be transmitted to vertebrates because this strain is unable to invade tick hemocytes or salivary gland tissues and remains strictly symbiont of *D. andersoni* ticks. This infection might be transmitted vertically (Baldridge et al., 2004).

Some rickettsiae are specifically associated with one kind of ticks while others are found in a large variety of arthropods. *R. conorii conorii* appears to associate mainly with *Rh. sanguineus* in the Mediterranean region and *Haemaphysalis leachi* and *Rhipicephalus simus* in Sub-Saharan Africa (Drancourt et al., 1992). In contrast, *R. rickettsii* is associated with different tick vectors from different genera such *Dermacentor andersoni*, *Dermacentor variabilis*, *Dermacentor nitens*, *Amblyomma cajennense*, *Amblyomma aureolatum*, *Amblyomma americanum*, *Amblyomma imitator* and *Rhipicephalus sanguineus*, and *Haemaphysalis leporispalustris*, all of which might occasionally be involved in the transmission of this pathogen to humans and animals (Parola et al., 2013). The development of molecular tools has allowed for the identification of new associations between arthropod species and some *Rickettsia* species for which other vectors and reservoir ticks were already well described. In addition to *Amblyomma* ticks, especially *Amblyomma*

variegatum and *Amblyomma hebraeum* (Jensenius et al., 2003), which are the tick-vectors and reservoirs of *R. africae*, this species was also detected in *Rhipicephalus* spp. and *Hyalomma* ticks (Parola et al., 2013). *R. felis* was detected in *Haemaphysalis flava*, *Haemaphysalis kitasatoe*, *Ixodidae ovatus*, *Ixodidae granulates*, *Rhipicephalus sanguineus* and *Amblyoma cajennense* (Cardoso et al., 2006; Ishikura et al., 2003; Oliveira et al., 2008; Tsui et al., 2007), (Abarca et al., 2013). *R. prowazekii*, which is usually transmitted by lice, was detected in *Hyalomma* ticks collected in Ethiopia (Reiss-Gutfreund, 1966) and *Amblyomma* ticks collected in Mexico (Medina-Sanchez et al., 2005). The infection of these arthropods seems to be facilitated by the co-feeding on the same host (Mediannikov et al., 2012b; Portillo et al., 2007). The role of these ticks as potential vectors was not yet confirmed. The risk of tick-borne rickettsiae in a given region could depend on the presence of specific tick species, the vector tick abundance, biological stages, the duration of tick attachment, human activities, and geographical and climate conditions.

3.2.2. Louse-borne rickettsiae

Lice are obligate blood-feeding insects that parasitize birds and mammals, and approximately 3200 species of lice have been described. Three species are known to infest humans: the body louse (*Pediculus humanus humanus*) and head lice (*Pediculus humanus capitis*), both from the family *Pediculidae*, and the crab or pubic louse (*Pthirus pubis*), which belongs to the family *Pthiridae*. The body louse is the principal vector of *R. prowazekii* (Bechah et al., 2008). Body lice live in clothes and multiply when conditions such as cold weather or a lack of hygiene are present. To date, human body louse infestations are increased among the poorest populations, such as homeless individuals in industrialized countries. The transmission occurs when infected lice fecal matter contaminates the lice feeding sites or when the conjunctivae or mucous membranes are exposed to the crushed bodies or feces of infected lice (Bechah et al., 2008). *R. prowazekii* infection can occur via aerosols of fecal dust, and this represents the main risk of contracting typhus for physicians who are in contact with infected lice-infested patients (Raoult et al., 2004). In laboratory conditions, body lice are able to acquire, maintain, and transmit *R. typhi* and *R. akari*, which are usually transmitted by fleas and mites, respectively, and the *R. rickettsii* and *R. conorii* agents, both of which are transmitted by ticks (Houhamdi et al., 2003; Houhamdi and Raoult, 2006; Weyer, 1952a, 1952b). *R. typhi* has been isolated from rodent lice (Traub et al., 1978) and human body lice during outbreaks of murine typhus in northern China and India (Kashmir State) (Kalra and Rao, 1951; Liu, 1944). Flying squirrel (*Glaucomys volans*) lice, mostly *Neohaematopinus sciuropteri*, were implicated in the transmission of *R. prowazekii* between squirrels and from squirrels to humans (Bozeman et al., 1975; Duma et al., 1981; Sonenshine et al., 1978). *Rickettsia* spp. were detected with molecular tools in *Linognathus* spp. and *H. eurysternus* lice collected from ruminants in Hungary (Hornok et al., 2010).

3.2.3. Flea-borne rickettsiae

Fleas belong to the *Siphonaptera* order of approximately 2500 described species, and they parasitize mammals and, more rarely, birds. *Xenopsylla cheopis*, the oriental rat flea, is the main vector of *R. typhi*, and transmission is affected by contact with rickettsia-containing flea feces during or after blood feeding via inhalation or conjunctival contamination and via flea bites (Azad, 1990). The primary reservoirs are commensal rats, mainly *Rattus rattus* and *Rattus norvegicus*, but various rodents and other wild and domestic animals, including domestic cats and opossums, have been reported to act as hosts. *R. typhi* has been detected in at least twelve species of fleas of the genera *Ctenocephalides*, *Ctenophthalmus*, *Echidnophaga*, *Leptopsylla*, *Monopsyllus*, *Nosopsylla*, *Pulex*, *Rhadinopsylla*, and *Xenopsylla* (Azad, 1990; Kim et al., 2010). To a lesser extent, *R. typhi* is transmitted transstadially and transovarially in *X. cheopis* fleas (Farhang-Azad et al., 1985).

Fleas are also the vectors of other *Rickettsia* spp. *R. felis* was detected in cat fleas (*Ctenocephalides felis*) (Parola, 2011); its vertical and horizontal transmission is well documented and supports the role of this host as both a vector and a reservoir (Hirunkanokpun et al., 2011). *R. felis* was detected in 14 other flea species, including *Anomiopsyllus nudata*, *Archaeopsylla erinacei*, *Diamanus montanus*, *Ctenocephalides canis*, *Ctenocephalides orientis*, *Ctenophthalmus* sp., *Echidnophaga gallinacean* (sticktight fleas), *Leptopsylla segnis*, *Polygenis atopus*, *Pulex irritans* (human flea), *Spilopsyllus cuniculi*, *Tunga penetrans*, *X. brasiliensis* and *X. cheopis* that have been collected from various host animals (dogs, rodents, monkey, opossums, shrew, foxes, hedgehogs, pigs, and humans). Rickettsial genotypes that are closely related to *R. felis*, including the *Rickettsia* sp. genotype 2125, were detected in *C. canis*, *C. felis*, *E. gallinacean*, *P. irritans*, *Synosternus pallidus* and in *X. cheopis* (Hornok et al., 2010; Jiang et al., 2013; Loftis et al., 2006; Nelder et al., 2009; Parola et al., 2003; Roucher et al., 2012) that were obtained from a large variety of domestic and peridomestic animals from human dwellings and from various regions of the world. *R. africae* was recently detected in *Ceratophyllus garei* fleas that were collected from reed warblers (*Acrocephalus scirpaceus*) and their nests in fishponds in Slovakia (Mediannikov et al., 2010). The infected fleas were likely transported to Central Europe via migratory birds that returned from Africa.

