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Gene Sequence-Based Criteria for Identification of New *Rickettsia* Isolates and Description of *Rickettsia heilongjiangensis* sp. nov.

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We propose genetic guidelines for the classification of rickettsial isolates at the genus, group, and species levels by using sequences of the 16S rRNA (*rrs*) gene and four protein-coding genes, the *gltA*, *ompA*, and *ompB* genes and gene D. To be classified as a member of the genus *Rickettsia*, an isolate should exhibit degrees of *rrs* and *gltA* homology with any of the 20 *Rickettsia* species studied of ≥98.1 and ≥86.5%, respectively. A member of the typhus group should fulfill at least two of the following four criteria: pairwise nucleotide sequence homologies with *rrs*, *gltA*, *ompB*, and gene D of either *Rickettsia typhi* or *Rickettsia prowazekii* of ≥99.4, ≥96.6, ≥92.4, and ≥91.6%, respectively. A member of the spotted fever group should either possess the *ompA* gene or fulfill at least two of the following four criteria: pairwise nucleotide sequence homologies with *rrs*, *gltA*, *ompB*, and gene D of any member of this group of ≥98.8, ≥92.7, ≥85.8, and ≥82.2%, respectively. The existence of a distinct "ancestral" group should be questioned. To be classified as a new *Rickettsia* species, an isolate should not exhibit more than one of the following degrees of nucleotide similarity with the most homologous validated species: ≥99.8 and ≥ 99.9% for the *rrs* and *gltA* genes, respectively, and, when amplifiable, ≥98.8, ≥99.2, and ≥99.3% for the *ompA* and *ompB* genes and gene D, respectively. By use of our classification scheme, "*Rickettsia heilongjiangii*" belongs to a new species for which we officially propose the name *Rickettsia heilongjiangensis* sp. nov.

The order *Rickettsiales* initially consisted of most of the bacteria associated with eukaryotic cells (63). On the basis of their 16S rRNA gene (*rrs*) sequences, *Rickettsiella grylli*, *Coxiella burnetii*, *Wolbachia persica*, *Rochalimaea* spp., *Grahamella* spp., *Bartonella* spp., *Eperithrozoon ovis*, and *Hemobartonella* spp. have been removed from the order *Rickettsiales* (5, 7, 41, 43, 62), which comprises only the genera *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*. Historically, the genus *Rickettsia* was divided into three groups on the basis of phenotypic criteria: the typhus group, the spotted fever group, and the scrub typhus group (63). In 1995, Tamura et al. (53) proposed reclassification of *Rickettsia tsutsugamushi* (33) into a new genus, *Orientia*, using 16S rRNA gene sequence analysis. The same year, Stothard and Fuerst (52) suggested that *Rickettsia bellii* (36) and *Rickettsia canadensis* (27) represented a phylogenetic line that predated the typhus-spotted fever group split and, thus, were included into a fourth group named the "ancestral" group. At present, the genus *Rickettsia* contains 21 validated species classified into three groups: (i) the ancestral group described above; (ii) the typhus group, which includes *Rickettsia prowazekii* (12) and *Rickettsia typhi* (34); and (iii) the spotted fever group, which consists of *Rickettsia rickettsii* (9), *Rickettsia conorii* (10), *Rickettsia africae* (20), *Rickettsia sibirica* (67), *Rickettsia slovaca* (48), *Rickettsia honei* (51), *Rickettsia japonica* (56), *Rickettsia australis* (35), *Rickettsia akari* (19), *Rickettsia felis* (6), *Rickettsia aeschlimannii* (1), *Rickettsia helvetica* (2), *Rickettsia massiliae* (3), *Rickettsia rhipicephali* (11), *Rickettsia montanensis* (63), *Rickettsia parkeri* (22), and *Rickettsia peacockii* (31). In addition to the 21 recognized species, more than 20 rickettsial isolates which have not been fully characterized or which have not received a species designation have been described, and the classification of these isolates is confusing (42).

The most widely accepted description of bacterial species is based on the results of DNA-DNA hybridization and the description of phenotypic characteristics in a polyphasic classification strategy (18, 61). However, when the 70% DNA-DNA relatedness cutoff (61) is applied to rickettsiae, *R. rickettsii*, *R. conorii*, *R. sibirica*, and *R. montanensis* would belong to the same species (28, 58); thus, classification of members of the genus *Rickettsia* is still based on the mouse serotyping method developed in 1978 (37). This test detects specific epitopes of the high-molecular-mass, surface-exposed protein antigens (rOmpA, rOmpB, and 120-kDa proteins) of rickettsiae. However, mouse immunization is not highly reproducible and requires a large amount of work because each new isolate must be compared to all previously described species. Therefore, new taxonomic methods for the classification of rickettsiae should be developed.

