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Genomics of *Rickettsiaceae*: An Update

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Abstract

Recent advancements in genomes sequencing of members of *Rickettsiaceae* family have led to set a new landmark in the study of these microorganisms. Genomic analyses of *Rickettsia* and *Orientia* reveal a history of genome reduction because of the interaction with intermediate and final hosts; the evidence shows that this is an ongoing process. The gene loss, the gain, and loss of plasmids in such an easy way, among other significant processes are the evidence of the evolutionary history of this bacterial group involving reductive processes. In particular, the integrative conjugative element called REIS, was necessary in the process of adaption to an intracellular lifestyle in eukaryotes. We present a genomic focusing on *Rickettsia* and *Orientia* species, due to the animal and human importance. In this analysis, the genomic evidence shows that genomes have been extensively shuffled; however, the existence of core genes has also been conserved.

Keywords: comparative genomics, *Rickettsia*, pathogens, reductive evolution

1. Introduction

The Rickettsiales are an order within α-proteobacteria that comprises obligate intracellular endosymbionts of arthropods and mammals. Some authors have proposed three pathogenic genera of *Rickettsiales*: (1) *Rickettsiaceae*; (2) *Bartonellaceae*; and (3) *Anaplasmataceae* [1].

More recently, taxonomy of Rickettsiales has changed based on molecular systematics, phylogenomics, and bioinformatics studies. Today, four taxonomic families are recognized: *Anaplasmataceae, Rickettsiaceae, Ca. Midichloriaceae*, and *Holosporaceae*, with *Rickettsiaceae* being the most well-known group for they are human and animal pathogens [1, 2].

Rickettsiaceae family comprises a large and extremely diverse group of strictly intracellular Gram-negative rod-shaped, non-sporulating, coccoid, and small bacteria. Many of them are obligate intracellular parasites that can infect eukaryotic organisms, including animals and man, through arthropod bites and can cause from mild to severe and even fatal diseases such as epidemic typhus and Rocky Mountain Spotted Fever (RMSF) [1, 3]. This family comprises exclusively two genera: *Rickettsia* and *Orientia*. Both genera contain many known and potential pathogens considered as causative agents of emerging and re-emerging human and animals diseases [1]. Genome size of bacteria of *Rickettsiaceae* are typically small (0.8–2.3 Mbp) mainly due to reductive evolution [4].

These genomes contain split genes, gene remnants, and pseudogenes because of different steps of the genome degradation process. In *Rickettsia*, genomics has revealed extreme genome reduction and massive gene loss compared to less virulent or endosymbiotic species [5].

The *Rickettsiaceae* family has 42 species, 2 belonging to the genus *Orientia* that have been sequenced, and 40 species of the genus *Rickettsia* of which 37 genomes has been sequenced.

This wealth of information reveals a large field of study in comparative genomics to understand the evolution from a free-living to an intracellular or endosymbiotic lifestyle.

Adaptation to intracellular or endosymbiont lifestyle of the family *Rickettsiaceae* is based on the genome degradation process as reducing genes. Additionally, to about 2135 [6] gene remnants and pseudogenes (1622), split genes, and horizontal transfer to other bacterial groups have also been observed. In fact, at least three events in *Orientia* received external genes and *Rickettsia* spp. in six occasions [6].

The generation of *de novo* genes in 17 cases of *Rickettsia* species has been reported, and at least two of them are functional [6]. *Rickettsia* spp. contain gene families, selfish DNA, repeat palindromic elements, genes encoding eukaryotic-like motifs, large fraction of high conserved noncoding DNA, and large fraction of mobile genetic elements (MGEs), including plasmids [5].

The study of the dynamics of genomes evolution of the family *Rickettsiaceae* with regard to new "omics" sciences are driven to understand this extraordinary bacterial group with importance for human and veterinary medicine.

2. Rickettsia

2.1. *Rickettsia* evolution

Apparently, the clade *Rickettsia* diverged from *Claudobacter* 1650-2, 390 million years ago [7]. Then, the primarily lineages infecting arthropods emerged approximately 525–425 million years ago [5, 8]. The emerging of this group has been suggested approximately 150 million years ago after several transitions from a likely free-living ancestor of Rickettsiales to an intracellular life. Nowadays, *Rickettsia* have been discovered in a different hosts as whiteflies, bruchid beetles, ladybird beetles, aphids, among others, which suggest that they are more common than expected [9–12].
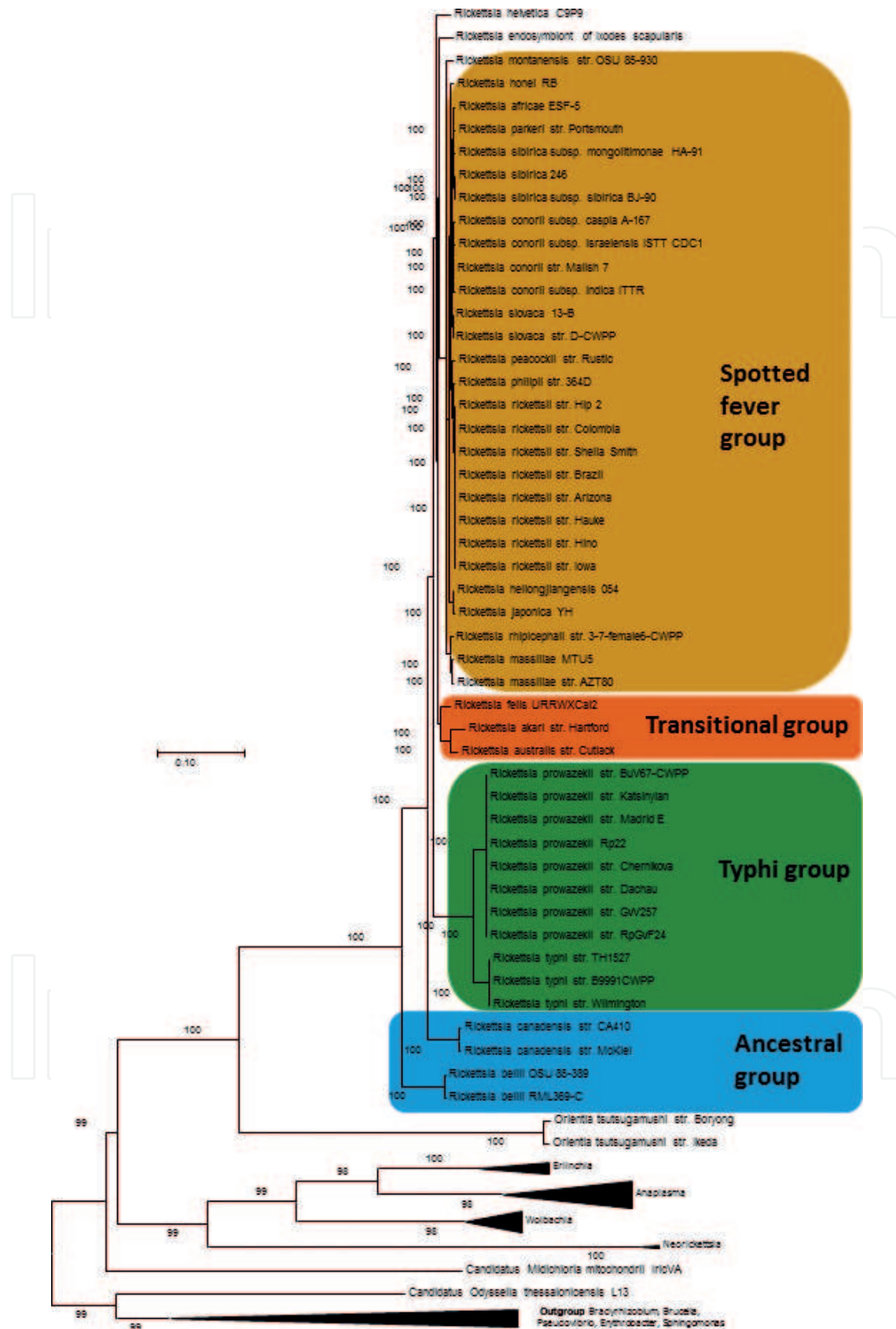
Figure 1. Phylogenetic approximation obtained from amino acid sequences with the online program PATRIC, with the default pipeline (www.patricbrc.org/).