3.2.4. Mite-borne rickettsiae

Mites comprise a large group of small to tiny arthropods distributed in three large orders, *Mesostigmata*, *Trombidiformes*, and *Sarcoptiformes*. *Lyponyssoides sanguineus*, a hematophagous mesostigmatid mite that parasites the house mouse (*Mus musculus*) might act as a vector for *R. akari*, the rickettsial pox agent (Paddock and Eremeeva, 2007). Transmission to humans occurs via the *L. sanguineus* nymph or adult bite. Transovarial and transstadial transmission occurs in *L. sanguineus*, suggesting that this is the primary reservoir host of *R. akari* (Paddock and Eremeeva, 2007). *Liponyssus bacoti* mites can become infected with *R. akari* and transmit transovarially to its progeny, but is not an efficient vector (Philip and Hughes, 1948). Other *Rickettsia* spp. have been detected in mites including two *Rickettsia* genotypes, similar to *R. australis* and to *Candidatus* Rickettsia asemboensis that were found in *L. bacoti* mites collected from six separate locations in Egypt (Reeves et al., 2007). *L. bacoti* infests rats, other rodents, and marsupials and can bite humans who live or work in rodent-infested buildings. *Leptotrombidium deliense* chigger mites that are known to be competent vectors for *Orientia tsutsugamushi*, the causative agent of scrub typhus, can harbor *R. felis* and one *Rickettsia* sp. that is closely related to *R. australis* (Tsui et al., 2007). Humans become infected when they accidently pass through zones where rodent-chigger cycles occur. *R. felis* was detected in *Mesostigmata* mites collected from trapped rodents in Taiwan (Tsui et al., 2007). A *Rickettsia* sequence was detected in *Tetranychus urticae*, plant-feeding mites, but the role of this bacterium is unknown (Hoy and Jeyaprakash, 2005).

3.2.5. Mosquitoes

Mosquitoes are insects that comprise more than 3500 species and subspecies, which are classified within the order *Culicidae* into two main subfamilies, *Anophelinae* and *Culicinae*; the latter subfamily includes the tribes *Culicini* and *Aedini* and several other tribes. Mosquitoes are among the most competent and dangerous vectors of various infectious diseases. Only the females require blood meals to produce their first batches of eggs, and they can transmit various pathogens. *Culex pipiens* mosquitoes were found to harbor

Wolbachia pipientis, a member of the family *Anaplasmataceae* within the order *Rickettsiales* (Hertig and Wolbach, 1924). Transovarially transmitted *Rickettsia* species were later described by electron microscopy in *Aedes polynesiensis*, the Polynesian tiger mosquito (Yen, 1975). In 2012, *R. felis* was detected with molecular tools in *Aedes albopictus*, Asian tiger mosquitoes, that were collected from Libreville, Gabon (Socolovschi et al., 2012c) and in *Anopheles gambiae* form S mosquitoes, the major vector in malaria infection, from Côte d'Ivoire (Socolovschi et al., 2012b). A novel *Rickettsia* species that is closely related to *R. felis* was detected in *An. gambiae* and in *Anopheles melas* (Mediannikov et al., 2013; Ndiath et al., 2011). *R. felis* infections were frequently observed in febrile patients in malaria-endemic regions (Mediannikov et al., 2013). These findings suggest that mosquitoes might act as vectors for *R. felis*. The identification of a common vector for *R. felis* and *Plasmodium* suggest that *R. felis* infection should be suspected in geographical areas where malaria is endemic (Mediannikov et al., 2013).

3.2.6. Other arthropods

Molecular detection techniques have revealed the great diversity of rickettsial hosts. Many *Rickettsia* species have been isolated in arthropods other than the well-known vectors cited above and even in hosts that have no relationships with vertebrates. Examples include *Rickettsia* species that were detected in flies (tsetse flies, *Culicoides sonorensis*, *Limonia chore*), non-hematophagous common booklice (*Liposcelis bostrychophila*), wasps (*Neochrysocharis formosa*), true bugs (*Acyrthosiphon pisum*, *Bemisia tabaci*, *Empoasca papayae*), springtails (*Onychiurus sinensis*), beetles (*Adalia bipunctata*, *Adalia decempunctata*, *Brachys tessellatus*, *Coccotrypes dactyliperda*, *Kytorhinus sharpianus*) leeches (*Hemiclepsis marginata*, *Torix tagoi*, *Torix tukubana*), and amoebae (*Nuclearia pattersoni*) (Mediannikov et al., 2012a; Perlman et al., 2006; Thepparit et al., 2011). The biological roles of some of these *Rickettsia* species were reported to include oocyte maturation, parthenogenesis induction, host feminization and the killing of males (Behar et al., 2010; Perotti et al., 2006; Thepparit et al., 2011).

3.3. Geographical distribution

Molecular assays found that *Rickettsia* species are globally distributed worldwide (Fig. 3). Some *Rickettsiae* are present on two continents, including *R. rickettsii* and *R. parkeri* in North and South America, *R. slovaca* in Europe and North Africa, and *R. conorii* caspia in Europe and Sub-Saharan Africa. Other *Rickettsia* spp. are present in several geographical areas, including *R. raoultii* in Europe, North Africa and Asia, *R. sibirica mongolitimonae* in Asia, Europe, and North and Sub-Saharan Africa, and *R. africae* in North and Sub-Saharan Africa, the West Indies, Western Asia, and Oceania. *R. felis* is globally distributed and *R. massiliae* is distributed in all continents except Australia (Parola et al., 2013). The geographical distribution of *Rickettsiae* is closely linked to that of their vectors. Tick species are highly dependent on their biotopes, and very few ticks, including *Rh. sanguineus*, are distributed worldwide. Fleas, lice and mites are globally distributed. Arthropods have relatively low mobility, and their dispersal is mainly linked to host animal migration during vector-borne pathogen infestation. *R. africae* was likely introduced to the West Indies, Madagascar and the Reunion islands by cattle from Senegal and Tanzania, respectively (Parola and Barre, 2004). Migratory birds' movements have been involved in the transfer of arthropods, especially the transport of infected arthropods from endemic areas to new regions (Socolovschi et al., 2012d). Examples include the transfer of *R. sibirica sibirica* from Asia to Spain (Palomar et al., 2012), *R. africae* from Africa to Slovakia (Sekeyova et al., 2012), *R. japonica* from East Asia to Northwestern Russia (Movila et al., 2011), and the dissemination of *R. aeschlimannii* from Africa across different European countries (Demoncheaux et al., 2012; Fernandez-Soto et al., 2006; Matsumoto et al., 2004; Psaroulaki et al., 2006; Rumer et al.,