In recent years, several genes have been sequenced for most of the known rickettsial isolates, including the panbacterial genes encoding 16S rRNA (*rrs*) (44) and citrate synthase (*gltA*)

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(46) and the *Rickettsia*-specific *ompA* (16) and *ompB* (45) genes and gene D (47), which encode the surface-exposed, high-molecular-weight proteins rOmpA, rOmpB, and PS120, respectively. The usefulness of DNA taxonomy has been recognized for living organisms (54), and Maiden et al. (26) have demonstrated the usefulness of sequencing multiple genes for taxonomic purposes. The Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology (50) emphasized the need to sequence a minimum of five genes, including protein-coding genes. In an attempt to obtain gene sequence-based data for the classification of *Rickettsia* at various taxonomic levels, including the genus, group, and species levels, we compared the published sequences of the complete *rrs*, *gltA*, and *ompB* genes and gene D as well as the 5′ end of the *ompA* gene of all validated *Rickettsia* species except *R. peacockii*, for which the *rrs*, *gltA*, and *ompA* sequences available in GenBank are only partial and the *ompB* and gene D sequences are not available. Our aim was to set up objective guidelines that would allow any scientist to classify bacterial isolates as members or not members of the *Rickettsia* genus at various taxonomic levels.

MATERIALS AND METHODS

Selection, editing, and comparison of nucleotides sequences. We used the nucleotide sequences available in GenBank of the *rrs*, *gltA*, *ompA*, and *ompB* genes and gene D of both validated and as yet unclassified *Rickettsia* species and of one member of each of the genera *Orientia*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*, which, together with the genus *Rickettsia*, constitute the order *Rickettsiales*. Sequences were aligned by using the multiple-sequence alignment program CLUSTAL W (55). Sequences were edited by removal of fragments at the 5′ and 3′ ends so that their lengths matched that of the longest sequence common to all species compared. By comparison with *R. rickettsii*, we used the sequences of fragments between bases 17 and 1,424 for the *rrs* gene (GenBank accession number L36217), bases 87 and 1,134 for the *gltA* gene (GenBank accession number U59729), bases 1 and 590 for the 5′ end of the *ompA* gene (U43804), bases 296 and 5,141 for the *ompB* gene (GenBank accession number X16353), and bases 33 and 2,979 for gene D (GenBank accession number AF163000). In order to avoid misclassifications linked to deletions and/or insertions that were not inherited from common ancestors, as has been observed for the *ompA* gene among *R. conorii* strains (16), but that were caused by various events, including errors in DNA replication, only pairwise transitions and transversions between sequences, not deletions and insertions, were taken into account to calculate the degree of sequence homology (in percent).

In order to estimate the neutrality of the nucleotide sequence variations within the four protein-encoding genes used in this study, we used the *Z* test for large data sets (29) within the MEGA (version 2.1) software package (21). We used this test to estimate the neutral evolution of our sequences by calculation of the differences in synonymous (d_S) and nonsynonymous (d_N) substitutions among them. The variance of the differences between the average d_S and the average d_N was estimated by bootstrap analysis. A bootstrap value of <0.05 indicated that the gene did not undergo neutral evolution.

Calculation of cutoff values at genus, group, and species levels. To calculate cutoff values at the genus and group levels, we first calculated the mean sequence homology between species accepted as belonging to the genus *Rickettsia* or to each group within this genus on the basis of phenotypic criteria. For the genus and the spotted fever group means, the standard deviation (SD) was calculated. The cutoff was then defined as the mean less 3 SDs. Thus, a strain exhibiting a degree of homology at least 3 SDs lower than the mean sequence divergence between each pair of species belonging to a given group would be likely (with more than 99% probability) not to belong to that group.