The genus *Rickettsia* comprises pathogenic bacteria causing RMSF, Mediterranean spotted fever, epidemic typhus, and murine typhus [13]. Traditionally, *Rickettsia* was divided into spotted fever and typhus as major groups; however, based on molecular phylogenetic analyses now, it is classified into four groups: (1) ancestral, (2) typhus, (3) transitional, and (4) spotted fever (**Figure 1**). Based on whole genome sequence analysis transitional group was suggested, however, this grouping has generated some controversy based on genetic and genomic criteria and is not widely accepted [5, 14]. The controversy generated by these bacteria classification is because traditional classification methods used in bacteriology are hard to apply to *Rickettsia* spp.

2.2. Genomic of *Rickettsia*

With the use of genome sequence techniques and the characterization of genomic sequences of microorganisms without the need of cultivation, the *Rickettsiaceae* diversity has been explored. Today, 79 genomic sequences of *Rickettsia* and 11 *Orientia* strains are known (**Table 1**).

The availability of complete genome sequences of different *Rickettsia* species led to perform comparative genomic approaches in order to understand bacterial evolution and pathogenesis [5]. Genome reduction is a trait observed in *Rickettsia* species, the gene loss has been an important and ongoing process in evolution of these bacteria. Some intragenus variations in size genome and gene content observed in *Rickettsia* are the consequences of the large diversity of host and infection strategies that these bacteria have developed [5].

The *Rickettsia* genomes exhibit a high degree of synteny punctuated by distinctive chromosome inversions, which goes diminishing as the phylogenetic relationship it is narrower (**Figure 2**).

In general, the genus *Rickettsia* maintains GC content, rRNA, tRNA, and pseudogenes, with only some exceptions (**Table 1**). The aggregate characteristics (number, length, composition, and repeat identity) of tandem repeat sequences of *Rickettsia* which often exhibit recent and rapid divergence between closely related strains and species, are very conserved [15].

2.2.1. Plasmids in Rickettsia

The gene acquisition and gene loss are the major mechanism of adaptation interactions between bacteria and their host, in either the pathogenic or endosymbiotic lifestyle of Rickettsiales and other bacteria. To accomplish this process the preferential vehicle are the plasmids, that encompass very large genetic regions, even more than 100 kilobases (kb) including several set of genes. Their frequent integration at or near tRNA loci suggests that many of them were introduced into bacterial genomes via phage-mediated transfer events. In pathogenicity, they are called "pathogenic islands" and in endosymbionts "symbiotic islands." Recently, the dogma that plasmids are not present in *Rickettsiaceae* was refuted, with the pulsed-field gel electrophoresis (PFGE) and Southern blot analyses of DNAs in different species that suggests that they may be widespread in the genus. Plasmid existence in spite of pressure exerted by reductive genome evolution suggests an important role in rickettsial biology [18].