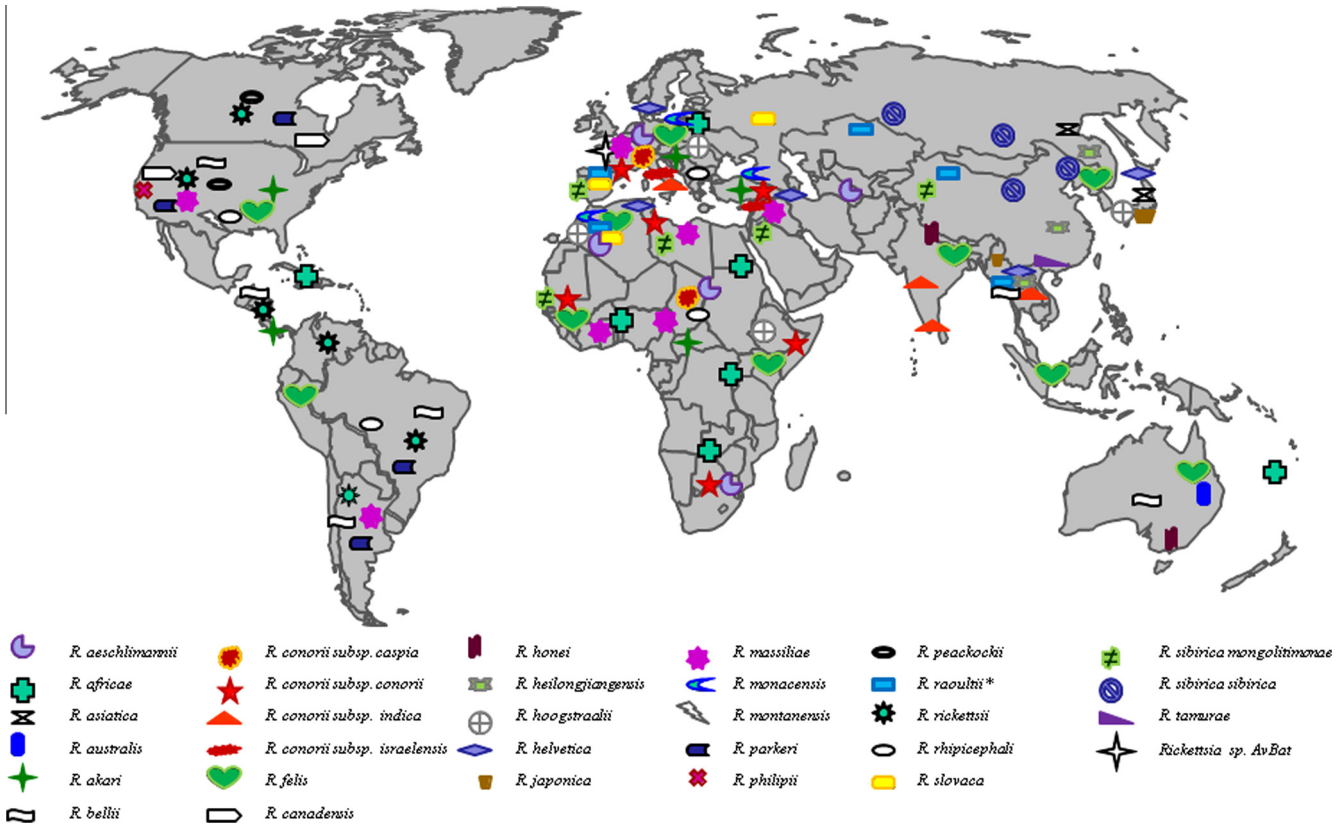

Fig. 3. Geographical distribution by continent of *Rickettsia* spp. isolated from arthropods.

2011). Interactions between rickettsial species in the same arthropod species might affect the ecology, distribution, and incidence of rickettsioses, as exemplified by the relationships between *R. peacockii* and *R. rickettsii* in Rocky Mountain wood ticks (*Dermacentor andersoni*) and between *R. amblyommii* and *R. rickettsii* in lone star ticks (*Amblyomma americanum*) (Niebylski et al., 1997; Paddock, 2009). Nonpathogenic or mildly pathogenic rickettsiae could exert negative effects on virulent species by competing for limited microhabitats within the tick host in a process termed rickettsial interference (Macaluso et al., 2002).

Epidemic and murine typhus are re-emerging diseases. It has been estimated that 30 million cases of epidemic typhus occurred in the Soviet Union and Eastern Europe between 1918 and 1922, causing 3 million deaths (Badiaga and Brouqui, 2012; Bechah et al., 2008; Raoult, 1998). This disease is associated with a high prevalence of body louse infestation and has recently re-emerged in situations of poverty and poor hygiene, including in jails and among homeless people. The majority of cases of epidemic typhus are reported in the rural highlands of Africa and in Central and South America and in refugee camps in central and eastern Africa (Angelakis et al., 2011; Badiaga and Brouqui, 2012; Bechah et al., 2008; Mumcuoglu et al., 1993; Raoult et al., 1998). Murine typhus is most prevalent in warm countries, and sporadic cases are reported in northern countries in travelers who visited endemic areas (Angelakis et al., 2010a, 2012a). Human infection is associated with the presence of rats and their fleas within indoor urban environments. The widespread distribution of murine typhus in many coastal areas is attributed to the introduction of infected rats and their fleas from ships. Worldwide, murine typhus has been documented in diverse geographic areas, including the Mediterranean, Africa, Southeast Asia, and the United States. Thousands of cases of murine typhus occur annually in the United States, and outbreaks have been reported in many countries (Azad, 1990). Murine typhus was diagnosed in 1.6% and 38% of patients who were hospitalized with fevers of unknown origin, in Sri Lanka (Angelakis et al., 2012a) and Indonesia (Azad, 1990), respectively. Murine typhus, which had not been reported in Japan since the 1950s, recently reemerged in that country (Sakaguchi et al., 2004).

4. Evolution

4.1. Genome reduction and increased pathogenicity

The genome sequences of 45 strains of rickettsiae are available, and these sequences exhibit wide variations in size and gene content (Georgiades et al., 2011) (Table 1). Like other intracellular bacteria, *Rickettsia* species display consistently small genome sizes as a result of genome reduction through gene loss (Merhej et al., 2009b; Moran, 2002; Toft and Andersson, 2010). The genome size ranges from 1.11 to 2.1 Mb for *R. typhi* and *R. prowazekii* and *Rickettsia* endosymbiont of *Ixodes scapularis*, respectively, with a mean gene count of 1236.54 ± 281.22 and a mean GC content of 31.5 ± 1.52 (Table 1). These variations in genome size could result from differential rates of gene loss and gene acquisition. The genome size and gene count were reduced during specialization for an intracellular lifestyle, limiting the capacity for horizontal DNA acquisition because few microorganisms typically live together in the same cell (defined as an allopatric lifestyle) (Georgiades et al., 2011; Merhej et al., 2009b). The early stages of adaptation to intracellular life might have occurred through horizontal transfer in protists that contained several organisms (Ogata et al., 2006b). Specialization to a restricted set of hosts was characterized by different steps of genome degradation, from reversible split genes to the generation of 'pseudogenes' and gene remnants (Bechah et al., 2010; Ogata et al., 2001; Sentausa et al., 2012a,e; Toft and Andersson, 2010). The group comprising *R. conorii*, *R. massiliae*, *R. rickettsii* and *R. africae* lost more than 250 genes after its association with ticks and its separation from *R. felis* and *R. akari* (Georgiades et al., 2011).