To test the validity of the criteria established in this way, the entire similarity matrix that included each appropriate species for each gene was recalculated iteratively, with each accepted species omitted in succession. In this manner, 104 independent "omit" matrices were determined. The means and SDs of these new omit similarity matrices were calculated and used for comparison with the values for the sequences of the omitted species to determine whether it could be classified correctly. The sequences of several species had sufficient divergence that their inclusion skewed the validity of the comparisons with sequences and tests for several calculations, suggesting that their omission would result in more valid classification criteria. When the inclusive similarity matrices were then recalculated by omitting the values only for the outlying species that introduced bias for that specific gene alignment, the criteria were retested and found to more frequently predict the correct taxonomic assignment for accepted species, group, and genera. Thus, the data for the sequences of the outlying species were omitted from the final matrices and calculations used to determine inclusion or exclusion cutoff criteria.

The variance of the p distance was calculated for each of the five genes studied by using the MEGA (version 2.1) software package (21) and was used to estimate the normality of the distribution of sequence similarities among the *Rickettsia* species studied (30). A variance of <0.05 indicated that the similarity values were not normally distributed.

In order to validate each cutoff, we applied it to the pairwise sequence homology rates among all species used to establish the cutoff as well as the species used as outgroups. We determined the sensitivity of a cutoff for a given group by dividing the number of pairwise sequence homology rates that were above the cutoff among members of this group by the total number of pairwise comparisons within this group. Conversely, we calculated the specificity of this cutoff by dividing the number of pairwise sequence homology rates that were above the cutoff among the species used as outgroups by the total number of pairwise comparisons among these species. However, as each of the typhus and ancestral groups contained only two validated species, we used the homology rate between the two species as cutoffs and could not perform any omit test.

The inclusion of a bacterial isolate into the genus *Rickettsia* was based on the cutoffs for *rrs* and *gltA*, as only the sequences of these two genes were available for the outgroup species, i.e., *Orientia*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia* (Table 1). A bacterial isolate not fulfilling the genus criterion was not processed for further classification.

The inclusion of a rickettsial isolate within the spotted fever group was prioritized on the basis of the presence of the *ompA* gene, because this gene has been demonstrated to be specific for the spotted fever group rickettsiae (16). In cases in which *ompA* was not detected, a rickettsial isolate was classified within the spotted fever group on the basis of similarities in *rrs*, *gltA*, *ompB*, and gene D. The cutoffs defining inclusion in the typhus group were calculated by using the *rrs*, *gltA*, *ompB*, and gene D sequences. For the ancestral group, only the *rrs* and *gltA* gene sequences were used, as the *ompA*, *ompB*, and gene D sequences are not available for these species (16, 45, 47).

As too few sequences from a given gene were available for various strains of a given species to establish any reliable variability measure, we could not calculate cutoffs at the species level. Instead, we selected as a cutoff value for each gene the highest degree of pairwise nucleotide sequence similarity observed among the 20 recognized species. In order to estimate the validity of these criteria, we applied them to sequences available in GenBank from strains of validated species not used to establish the criteria.

Finally, in order to estimate the applicability of our genetic criteria, we applied them to seven rickettsiae previously classified as members of the spotted fever group, i.e., "*Rickettsia mongolotimonae*" (66), which is phylogenetically closely related to *R. sibirica* on the basis of genotypic and phenotypic criteria (66); BJ-90 (70), which is considered an *R. sibirica* strain or subspecies on the basis of genotypic data; strain S (14), which has not been found to be phenotypically and genotypically different enough from *R. africae* to be classified as a new species; two members of the *R. conorii* complex, Israeli spotted fever rickettsia (17) and Astrakhan fever rickettsia (15), both of which are considered at present to belong to the species *R. conorii* (59); Bar 29 (4), which is considered to belong to *R. massiliae*; and "*R. heilongjiangii*" (69), a Chinese strain most closely related to *R. japonica* but considered a separate species on the basis of epidemiological characteristics and the mouse serotyping assay.

PCR amplification and DNA sequencing of missing gene sequences. As the *ompB* sequence from BJ-90 and the gene D sequence from "*R. heilongjiangii*" were missing, we amplified and sequenced them using the primers and methods described previously (45, 47). As no DNA was available from "*Rickettsia hulinii*" (69), another Chinese strain for which the gene D sequence was also missing, we did not include it in our study.

Phylogenetic analysis. In order to compare the taxonomic classification obtained from our genetic criteria to that deduced from phylogenetic analysis, we inferred from the same sequence alignments the phylogenetic relationships among the rickettsiae studied using the maximum-parsimony and neighbor-joining methods within the MEGA (version 2.1) software package (21) and the maximum-likelihood method within the PHYLIP software package (40). Bootstrap replicates obtained from 100 trees were performed to estimate the node reliabilities of the phylogenetic trees obtained by the three methods (8).