Taxon_ID	Species	Diseases	Genome _pb	Gene No.	GC	16S_rRNA	23S_rRNA	tRNA	Other RNA	Pseudo_Gene	HGT%
644736402	R_africae	African Tick-Bite Fever	1290917	1077	0.32	1	1	33	0	0	0.56
640753043	R_akari	Rickettsial pox	1231060	1295	0.32	1	1	33	0	0	2.55
2627853922	R_argasii		1437875	1740	0.32	1	1	33	10	0	0.98
2512564059	R_australis		1323280	1297	0.32	1	1	33	0	0	0
2551306119	R_australis		1320572	1556	0.32	1	1	33	5	0	0.58
637000242	R_bellii		1522076	1470	0.32	1	1	34	4	0	0.88
640753044	R_bellii	Epidemic typhus	1528980	1513	0.32	1	1	34	0	0	1.92
2654587808	R_bellii		1543880	1558	0.32	1	1	34	5	0	0.06
2630968994	R_bellii		1618071	1712	0.32	1	1	34	5	0	4.15
640753045	R_canadensis	Epidemic typhus	1159772	1129	0.31	1	1	33	0	0	3.19
2512564031	R_canadensis		1150228	1052	0.31	1	1	33	0	0	0
2548876919	R_conorii	Spotted fever	1249482	1498	0.32	1	1	33	6	0	0.2
637000243	R_conorii	RMSF	1268755	1416	0.32	1	1	33	4	2	0.21
2548877049	R_conorii		1252815	1558	0.32	1	1	33	5	0	0.06
2548877043	R_conorii		1260331	1563	0.33	1	1	33	6	0	0.06
2627853914	R_endosymb._I_pacificus		1564726	1849	0.32	1	1	33	6	0	0.7
647533204	R_endosymb._I_scapularis		2100092	2160	0.32	1	1	33	7	0	4.31
2627853651	R_felis		1579101	1775	0.32	1	1	32	5	0	2.65
637000244	R_felis	FbSF, Rickettsiosis	1587240	1552	0.33	1	1	33	4	0	4.45
2648501148	R_felis		1545289	2085	0.32	0	1	33	5	0	0.19
2654587834	R_felis		1485193	1549	0.32	1	1	33	5	0	0
650716082	R_heilongjiangensis		1278471	1333	0.32	1	1	33	0	0	0.08
2597489904	R_helvetica		1417015	1661	0.32	1	1	33	7	0	1.38
2548877054	R_honei		1268758	1510	0.32	1	1	33	5	0	0.07
2630968761	R_hoogstraalii		2303093	2634	0.32	1	1	36	107	0	1.03
2639762805	R_hoogstraalii		1484812	1628	0.32	1	1	33	5	0	0.43
2558860235	R_japonica	Spotted fever	1276675	1439	0.32	1	1	33	5	0	0
2511231062	R_japonica	Spotted fever	1283087	1009	0.32	1	1	33	2	0	0
2627853941	R_massiliae		1266919	1484	0.33	1	1	33	5	0	0.2
641228503	R_massiliae		1376184	1435	0.33	1	1	33	3	416	0.84
2512564029	R_massiliae		1278719	1243	0.33	1	1	33	0	0	0.08
2512564067	R_montanensis		1279798	1253	0.33	1	1	33	0	0	0.08
2512564062	R_parkeri		1300386	1354	0.32	1	1	33	0	0	0.3
2654587812	R_parkeri		1309691	1516	0.32	1	1	34	7	0	0
2654587805	R_parkeri		1300383	1500	0.32	1	1	33	7	0	0
2654587813	R_parkeri		1300534	1504	0.32	1	1	33	7	0	0
644736403	R_peacockii	RMSF, Typhus	1314898	984	0.33	1	1	34	0	0	0.81
2512564043	R_philipii		1287740	1380	0.32	1	1	33	0	0	0.22
2541047049	R_prowazekii		1109301	956	0.29	1	1	33	0	0	0.21
646862338	R_prowazekii	RMSF, Typhus	1111612	990	0.29	1	1	33	2	0	1.01
2512564053	R_prowazekii		1109804	881	0.29	1	1	33	0	0	0.23
2684623071	R_prowazekii		1111769	919	0.29	1	1	33	5	0	0
2512564077	R_prowazekii		1112101	870	0.29	1	1	33	0	0	0.23
2512564080	R_prowazekii		1111445	879	0.29	1	1	33	0	0	0.23
2512564076	R_prowazekii		1111969	865	0.29	1	1	33	0	0	0.23
2541047975	R_prowazekii		1109257	1006	0.29	0	0	33	0	0	0.2
2512564054	R_prowazekii		1111454	880	0.29	1	1	33	0	0	0.23
2541047974	R_prowazekii		1113970	946	0.29	0	0	33	0	0	0.11
637000245	R_prowazekii	RMSF, Typhus	1111523	875	0.29	1	1	33	4	0	0.11
2512564070	R_prowazekii		1109051	875	0.29	1	1	33	0	0	0.23
2554235386	R_prowazekii		1111520	974	0.29	1	1	33	0	0	0.21
2671180980	R_rhipicephali		1448632	1672	0.32	1	1	33	6	0	0.24
2512564064	R_rhipicephali		1305467	1302	0.32	1	1	33	0	0	0
2627854257	R_rickettsii		1269809	1472	0.32	1	1	35	6	0	0
2512564049	R_rickettsii		1270083	1387	0.32	1	1	34	0	0	0.14
641522647	R_rickettsii	RMSF	1268175	1421	0.32	1	1	34	0	0	0.77
2512564034	R_rickettsii		1255681	1369	0.32	1	1	34	0	0	0.15
2512564047	R_rickettsii		1270751	1345	0.32	1	1	34	0	0	0.07
2512564048	R_rickettsii		1286792	1388	0.33	1	1	37	0	0	0.79
640753046	R_rickettsii	RMSF	1257710	1382	0.32	1	1	34	0	0	0.65
2512564040	R_rickettsii		1269774	1377	0.32	1	1	34	0	0	0.07
2512564030	R_rickettsii		1267197	1380	0.32	1	1	34	0	0	0.14
2627853817	R_rickettsii		1257005	1453	0.32	1	1	34	6	0	0.07
2548876780	R_sibirica	RMSF, Typhus	1254734	1474	0.32	1	1	33	5	0	0.2
2548876922	R_sibirica		1252337	1519	0.32	1	1	33	5	0	0.07
638341178	R_sibirica	North Asian tick typhus	1250021	1270	0.32	1	1	33	0	0	0.08
2512564061	R_slovaca		1275720	1383	0.33	1	1	33	0	0	0.07
2511231061	R_slovaca	Headache, Malaise	1275089	1322	0.33	1	1	33	2	172	0
2582581025	R_sp. ISO7		1657692	1710	0.32	1	1	33	1	31	1.17
2551306549	R_sp. MEAM1		1105028	1395	0.32	3	4	31	5	0	5.88
2518645505	R_sp. PRT001		1317139	1541	0.31	0	3	13	13	0	51.2
2513020046	R_sp. PRT001		934551	1079	0.29	0	0	12	0	0	61.54
2617271228	R_tamurae		1453216	1670	0.32	1	1	33	10	0	0.78
637000246	R_typhi	Typhus	1111496	920	0.29	1	1	33	4	42	2.61
2512564075	R_typhi		1112957	875	0.29	1	1	33	0	0	0.23
2512564057	R_typhi	Typhus	1112372	874	0.29	1	1	33	0	0	0.34
2585427698	R_bacterium Os18		1469252	1485	0.29	1	1	38	3	0	7.21
2599185121	R_ bacterium Ac37b		1917613	1886	0.31	1	1	38	3	0	34.73
2630968804	R_bacterium SCGCAAA041		822563	896	0.33	1	0	13	2	0	21.76
2645727592	Orientia chuto		1092196	1124	0.29	1	1	33	3	0	1.07
2627853971	Orientia tsutsugamushi		1997698	3013	0.3	1	1	28	24	0	0.23
640427127	Orientia tsutsugamushi	Scrub typhus	2127051	2180	0.31	1	1	35	1	959	2.66
2648501828	Orientia tsutsugamushi		2221260	2867	0.3	1	1	34	32	0	0.24
642555145	Orientia tsutsugamushi	Scrub typhus	2008987	2004	0.31	1	1	34	0	0	1.85
2627853710	Orientia tsutsugamushi		1689193	2232	0.31	0	1	28	30	0	1.08
2627853684	Orientia tsutsugamushi		2460104	3094	0.31	1	1	35	39	0	0.26
2639762819	Orientia tsutsugamushi		3033399	4269	0.3	2	1	57	30	0	0.19
2627853773	Orientia tsutsugamushi		712858	942	0.3	1	0	10	7	0	0.11
2645727557	Orientia tsutsugamushi		1478442	1643	0.3	1	1	35	35	0	0.61
2627854151	Orientia tsutsugamushi		1454354	1624	0.3	1	1	34	22	0	0.43
RMSF = Rocky Mountain Spotted Fever		**FbSF = Flea-borne Spotted Fever**									