Genome reduction is associated with increased pathogenicity (Fournier et al., 2009; Merhej et al., 2009b; Ogata et al., 2001) and with an allopatric intracellular lifestyle (Andersson and Kurland, 1998; Bechah et al., 2010; Blanc et al., 2007b; Darby et al., 2007; Tamas et al., 2008). Comparisons of pathogenic genomes to those of related less pathogenic or nonpathogenic strains or species have been conducted in an attempt to identify the molecular genetic basis of TG virulence traits. A comparison of *R. conorii* and *R. prowazekii* revealed that the *R. conorii* genome (1374 open reading frames (ORFs)) contains 552 ORFs that lack clear orthologues in the *R. prowazekii* genome (834 ORFs) (Ogata et al., 2001). Two hundred and twenty-nine (41%) of the *R. conorii*-specific ORFs exhibit significant sequence similarities to intergenic regions of the *R. prowazekii* genome, suggesting that the difference between the two genomes is mainly due to accelerated genome reduction in *R. prowazekii*. The presence of pathogenicity islands, which are clusters of virulence trait-encoding genes acquired through lateral transfer (Hacker and Kaper, 2000), is a common feature in many pathogens, but there was no evidence for pathogenicity islands in TG genomes. Genes for virulence, including the *rickA* and *sca2* genes, that are involved in actin-based motility, adherence and host-cell invasion (Baldridge et al., 2010; Cardwell and Martinez, 2009; Gouin et al., 2004; Haglund et al., 2010; Kleba et al., 2010) were found to be intact in most low pathogenic SFG rickettsiae but were absent or split in *R. prowazekii* (Balraj et al., 2009; Ogata et al., 2001). The TG genomes seem to have undergone a massive gene loss that was not balanced by the acquisition of new genes, likely as a result of the bacterial isolation that limits contact with sources of foreign genetic material. Comparative genomics of *Rickettsia* spp. indicated the loss of genes encoding transcriptional regulators, rather than the acquisition of genes for virulence, as a possible factor involved in the emergence of pathogenicity (Fournier et al., 2009). The higher pathogenicity of *R. prowazekii*, compared with that of *R. typhi*, is unexplained, because these two species are very closely related. The only specificity of *R. prowazekii* was a lack of motility-associated genes that have been identified in SFG and *R. typhi* and have been paradoxically considered as virulence factors in *Shigella* spp. and *Listeria* spp. (Frischknecht and Way, 2001; Pollard and Borisy, 2003).

4.2. Sympatric lifestyle and lateral transfer

In a parallel to gene loss, horizontal DNA acquisition seems to have largely contributed to genome expansion in certain *Rickettsia* spp. in accordance with a sympatric way of life. Traces of lateral sequence acquisitions were evident in *R. felis* (Merhej et al., 2011), *R. akari* (Georgiades et al., 2011), *R. massiliae* (Blanc et al., 2007a) and *R. bellii* (Ogata et al., 2006b). Lateral transfers of leucine rich repeats were demonstrated from eukaryotes to *R. felis* and *R. bellii* (Georgiades et al., 2011). *R. felis* seems to have undergone fewer gene losses than other SFG lineages (Blanc et al., 2007b; Georgiades et al., 2011). *R. felis* exhibits many insertion sequences and phage-related genes (Ogata et al., 2005a) and sequences that were laterally acquired through transduction and recombination (Merhej et al., 2011). Pilus-like structures that permit conjugation have been observed in *R. bellii*, *R. felis* and *R. massiliae* (Blanc et al., 2007a; Ogata et al., 2005b; Ogata et al., 2006a; Weinert et al., 2009). These genomes have been found to encode a complete set of the type IV secretion system (T4SS), while other rickettsial genomes contain a more or less complete set (Gillespie et al., 2010) (Ogata et al., 2006b). The T4SS is a family of multiprotein

Table 1
Characteristics of the sequenced *Rickettsia* genomes.

Organism	Chromosome	Plasmid	Size (Mb)	GC%	Genes	Protein	References
Spotted fever group							
Rickettsia conorii str. Malish 7	NC_003103		1.27	32.4	1414	1374	Ogata et al. (2000, 2001)
Rickettsia conorii subsp. caspia A-167	PRJNA156941		1.26	32.5			Sentausa et al. (2012b)
Rickettsia conorii subsp. indica ITTR	PRJNA89511		1.25	32.4			Sentausa et al. (2012c)
Rickettsia conorii subsp. israelensis ISTT CDC1	PRJNA156943		1.25	32.5			Sentausa et al. (2012d)
Rickettsia rickettsii str. Iowa	NC_010263		1.27	32.4	1493	1384	
Rickettsia rickettsii str. Hlp2	NC_016915		1.27	32.5	1345	1308	
Rickettsia rickettsii str. 'Sheila Smith'	NC_009882		1.26	32.5	1379	1343	
Rickettsia rickettsii str. Arizona	NC_016909		1.27	32.4	1380	1343	Cunha (2008) and Ellison et al. (2008)
Rickettsia rickettsii str. Brazil	NC_016913		1.26	32.5	1369	1332	
Rickettsia rickettsii str. Colombia	NC_016908		1.27	32.5	1387	1350	
Rickettsia rickettsii str. Hauke	NC_016911		1.27	32.5	1377	1340	
Rickettsia rickettsii str. Hino	NC_016914		1.27	32.5	1372	1335	
Rickettsia africae ESF-5	NC_012633	NC_012634	1.29	32.4	1167	1041	Fournier et al. (2009)
Rickettsia parkeri Portsmouth	NC_017044		1.3	32.4	1355	1318	Paddock et al. (2008)
Rickettsia slovaca str. D-CWPP	NC_017065		1.28	32.5	1386	1347	Fournier et al. (2012)
Rickettsia slovaca 13-B	NC_016639		1.28	32.5	1323	1112	
Rickettsia heilongjiangensis 054	NC_015866		1.28	32.3	1338	1297	Duan et al. (2011)
Rickettsia japonica YH	NC_016050		1.28	32.4	1010	971	Dong et al. (2012b)
Rickettsia peacockii str. Rustic	NC_012730	NC_012732	1.31	32.6	984	947	Felsheim et al. (2009)
Rickettsia massiliae MTU5	NC_009900	NC_009897	1.38	32.5	1436	980	Blanc et al. (2007a)
Rickettsia massiliae str. AZT80	NC_016931	NC_016939	1.28	32.6	1243	1207	
Rickettsia rhipicephali str. 3-7-female6-CWPP	NC_017042	NC_017055	1.31	32.4	1302	1266	
Rickettsia montanensis str. OSU 85-930	NC_017043		1.28	32.6	1254	1217	
Rickettsia helvetica C9P9 dong	NZ_CM001467	NZ_CM001468	1.42	–	1428	1393	Dong et al. (2012a)
Rickettsia akari Hartford	NC_009881		1.23	32.3	1293	1258	
Rickettsia felis URRWXCal2	NC_007109	NC_007110 NC_007111	1.59	32.6	1551	1512	Behar et al. (2010); Ogata et al. (2005a)
Rickettsia australis str. Cutlack	NC_017058	NC_017041	1.32	32.3	1297	1261	Dong et al. (2012a)
Candidatus Rickettsia amblyommii str. GAT-30V	NC_017028	NC_017020 NC_017021 NC_017029	1.48	32.4	1427	1390	Baldridge et al. (2010)
Rickettsia philipii 364D	NC_016930		1.29	32.5	1380	1344	
Rickettsia endosymbiont of *Ixodes scapularis*	CM000770	CM000771, CM000772, CM000773	2.1	33.3	2283	2117	Gillespie et al. (2012)
Typhus group							
Rickettsia typhi str. B9991CWPP	NC_017062		1.11	28.9	875	839	McLeod et al. (2004)
Rickettsia typhi str. TH1527	NC_017066		1.11	28.9	874	838	
Rickettsia typhi str. Wilmington	NC_006142		1.11	28.9	918	837	
Rickettsia prowazekii str. RpGvF24	NC_017057		1.11	29	870	834	Andersson et al. (1998); Bechah et al. (2010)
Rickettsia prowazekii str. BuV67-CWPP	NC_017056		1.11	29	879	843	
Rickettsia prowazekii str. Chernikova	NC_017049		1.11	29	881	845	
Rickettsia prowazekii str. Dachau	NC_017051		1.11	29	875	839	
Rickettsia prowazekii str. GvV257	NC_017048		1.11	29	865	829	
Rickettsia prowazekii str. Katsinyian	NC_017050		1.11	29	880	844	
Rickettsia prowazekii str. Madrid E	NC_000963		1.11	29	888	835	
Rickettsia prowazekii str. Rp22	NC_017560		1.11	29	999	950	
R. canadensis group							
Rickettsia canadensis str. McKiel	NC_009879		1.16	31.1	1126	1090	Eremeeva et al. (2005)
Rickettsia canadensis str. CA410	NC_016929		1.15	31	1052	1016	
R. bellii group							
Rickettsia bellii OSU 85-389	NC_009883		1.53	31.6	1511	1475	Ogata et al. (2006b)
Rickettsia bellii RML369-C	NC_007940		1.52	31.6	1469	1429	

complexes that has been involved in pathogenesis, especially in the transport and secretion of virulence factor secretion and the control of the intracellular survival of many bacterial species within a host cell (Christie, 2001; Saisongkorh et al., 2010). The T4SS seems to function in conjugative DNA transfer. These transfers were likely facilitated by co-infections of the same vector by other organisms (Merhej et al., 2011). The genomes of *R. bellii*, *R. felis* and the *Rickettsia* sp. endosymbiont of *Ixodes scapularis* exhibit the highest proportions of transposases among *Rickettsia* (Category L in Table 2). The presence of a large number of transposases that promote the translocation of genomic sequences within and between species might explain the extensive genome shuffling that accompanied the divergence of these species (Ogata et al., 2005a, 2006b). Horizontal DNA transfer and genome shuffling seem to be related to a variety of host-rickettsia relationships, including the symbiosis development and the emergence of pathogenicity in certain SFG species.

Plasmids have been identified in *R. africae*, *R. peacockii*, *R. massiliae*, *R. rhipicephali*, *R. felis*, *R. australis* and *Candidatus* Rickettsia amblyommii (Baldridge et al., 2010; Fournier et al., 2008; Ogata et al., 2005b) (Table 1). Plasmids generally carry genes that are nonessential to bacterial replication but that enhance bacterial diversity and provide the host bacterium with functions such as drug resistance or traits for environmental adaptation that affect pathogenicity (Ochman et al., 2000; Raskin et al., 2006). *R. felis* exhibits two circular plasmids of 63 and 39 Kb, named pRF and

Table 2
Gene count in each COGs functional category for each studied *Rickettsiae* genome. Statistics were computed from the Integrated Microbial Genomes database (Markowitz et al., 2012) http://img.jgi.doe.gov/.

		COG's functional categories	*Candidatus R. amblyommii* GAT 30V	*R. philipii* 364D	*R. rickettsii* Iowa	*R. rickettsii* Hlp 2	*R. rickettsii* Sheila Smith	*R. rickettsii* Arizona	*R. rickettsii* Brazil	*R. rickettsii* Colombia	*R. rickettsii* Hauke	*R. rickettsii* Hino	*R. conorii* Malish 7	*R. africae* ESF 5	*R. parkeri* Portsmouth	*R. sibirica* 246	*R. slovaca* D CWPP	*R. slovaca* 13 B	*R. japonica* YH	*R. heilongjiangensis* 054	*R. peacockii* Rustic	*R. massiliae* MTU5	*R. massiliae* AZT80	*R. rhipicephali* 3 7 female6 CWPP	*R. montanensis* OSU 85 930	*R. akari* Hartford	*R. felis* URRWXCal2	*R. australis* Cutlack	*R. typhi* B9991CWPP	*R. typhi* TH1527	*R. typhi* Wilmington	*R. prowazekii* Madrid E	*R. prowazekii* Rp22	*R. canadensis* McKiel	*R. canadensis* CA410	*R. bellii* OSU 85 389	*R. bellii* RML369 C	*Rickettsia endosymbiont of Ixodes scapularis*
Information storage and processing	J	Translation, ribosomal structure and biogenesis	126	128	125	128	125	128	129	128	128	128	126	126	127	125	129	128	127	127	126	128	127	126	127	126	132	130	126	126	123	124	122	124	126	127	129	122
	A	RNA processing and modification	2	2	0	2	1	1	1	2	1	1	2	1	2	1	2	2	1	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	2	0
	K	Transcription	47	41	30	37	32	37	37	37	37	37	31	31	36	30	37	34	32	36	27	40	39	40	36	27	44	37	26	26	25	22	26	26	29	39	40	37
	L	Replication, recombination and repair	94	71	69	72	70	72	72	72	72	72	71	68	75	69	73	64	64	69	87	109	73	78	74	72	159	92	60	60	59	58	60	65	63	86	106	315
Cellular processes and signaling	D	Cell cycle control, cell division, chromosome partitioning	24	20	18	20	18	19	19	19	19	19	19	19	18	17	20	20	19	21	20	20	22	22	22	18	25	21	15	15	15	15	15	16	16	19	21	19
	V	Defense mechanisms	18	24	22	21	20	23	22	24	21	23	25	14	22	24	24	13	12	24	13	26	15	18	16	14	24	18	9	9	9	10	9	14	15	20	26	16
	T	Signal transduction mechanisms	39	28	23	24	23	23	23	24	23	23	26	21	24	22	26	19	20	23	19	28	26	27	25	21	35	25	18	18	20	16	18	20	21	35	37	38
	M	Cell wall/membrane/envelope biogenesis	82	82	73	78	73	78	78	78	77	78	79	75	81	79	89	77	76	83	72	80	79	78	80	76	84	82	77	77	74	76	74	71	77	80	84	76
	N	Cell motility	4	5	3	3	4	3	3	3	3	3	6	2	2	4	5	4	4	5	4	5	4	5	4	2	6	3	2	2	2	2	2	2	3	7	6	5
	U	Intracellular trafficking, secretion, and vesicular transport	50	45	41	43	42	43	43	43	43	43	45	39	44	43	44	39	41	42	40	49	43	46	45	39	48	42	39	39	39	38	40	39	42	45	47	52
	O	Posttranslational modification, protein turnover, chaperones	58	55	55	56	54	58	58	58	58	58	60	52	54	55	56	54	54	59	53	58	55	53	56	50	61	54	53	52	49	52	51	52	57	52	53	52
Metabolism	C	Energy production and conversion	73	77	74	76	74	77	77	77	77	78	79	74	75	78	75	71	74	76	69	78	73	72	76	77	78	78	75	75	71	74	73	69	72	76	79	74
	G	Carbohydrate transport and metabolism	25	23	16	23	17	23	24	24	23	23	19	17	25	19	27	25	24	25	18	22	24	23	26	17	26	28	24	24	18	18	18	15	20	20	23	19
	E	Amino acid transport and metabolism	33	37	35	35	35	36	37	36	36	36	38	32	36	36	39	31	31	32	30	41	31	30	33	33	40	34	31	31	31	30	30	28	30	34	38	41
	F	Nucleotide transport and metabolism	17	22	19	22	21	22	21	22	22	22	22	18	21	20	22	17	17	17	17	19	19	17	18	18	22	18	17	17	17	17	17	16	16	18	19	17
	H	Coenzyme transport and metabolism	33	36	33	35	33	36	37	36	37	37	35	29	35	35	33	31	29	33	28	41	33	32	32	32	41	33	30	30	30	29	30	29	30	36	36	43
	I	Lipid transport and metabolism	29	32	32	35	28	34	33	34	34	34	39	28	37	40	32	27	26	35	26	40	27	29	31	33	35	32	29	29	30	30	28	29	27	34	36	25
	P	Inorganic ion transport and metabolism	27	30	30	27	29	28	28	28	28	28	30	28	28	30	28	27	25	28	28	30	28	28	28	29	30	29	25	25	26	27	25	25	25	29	32	27
	Q	Secondary metabolites biosynthesis, transport and catabolism	11	10	11	12	9	12	12	12	12	12	12	10	13	11	10	9	9	11	10	12	9	10	13	11	12	11	10	10	10	10	10	11	10	11	11	14
Poorly characterized	R	General function prediction only	87	85	72	81	71	78	80	79	78	78	80	74	82	80	87	78	60	81	59	96	70	79	81	71	110	74	54	54	54	58	56	55	59	102	106	72
	S	Function unknown	80	81	67	79	68	79	76	78	78	78	70	69	78	70	79	71	68	81	63	84	80	85	81	65	101	78	55	55	49	50	51	51	56	90	91	82