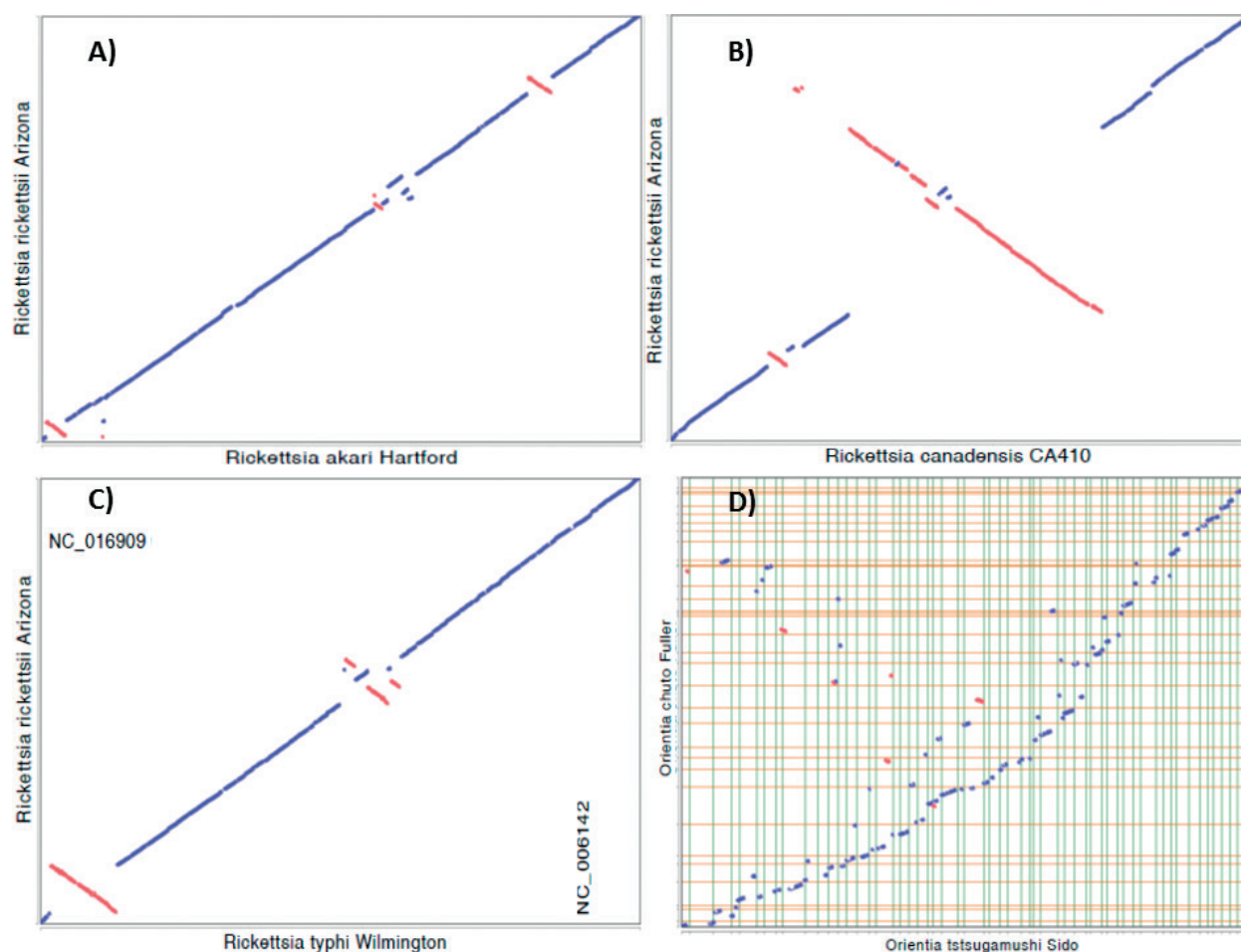**Table 1.** *Rickettsiaceae* family genomes data reported on img.jgi.doe.gov.

Figure 2. Comparison of pairwise syntenic dot plots of the nucleotide sequences: (A) *Rickettsia rickettsii* Arizona vs. *R. akari*; (B) *R. rickettsii* vs. *R. canadensis* CA410; (C) *R. rickettsii* vs. *R. typhi* Wilmington; and (D) *Orientia chuto* Fuller vs. *O. tsutsugamushi* Sido.

In the most relevant study of plasmids in *Rickettsia* with 26 species, the authors found that 11 species had 1 to 4 plasmid(s) with a size ranging from 12 to 83 kb, and contained 15 to 85 genes. They elucidated that pRICO, the last common ancestor of the current rickettsial plasmids, was vertically inherited mainly from *Rickettsia/Orientia* chromosomes and diverged vertically into a single or multiple plasmid(s) in the species [3].

Out of 747 protein-coding genes, 65% were full-length genes and 35% were partially degraded. Degradation levels varied among plasmids, ranging from 16 to 40% in larger plasmids (size >47 kbp) and 44 to 59% in smaller plasmids [3].