pRFδ, respectively. The predicted proteins encoded in the *R. felis* plasmids include ankyrin-repeat-containing proteins, thymidylate kinase, hyaluronidase, small heat-shock proteins, and several homologs for conjugative DNA transfer machinery. An analysis of the *R. felis* genome provided evidence for gene transfers between the chromosome and plasmids (Ogata et al., 2005a). Plasmids might be of significance in rickettsial evolution and epidemiology by conferring genetic plasticity and traits for host adaptation via lateral sequence transfer to counteract the reductive genome evolution typical of obligate intracellular bacteria (Baldridge et al., 2010). The presence of plasmids in *Rickettsia* spp. and the co-infections of single arthropods with different bacteria are consistent with the possibility that the arthropod has been an active arena of plasmid-mediated DNA exchange (Weinert et al., 2009). The absence of plasmids in mild SFG pathogens such as *R. parkeri*, and in the major pathogens of SFG, *R. conorii* and *R. rickettsii*, has left unresolved the question of the true function of plasmids in pathogenicity.

Protists seem to have served as the first places for the development of a sympatric lifestyle that facilitates horizontal DNA transfer. The *R. bellii* group exhibits relatively large genomes and shows a high coding capacity (85%), which is close to that of the *Escherichia coli* genome (87%) (Fig. 4) and consistent with a minor gene loss during evolution with a limited number of pseudogenes (only 100 in the genome) and a large conservation of ancestral genes (up to 89% of the 1252 genes of the "mother" of *Rickettsia* are present in the genome of *R. bellii*) (Blanc et al., 2007b). Amoebal symbionts related to members of *Rickettsiales* have been identified (Fritsche et al., 1999), and *R. bellii* can survive for at least six weeks in *Acanthamoeba polyphaga* (Ogata et al., 2006b), suggesting that the ancestor of *Rickettsiales* might have lived within amoebae. Amoeba seems to have acted as an evolutionary "training ground" for *Rickettsia* (Barker and Brown, 1994; Molmeret et al., 2005), in which *Rickettsia* acquired the ability to infect higher eukaryotic cells (Raoult and Boyer, 2010). Gene exchanges within amoeba might have significantly contributed to *Rickettsia* evolution by conferring a selective advantage during adaptation to the intracellular environment of eukaryotic cells and the development of the symbiotic properties that are particularly observed in the *R. bellii* group (Perlman et al., 2006; Renvoise et al., 2011).

4.3. Core genome and phylogenomic analysis

Rickettsia spp. differ in their gene content because of differential rates of gene gain and loss. Phylogenomic analyses based on gene content showed an unusual clustering that redistributes and mixes species from distinct phylogenetic groups (Fig. 5). Lost gene functions cover a wide spectrum of cellular activities, including information storage and processing, transport systems, energy metabolism, small molecule biosynthesis, and DNA metabolism (Georgiades et al., 2011; Wu et al., 2004). The core genome comprises 566 genes and mainly contains genes involved in translation, ribosomal structure and biogenesis, cell wall/membrane/envelope biogenesis, replication, recombination and repair and energy production and conversion (Fig. 6). Clustering analyses of the functional content place *R. felis* and *R. massiliae* outside the SFG and close to *R. bellii*, which reveals the role played by lateral transfer in the evolution of their genomic content (Fig. 5). *R. canadensis* represents a distinct lineage of rickettsiae (Merhej et al., 2009a), but the phylogenomic analysis places it close to the TG. *R. canadensis* are transmitted by the ticks *Haemaphysalis leporispalustris* (McKiel et al., 1967). It has small repetitive DNA elements that are similar to those of the SFG but it has a tiny genome, functional content and GC% that are similar to that of the typhus group (Tables 1 and 2, Fig. 5) (Eremeeva et al., 2005). These data suggest that *Rickettsia* species that have the same host might converge with regard to their gene content as they adapt to confront similar environmental challenges. The habitat of a bacterial species seems to play a major role in bacterial evolution. The co-habitation within the same host, which is possible for *R. felis* and *R. typhi*, led to genetic exchange and the modification of gene content (Merhej et al., 2009b).

4.4. Toxin-antitoxin systems

Toxin-antitoxin systems (TAS) represent an evolutionary strategy used by *Rickettsia* species to adapt to their environments and manipulate their hosts (Renvoise et al., 2011). TAS are small genetic modules found in bacterial genomes that encode a stable toxin and its neutralizing, short-lived antitoxin, which can be either an antisense RNA or a protein (Van, 2010). These genes,

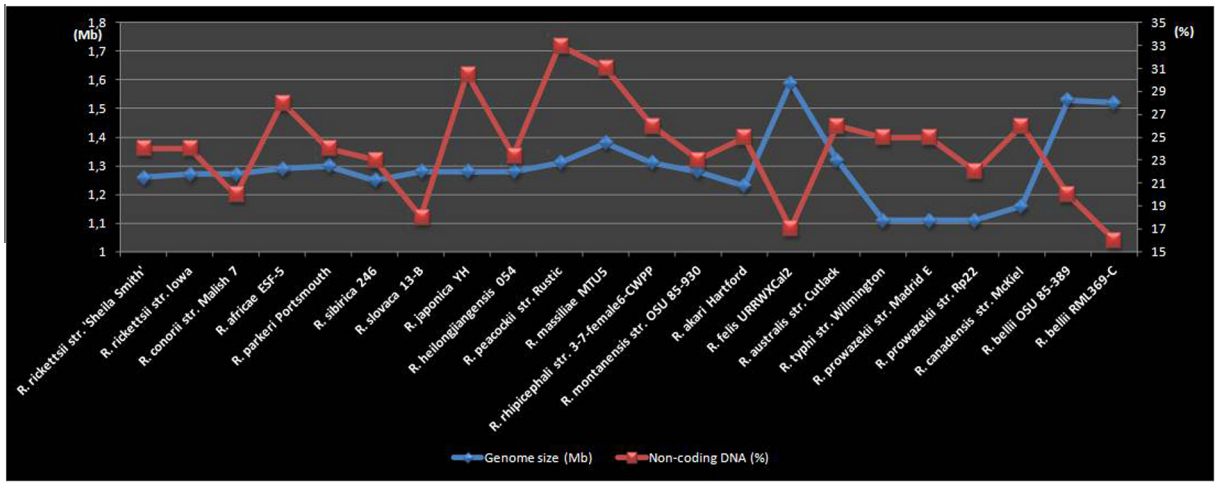

Fig. 4. Protein coding capacity of *Rickettsia* genomes. The genome size in Mb (vertical axis on the left) and the percentage of non-coding DNA regions (vertical axis on the right) are indicated for each species.

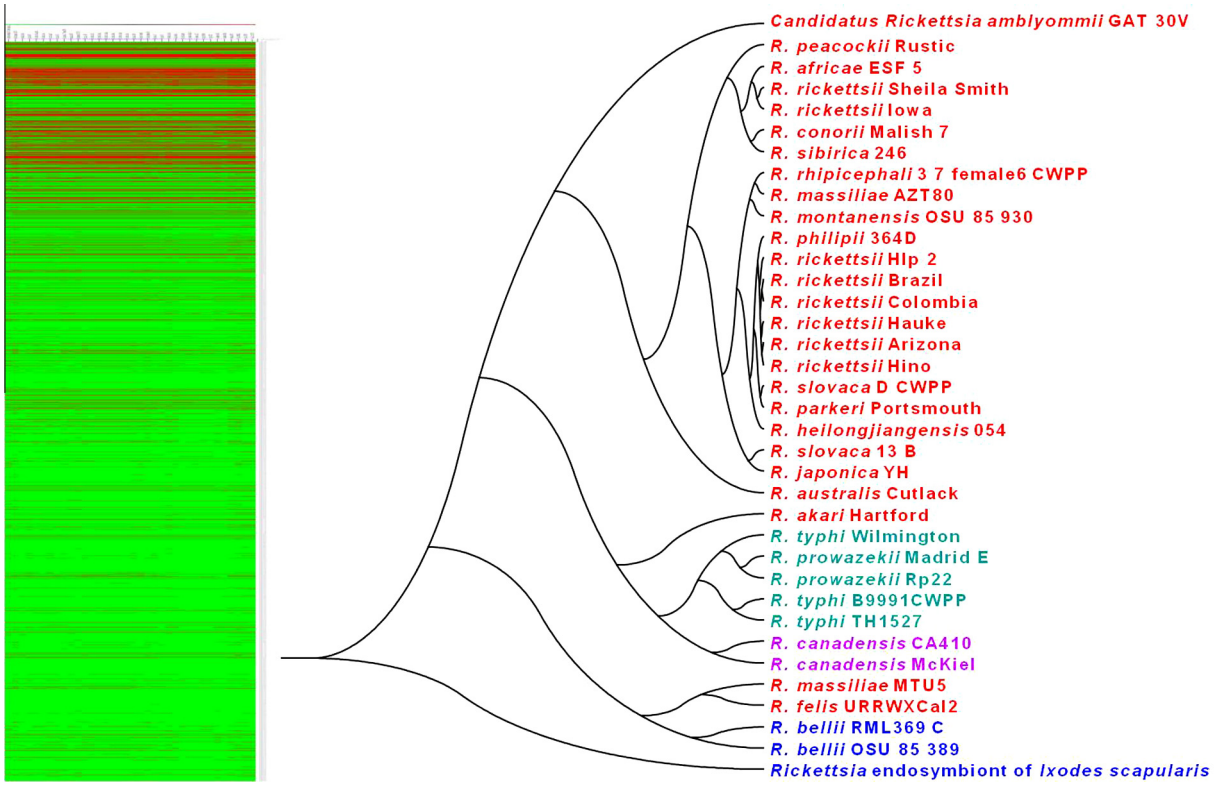

Fig. 5. Cluster analysis of the functional content of rickettsiae genomes. Hierarchical clustering by average linkage was carried with Tmev software (Saeed et al., 2003). The columns correspond to the *Rickettsia* genomes, and the rows correspond to the studied COGs selected genes ($n = 5131$). The variations in the COGs content are depicted with a color scale, in which the shades of red represent the presence and the shades of green represent the absence of the corresponding COG. The dendrogram on the right represents the correlation distances between the rickettsia genomes based on their COGs content. SFG species are represented in red, TG species are in green, the *R. bellii* group is in blue and the *R. canadensis* group in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which were originally identified in bacterial plasmids, ensure stable plasmid inheritance by a mechanism known as "postsegregational killing" (Gerdes et al., 2005). TAS appear to participate in the stringent response pathway cascade (Gerdes, 2000; Gerdes et al., 2005) and play roles in apoptosis, starvation-induced stasis and large genomic fragment stabilization (Van, 2010; Yamaguchi and Inouye, 2011). Recent genome surveys showed that many *Rickettsia* spp. contain TAS in their bacterial chromosomes, but TG rickettsiae are devoid of such modules (Audoly et al., 2011; Ogata et al., 2005a, 2006b; Socolovschi et al., 2013). The TAS count correlates significantly with vertical transmission and the presence of an eschar and inversely with mortality (Socolovschi et al., 2013). The *R. bellii* genome had the highest TAS count. Rickettsial TAS might contribute to global metabolic regulation and eventually lead to the selective killing (a primitive form of bacterial apoptosis) or reversible stasis of bacterial subpopulations during periods of starvation or other stresses (Engelberg-Kulka and Glaser, 1999; Gerdes, 2000). TAS seems to be essential to ensuring a stable