It has been observed that plasmids are lost during long-term serial passage in cultured cells, which complicate studies of ancestry to elucidate a single or multiple ancestors. Nevertheless plasmids clustered into four putative groups (I–IV) (**Figure 3**): group I included four large and three small plasmids of five species: pRra2 in *R. raoultii*, pRhe in *R. helvetica*, pRfe, pRfeI1, and pdRfe in *R. felis*, pRam32 in Candidatus *R. amblyomii*, and pRau in *R. rhipicephalii*; group II clustered two large and four small plasmids belonging to five species: pReis1 and pReis2 in *R. endosymbiont* of *Ixodes*, pRaf in *R. africae*, pRam23 in C. *R. amblyomii*, pRmo in *R. monacensis*, and pRpe in *R. peacokii*; group III contain five small plasmids of four species:
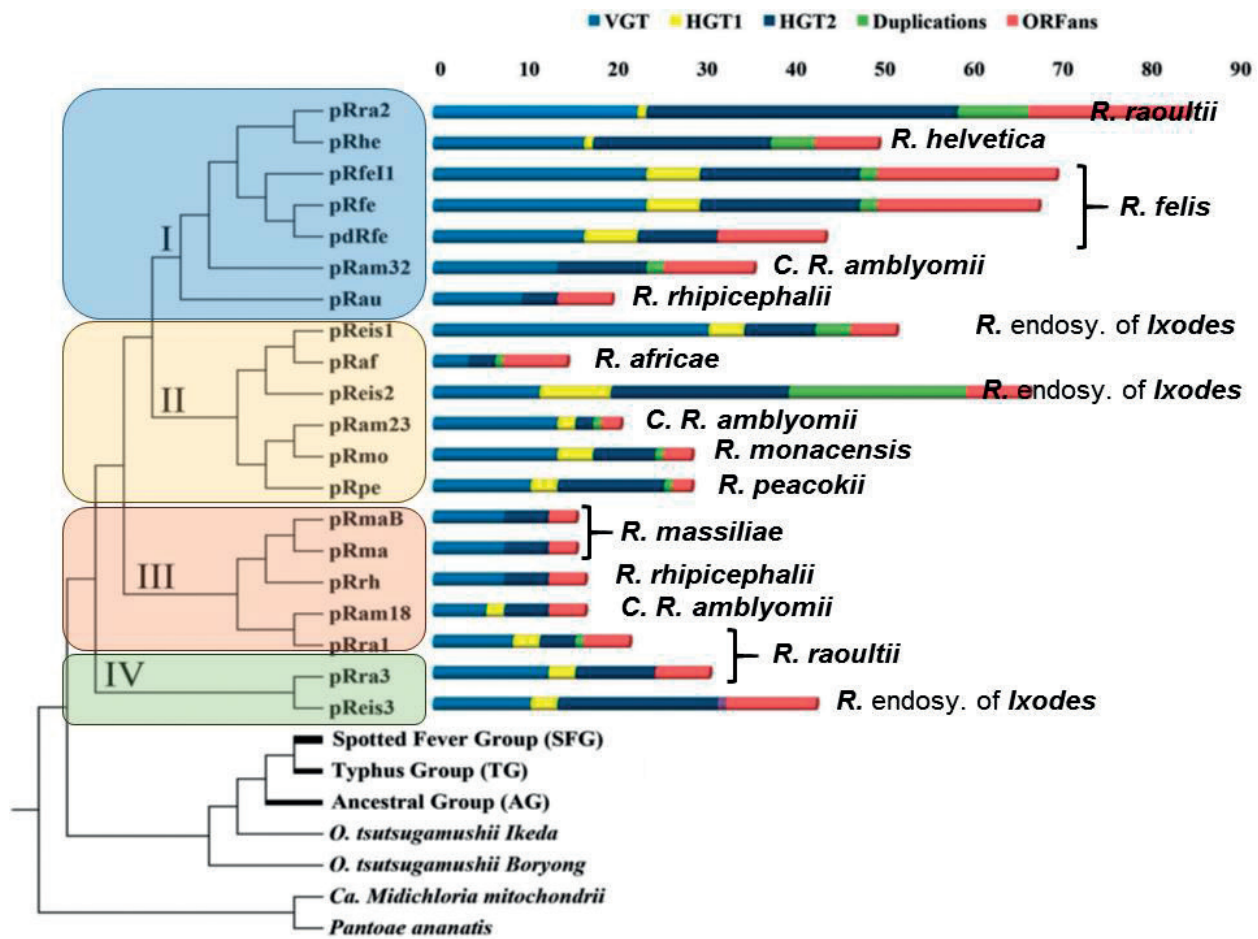
Figure 3. Evolutionary events that shaped rickettsial plasmids. Plasmid supertree obtained from 10 genes of *Rickettsia* and *Orientia* species. The genes used were hsp, dnaA, sca12, transposase 1 and 2, phospholipase, traD, leucine repeat, helix-turn-helix, and thymidylate kinase. Figure based on [3].

pRam18 in C. *R. amblyomii*, pRrh in *R. rhipicephalii*, pRra1 in *R. raoultii* and pRma and pRmaB in *R. massiliae*; and group IV gathered one large and one small plasmids from two species: pReis3 in *R.* endosymbiont of Ixodes and pRra3 in *R. raoultii*. At inter-species level, plasmids of the same group showed variable sequence conservations [6].

Rickettsia plasmids are a mirror of the evolutionary history of this bacterial group involving reductive processes, duplication events, and horizontal acquisition of genes necessary to adapt to an intracellular lifestyle in eukaryotes. It is now necessary to determine their distribution, evolution, and their role in host adaptation and virulence [16].

2.2.2. Gene loss and evolution of Rickettsia

The mechanism of gene loss it has been a fairly widespread strategy in the evolution of the Rickettsiales genomes, and was discovered in the *Rickettsia* endosymbiont *Ixodes scapularis* (REIS). It was found that proliferation of mobile genetic elements, in particular, an integrative conjugative element RAGE (for Rickettsiales Amplified Genetic Element) is present in chromosome and plasmids [6].

REIS encodes nine conserved RAGEs that include F-like type IV secretion systems similar to other in *Rickettsia* genomes. These comprise 35% of the total genome, making REIS one of the most plastic and repetitive bacterial mobile elements. The presence of REIS provides the most convincing evidence that conserved rickettsial genes associated with an intracellular lifestyle were acquired via MGEs, especially the RAGE. This, probably through a continuum of genomic invasions, provides insights about the origin of mechanisms of rickettsial pathogenicity [17].

The RAGEs are the fusion of *tra*-like family genes that encoding the conjugal transfer protein. Inserted genes can be found between *traA* and *traD* genes. We present a phylogeny with 60 sequences of *traD* genes of 16 genomes species (**Figure 4**), including ancestral, transitional, and spotted fever group.

Rickettsia spp. share 1027 genes that probably were vertically transferred from "proto-*Rickettsia*" *R. bellii* maintained all these genes and other species lost a large part of them, like *R. prowazekii* and *R. typhi* (128 lost genes). It is well supported that differential gene loss contributes to creation of new rickettsial species [6].

In conclusion, the loss of regulatory genes causes an increase of virulence in rickettsial species in ticks and mammals, and the *tra* operon is presumably involved [18].

2.2.3. Phylogeny and taxonomy of Rickettsia

The taxonomy of *Rickettsia* was historically based on the phenotypic criteria, the phylogenetic approaches with gene 16S rRNA defined three groups typhus group (TG) which includes: *R. prowazekii* and *R. typhi*; classic spotted fever group (SFG), which includes a large collection of mostly tick-borne rickettsials; and an ancestral group (AG), which included *R. bellii* and *R. canadensis* [19], even thus they remain unresolved clades at species level.

Other evolutionary gen reconstructions are inconsistent when using different portions of the genome [20]. An analysis based on the whole genome sequence analysis (WGSA) allows emerging of transitional group (TRG) consisting of *Rickettsia felis* which was primarily associated with *Ctenocephalides felis* and the sister group to the neighboring: *R. akari* [19, 20].

In different studies of *Rickettsia* using WGSA, we can observe resolved trees with single topology, which is supported by multiple sources of phylogenetic signal, which describes the evolutionary history of the core genome [20].