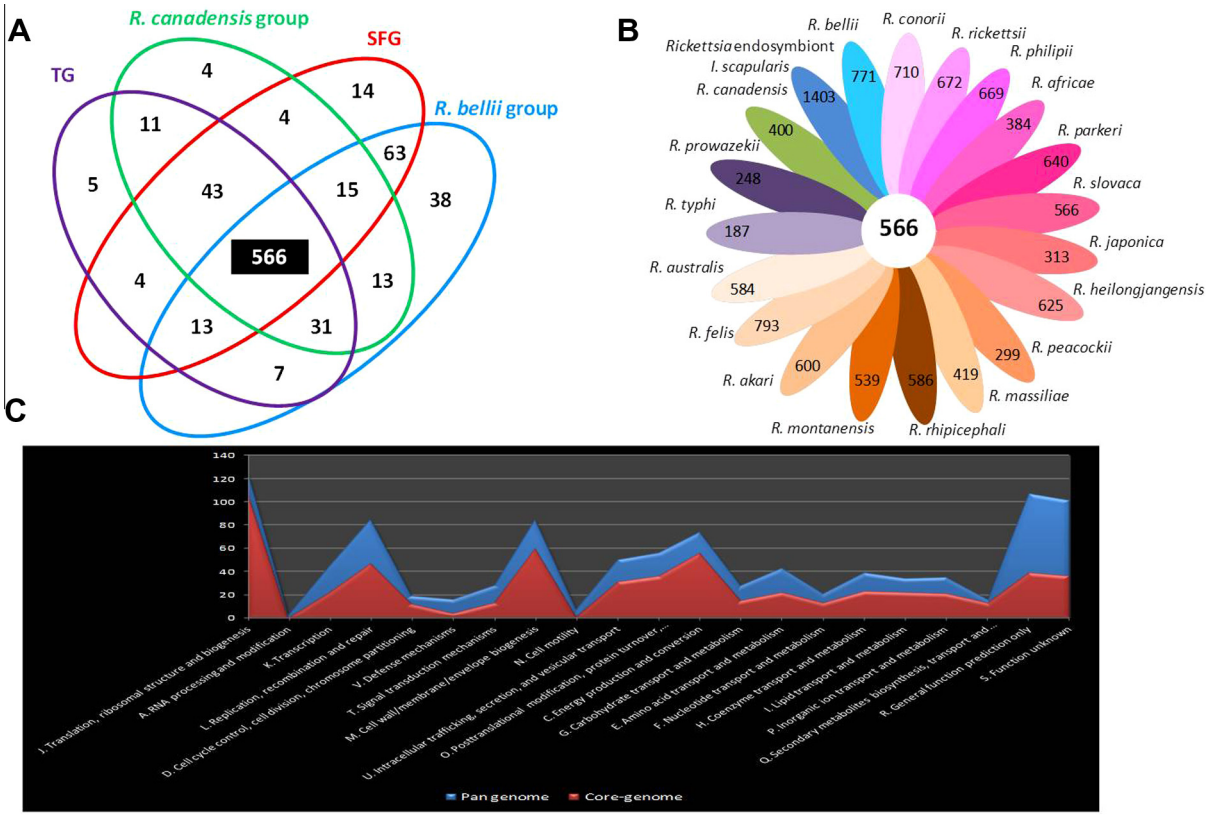

Fig. 6. Comparison of rickettsial gene content. (A) Venn diagram showing the pan-genome of *Rickettsia* spp. The number highlighted in black is the core genome. (B) Core genome of *Rickettsia* spp. The number in the middle circle is the number of core functions. The numbers on each petal corresponds to the number of additional functions present in each species. (C) Functional characterization of the core and pan-genomes of *Rickettsia* spp.

association between *R. bellii* and its hosts for a long period of time (Ogata et al., 2005a, 2006b; Socolovschi et al., 2013). TAS constitutes a promising topic for future research on pathogenicity (Georgiades and Raoult, 2011).

4.5. The adaptive mutation concept

Genome reduction is thought to result from a combination of factors, including genetic drift associated with a host-restricted lifestyle (small population sizes and bottlenecks) (Andersson and Kurland, 1998; Moran, 1996) and mutational bias that favors deletions over insertions (Andersson and Andersson, 1999; Moran et al., 2009; Nilsson et al., 2005). Genome reduction becomes extreme in the TG genomes, and the highest number of gene losses occurred in the branch leading to the TG species, yielding the smallest genomes among *Rickettsia* spp. (Table 1) with a low coding capacity (approximately 75%), large amounts (up to 24%) of noncoding DNA and pseudogenes and a low G + C content (up to 29%) (Andersson and Andersson, 1999) (Fig. 4). These genomic features can be ascribed to the biology of the different vectors and the epidemiology of epidemic rickettsiosis from TG compared with the SFG rickettsioses. While hard ticks typically have a 3-host life cycle (larva, nymph, and adult) and feed two or three times during their life of approximately 2–3 years (Parola and Raoult, 2001), lice feed on blood several times daily, 4–6 times per day, and have a lifespan of approximately 4–12 weeks, which is shortened to two weeks after ingesting infected blood (Azad and Beard, 1998). Add to this, hard ticks commonly ensure the vertical maintenance of SFG rickettsia over generations, possibly because of the low multiplication of bacteria. Lice are intermittent feeders that provide rickettsiae the potential to spread rapidly among populations responsible of the outbreaks of epidemic typhus that can infect hundreds of thousands persons. The number of *Rickettsia* spp. generated over time is likely more important in lice than in ticks, and more mutations could occur over the number of duplications. Population bottlenecks and consequent inefficient selection led to the accumulation of deleterious mutations.

The gene degradation mechanism seems to be enhanced by a shift towards a higher A + T nucleotide composition in combination with the loss of DNA repair systems during reductive evolution (Andersson and Kurland, 1998; Moran, 1996). As in other intracellular bacterial genomes, the reduced genome size of *Rickettsia* spp. correlates with a low GC content (Merhej et al., 2009b). Mutations have been shown to have a consistent bias towards adenosine-thymine across bacterial species (Hershberg and Petrov, 2010; Hildebrand et al., 2010). The mutation rate of guanine-cytosine (GC) to adenosine-thymine (AT) transitions is three-fold higher than that for AT to GC (Rocha and Feil, 2010; Sueoka, 1988). Spontaneous DNA damage, such as the deamination of C3U and 5-meC3T or the oxidation of G to form 7,8-dihydro-8-oxoG (8-oxoG), underlie this mutational bias (Coulondre et al., 1978; Duncan and Miller, 1980; Michaels and Miller, 1992). The lack of some or all of the relevant repair genes, including *ung*, *mutM*, and *mutY*, might be partially responsible for the reduced GC content (Ciccarelli et al., 2006; Itoh et al., 2002; Lind and Andersson, 2008). In the absence of the repair systems that would normally counteract the intrinsic forces of deamination and oxidation, the underlying mutational bias could lead to a reduced GC content.

Homopolymeric DNA tracts, combined with DNA polymerase infidelity and the subsequent deletion of repair systems, seem to play a major role in the development of high pathogenicity. The increased mutation rate that leads to AT-rich genomes represents a crucial point in the stepwise process of gene loss. A high AT content results in an increased density of homopolymers, providing

hotspots for mutations and leading to high rates of indels through replication slippage, frameshifts in coding genes that result in gene inactivation and DNA losses via larger deletions of neutralized DNA (Moran et al., 2009; Tamas et al., 2008). This phenomenon is important when it occurs in repair genes, leading to profound changes in lifestyle evolution (Date et al., 2003). The genome of the avirulent strain *R. prowazekii* Madrid E exhibits mutation hotspots in the homopolymeric tracts of eight genes, such as recO, which lead to the inactivation of the gene. RecO inactivation has been proposed to act as a trigger for the loss of virulence in the MadridE vaccine strain. Passage of the avirulent Madrid E strain in cells or experimental animals (with UV exposure) was associated with recO reactivation, followed by the repair of degraded genes and reversion to a virulent phenotype (Bechah et al., 2010). This example highlights the adaptive mutation concept (Bechah et al., 2010). Adaptive mutations seem to play a major role in the evolution of TG genomes and the emergence of pathogenicity.

5. Conclusions

Rickettsia spp. are more ubiquitous in the environment than initially thought, and they affect their hosts as both harmful and probably as symbiotic beneficial agents. A challenge in microbial research is to understand the evolution of these bacteria and the emergence of symbiotic or pathogenic traits. Assessments of the broad diversity of the genus remain difficult because of the fastidious characteristics of these bacteria. Applications of genotyping methods have successfully led to insights into the epidemiology and molecular evolution of the genus. Access to the genome sequences of many bacteria, including rickettsial genomes, has radically changed microbial research and has led to a transition of the field into the post-genomic era (Merhej et al., 2013). The analysis of rickettsial genomes revealed a reductive evolution due to gene loss as a result of an allopatric lifestyle, and some *Rickettsia* spp. widely exchanged genetic material with sympatric bacteria and eukaryotic hosts. The losses of transcriptional regulators and the adaptive mutations that occur in the hotspots of genes for repair and the TAS, which are involved in apoptosis and starvation-induced stasis, are particularly associated with pathogenic rickettsiae. These findings represent a promising topic for future studies on the emergence of pathogenicity.

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