Unfortunately, we cannot have always the powerful tool of WGSA, so the search of new molecular markers is necessary to provide a well-supported phylogenetic approach, at least at species level. As we can see in **Figure 5**, a reliable phylogeny can be obtained using several sequences of all *Rickettsia* species and the conserved gen *rpoB*, offering high-resolution clades at species level.

2.2.4. Comparative genomics of Rickettsia

In the existing 82 sequenced genomes of *Rickettsia* species, 77 belong to 37 species and 5 in *Candidatus* status. In general, the genomes size is constant, between 0.8 and 2.3 Gb; and the average size is 1.3 Gb.

The genomes shows 865 genes as minimum and a maximum of 2634 genes, the average is 1360 genes; the GC content is very constant among genomes with 33%. Only four genomes have
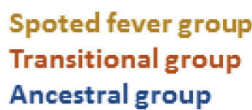
Figure 4. The evolutionary history of gen TraD was inferred by using MEGA7 using ML method and GTR model, with the highest log likelihood (−237.1216). The analysis involved 60 nucleotide sequences from genomes of the IMG (img. jgi.doe.gov) of 16 species and tree outgroup from NCBI.
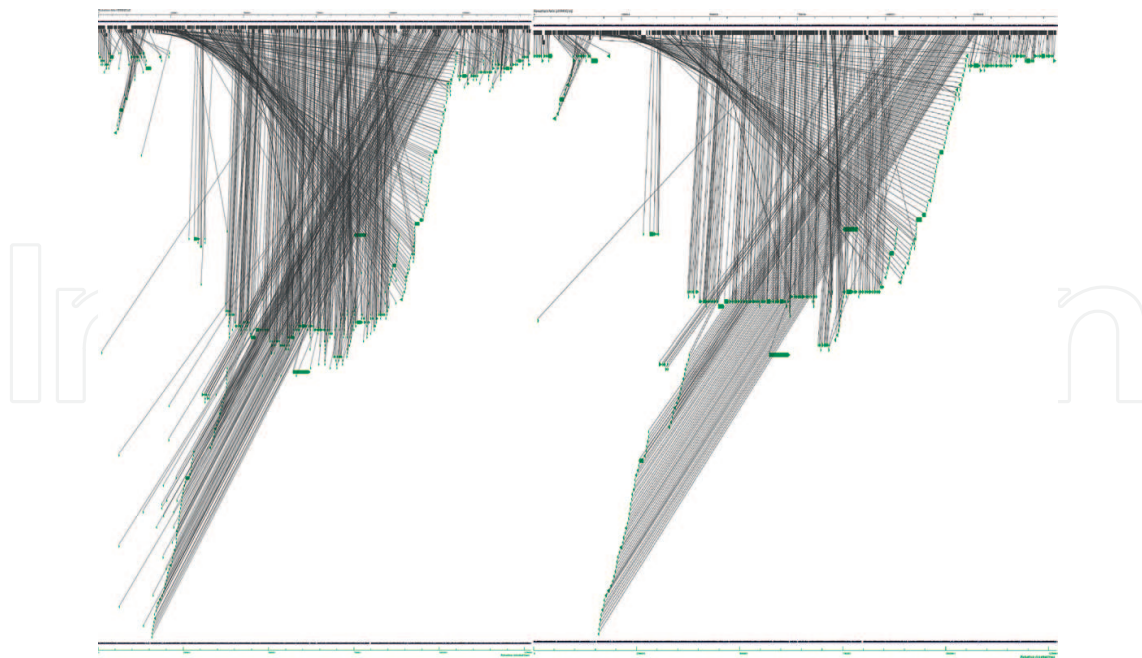
Figure 5. *rpoB* Phylogeny of *Rickettsia* and *Orientia* was inferred by using MEGA7 using ML method and GTR model, with the highest log likelihood (−14425.5668). The analysis involved 87 nucleotide sequences obtained from genomes of the IMG site img.jgi.doe.gov. There were a total of 2721 positions in the final dataset.

pseudogenes and the average of horizontal gene transfer is 2.87% (**Table 1**). The presence of conjugative elements in some of these genomes correlates with an increased number of transposons, breakpoints, and a general breakdown in genome synteny, which is very conserved

Figure 6. Genomic rearrangements with different cutoff: (A) 100 bp; (B) 1000 pb.

in nearby groups, with some inversions. However, as they move away phylogenetically, more inversions are observed and still synteny is conserved (**Figure 2**).

The genomic and metabolic impairment of *Rickettsia* genomes is mainly due to population bottlenecks in free live style and genome size reduction is related to the gene loss, split genes, and pseudogene formation during endosymbiosis. The presence of plasmids and their sporadically integration into the chromosome leading to emergence of pathogenicity and loss of regulation are also factors that influence in *Rickettsia* genomes variability [5].

When comparing synteny between *Rickettsia rickettsii*, member of spotted fever group, and *R. felis*, member of transitional group, (**Figure 6**) a panoramic view of the genome dynamics at large scale can be observed. A point cut of 100 pb events shows a significant difference respect to 1000 pb. This difference is also observed in the gen itself and the alignment with the Vista tool in the IMG site (img.jgi.doe.gov/cgi-bin/w/main.cgi?section=Vista&page=vista).

The comparative genomic studies reveal the relation between small size and more virulent species strains, this fact supports *Rickettsia* virulence and is the result of a reduction genome ongoing process. The reconstructions of inactive genes revealed that deletions strongly predominate over insertions with an excess of GC-to-AT substitutions, which explain the low GC content (32% in genome average) [21].

3. *Orientia*

This genus comprises *Orientia tsutsugamushi*, the causative agent of scrub typhus or Tsutsugamushi disease, and the novel species *Orientia chuto* identified in Dubai, in the United

Arab Emirates [22, 23]. Humans are the final host of the bacteria and the symptoms include a simple febrile illness to a life threatening fatal infection (meningitis, eschar, disseminated intravascular coagulation) and complicated with dysfunction in several organs [24].

O. tsutsugamushi is widely spread in the Asia-Pacific region comprising Siberia, Japan, Korea, Papua New Guinea, Thailand, Philippines, the Kamchatka Peninsula in the east, Pakistan in the west, and down to Australia in the south [22, 25].

These bacteria are an obligate intracellular Gram-negative rod-shaped and its vector is *Leptotrombidium* spp. species mite populations, where vertically is maintained. Transmission to humans occurs by the bite of infected larval-stages mites called chiggers [26]. Although, some other vectors have been reported, including ticks of rodents from different geographic origins [27, 28]. Vertical or transovarial transmission of *Orientia* spp. would be essential to the maintenance of the infection due to mites have a role as vectors and reservoir [25].

In the recent years, a dramatic variation in phenotypes and genotypes of *O. tsutsugamushi* has been observed in humans, animal host, and vector mites using immunological and molecular methods [25].

3.1. Genotyping of *Orientia*

Strain classification of *Orientia* and serotyping were performed based on the immunodominant 56 kDa type-specific antigen (TSA) located on the surface of the bacteria [29].

With this method, three antigenic prototypes were primarily described: Karp, Kato, and Gilliam; and then many more variations of different serotypes were described in several countries [30, 31]. As primary attempts, genotyping was made by sing RFLP (Restriction Fragment Length Polymorphism) to identify unique isolates or directly by sequence analysis of the TSA gene by PCR. A comparison between nested polymerase chain reaction (nPCR) of 56-kDa antigen gene, the most used molecular technique for confirmation of scrub typhus and genotyping of *O. tsutsugamushi,* and single-step conventional PCR (cPCR) revealed that nPCR products have more variation among strains than cPCR, which emphasizes cPCR advantages [32, 33].

The antigenic variation of the strains Karp, Kato, and Gilliam, subsequent strains, and recently isolates discovered depends on the diversity of the TSA located on the surface of *O. tsutsugamushi [34].*

Genome of *O. tsutsugamushi* strain Boryong has 2,217,051 bp, with 2179 potential protein-coding sequences and 963 sequences of fragmented genes, which represent a coding capacity of only 49.6%. A core genome is composed of 512 genes that share with seven *Rickettsia* species (**Figure 7**). The fragmented regions have a significant interest since they correspond to repeated DNA regions distributed throughout the whole genome. The absent of collinearity with other *Rickettsia* genomes and the no systematic pattern in the GC plot suggest that the genome has been extensively shuffled [35].

3.2. Comparative genomics

Nakayama et al. [36] compared two genomes of *O. tsutsugamushi*: Boryong and Ikeda strains. Both genomes recently reported and isolated in Korea and Japan [35, 37]. In this
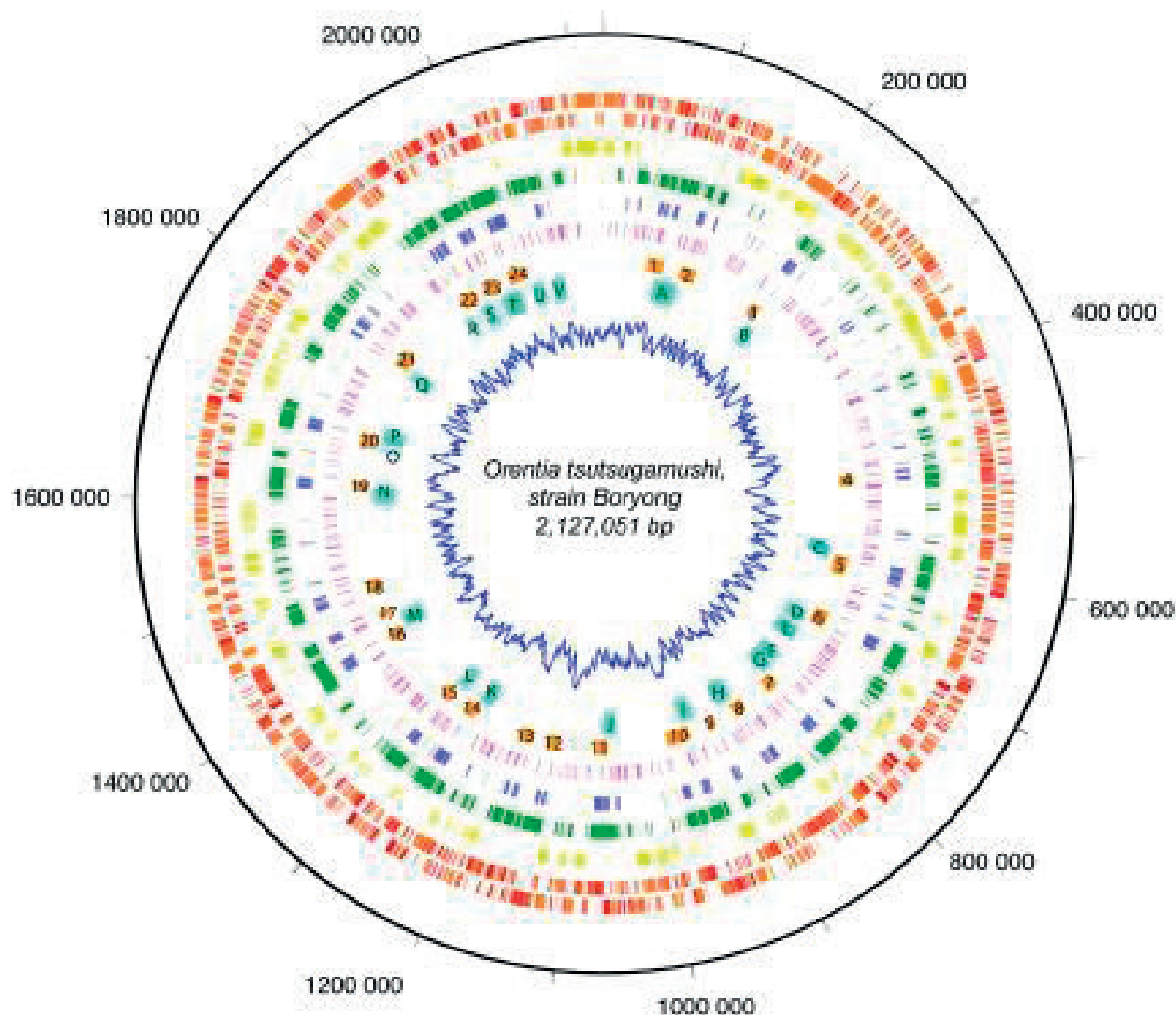
Figure 7. The mapping of different regions in the circular genome of *O. tsutsugamushi*. Figure reproduced from [35].

comparative analysis, a phylogenetic relationship of *O. tsutsugamushi strains* was reconstructed using 11 conserved genes in *O. tsutsugamushi* and closely related *Rickettsia* species. The multilocus sequencing analysis of 10 *O. tsutsugamushi* strains representing each TSA subtype revealed the distribution of strain-specific sequences identified in Boryong or Ikeda among the *O. tsutsugamushi* strains.

The analysis revealed an extensive reductive genome evolution and a significant amplification of repetitive sequences. In fact, the repetitive sequences identified in Ikeda strain were classified in three types: (1) Integrative and conjugative element (ICE) named OT amplified genetic element (OtAGE); (2) Transposable elements (TE); and (3) Short repetitive sequences of unknown origin (short repeat). Both genomes of *Orientia* contain the same set of repetitive sequences, which have been amplified in both strains and caused an extensive genome shuffling. Additional to this, the existence of core genes set of family Rickettsiaceae is also highly conserved. It seems that the extensive genome rearrangements generated by repetitive sequences have occurred between the two strains, although the high complex and repeat-rich feature of the *Orientia* genomes and some genomics differences still have to be clarified [36].
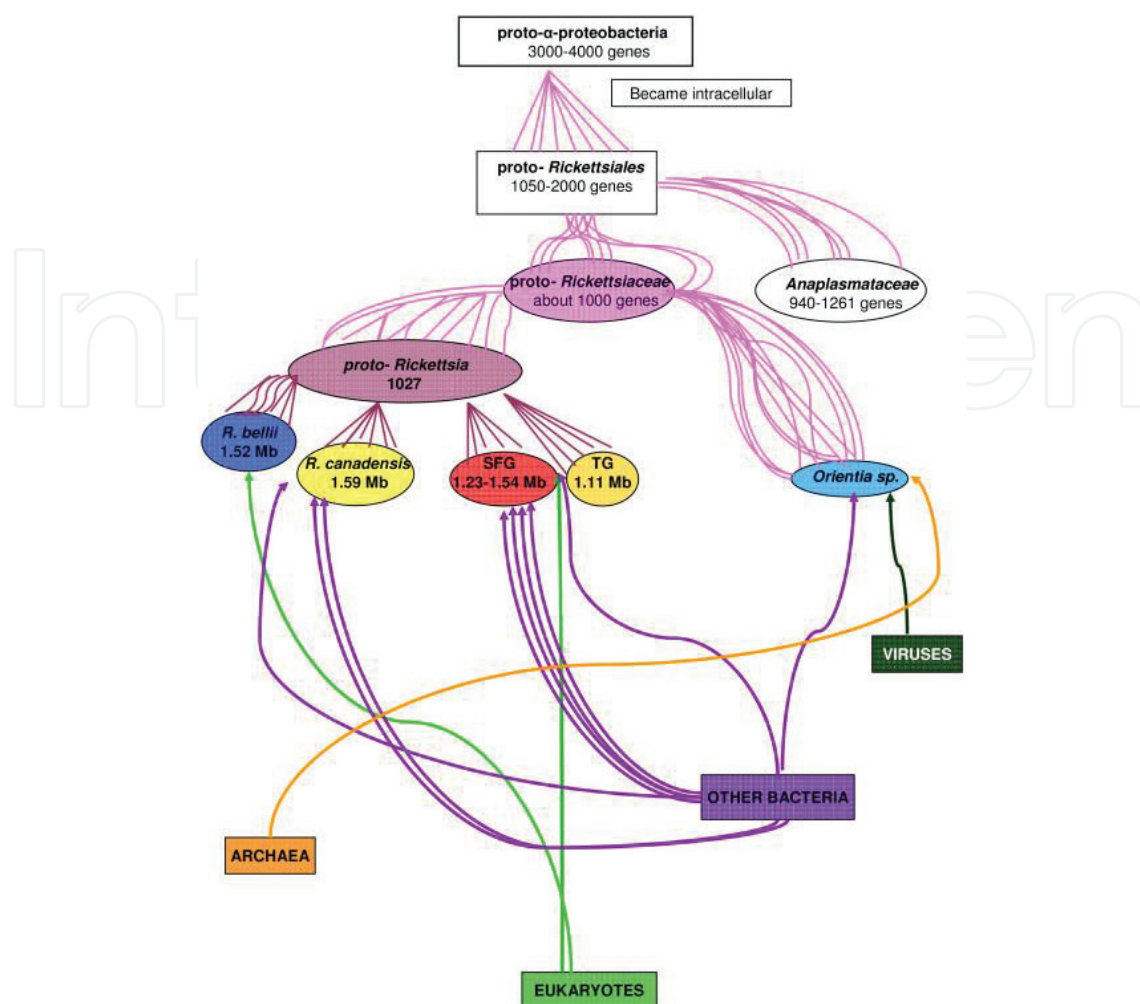
Figure 8. Probable gene gain events occurred in *Rickettsia* and *Orientia. Orientia* would have been gained genes from viruses, other bacteria and even archaea. Figure taken from [38].

3.3. Gene loss, gene gain, and evolution

Reductive evolution can be studied in members of *Rickettsiales*, because genome degradation is a process that occurs in members of this order. Gene loss has shaped the content of some *Rickettsiales* genomes, and horizontal gene transfer (HGT) has played an important role in the genome evolution of these bacteria [38].

An evolution study based on gene loss and HGT events in *Rickettsia* spp., *Anaplasma* spp. and *Orientia* spp. showed that three possible HGT event occurred from various organisms to *Orientia* and six events to *Rickettsia* spp., and three possible HGT event from *Rickettsia* and *Orientia* to other bacteria (**Figure 8**) [38].

Gene gain is a known event that has occurred throughout rickettsial evolution. In *O. tsutsugamushi* Ikeda one HGT event was identified and none in *O. tsutsugamushi* Boyrong. Many of the genes transferred by HGT were gained ancestrally, and include transposases and ankyrin repeat-containing proteins that appear to have been transferred from viruses and protist to *Orientia* species; the genes donated by *Orientia* were gained by *Firmicutes* spp., *Bacteroidetes* spp., and *Gamma-proteobacteria* spp. [38].

The whole genome analysis of *O. tsutsugamushi* Boryong has revealed the presence of type IV secretion system histidine kinases, SpoT, Tra, and ankyrin repeat- and tetratricopeptide repeat (TPR) containing proteins. Histidine kinases are proteins that act as sensor and signal transduction in response to changes in the environment; SpoT family proteins have a role in the response to energy starvation; Tra family proteins participate in gene transfer between rickettsia and other bacteria [39].

In a shotgun proteomics analysis using SDS-PAGE and LC-MSMS, many expressed proteins and the protein profiles were identified. 584 out of 1152 proteins of *O. tsutsugamushi* were identified by trypsin and Lys-C digestion and LC-MSMS, which corresponds to 49.4% proteins, annotated on the genome of the bacteria. It seems that during evolution the obligate intracellular bacteria lacked some proteins of important function (i.e., metabolism) and conserved proteins that allow them to survive in the host cells [39].

4. Conclusions

Rickettsiaceae family comprises widely distributed and genomically diverse microorganisms. Genome analysis of the members of the family has revealed an extraordinary evolution process throughout the time driven by the constant interactions with host cells and other bacteria. Recently, genomics analyses have revealed the presence of core genes in this family, as well as genes encoding proteins with significant function in *Orientia* spp.

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