TABLE 1. Accession numbers of gene sequences used in the present study and their original sources

Species	Strain	GenBank accession no.				
		16SrDNA	*gltA*	*ompA*	*ompB*	Gene D
Validated species						
R. prowazekii[a]	Breinl, ATCC VR-142T	M21789	M17149	NA[b]	AF123718	AF200340
R. typhi[a]	Wilmington, ATCC VR-144T	L36221	U59714	NA	L04661	AF188482
R. bellii[a]	369L42-1	L36103	U59716	NA	NA	NA
R. canadensis[a]	2678	L36104	U59713	NA	NA	NA
R. helvetica[a]	C9P9	L36212	U59723	NA	AF123725	AF163009
R. rickettsii[a]	R (Bitteroot), ATCC VR-891T	L36217	U59729	U43804	X16353	AF163000
R. conorii[a]	Seven (Malish), ATCC VR-613T	AF541999	U59730	U43806	AF123721	AF163008
R. africae[a]	ESF-5	L36098	U59733	U43790	AF123706	AF151724
R. sibirica[a]	246, ATCC VR-151T	L36218	U59734	U43807	AF123722	AF155057
R. honei[a]	TT-118, ATCC VR-599T	L36220	U59726	U43809	AF123724	AF163004
R. slovaca[a]	13-B	L36224	U59725	U43808	AF123723	AF155054
R. parkeri[a]	Maculatum 20	L36673	U59732	U43802	AF123717	AF155059
R. japonica[a]	YM	L36213	U59724	U43795	AF123713	AF155055
R. akari[a]	MK (Kaplan), ATCC VR-148T	L36099	U59717	NA	AF123707	AF213016
R. australis[a]	Phillips	L36101	U59718	AF149108	AF123709	AF187982
R. felis[a]	URRWXCal2, ATCC VR-1525	L28944	AF210692	AF210694	AF210695	AF196973
R. massiliae[a]	MtulT	L36214	U59719	U43799	AF123714	AF163003
R. montanensis[a]	M/5–6	L36215	U74756	U43801	AF123716	AF163002
R. rhipicephali[a]	3–7–6	L36216	U59721	U43803	AF123719	AF155053
R. aeschlimannii[a]	MC16T	U74757	U59722	U43800	AF123705	AF163005
Species used as outgroup at genus level						
Orientia tsutsugamushi	Gilliam	L36222	NA	NA	NA	NA
Anaplasma phagocytophilum	Webster	AY055469	AF304136	NA	NA	NA
Ehclichia chaffeensis	Arkansas	AF147752	AF304142	NA	NA	NA
Neorickettsia sennetsu	Miyayama	M73225	AF304148	NA	NA	NA
Wohlbachia pipientis	Isolate from *Folsomia candida*	AF179630	AF332584	NA	NA	NA
Strains used to test criteria at species level						
R. prowazekii	Madrid E	NA	AJ235273	NA	NA	NA
R. prowazekii	Virginia	NA	NA	NA	AF211821	NA
R. prowazekii	Florida	NA	NA	NA	AF211820	NA
R. conorii	Moroccan, ATCC VR-141	L36105	Identical to U59730	U45244	AF123721	AF163008
R. conorii	Indian tick typhus rickettsia, ATCC VR-597	L36107	Identical to U59730	U43794	AF123726	AF163005
R. massiliae	GS	NA	NA	U43793	NA	NA
R. honei	RB	NA	NA	NA	AF123711	NA
R. australis	PHS	NA	NA	AF149108	NA	NA
Unvalidated rickettsial strains						
"R. mongolotimonae"	HA91, ATCC VR-1526	L36219	U59731	U43796	AF123715	AF151725
BJ-90		AF178036	AF178035	AF179365	AY331393	AY331397
Strain S		U25042	U59735	U43805	AF123720	AF163001
Israeli spotted fever rickettsia	ISTT CDC1	L36223	U59727	U43797	AF123712	AF155058
Astrakhan fever rickettsia	A-167	L36100	U59728	U43791	AF123708	AF163007
Bar 29		L36102	U59720	U43792	AF123710	AF155056
"R. heilongjiangii"	054, ATCC VR-1524	AF178037	AF178034	AF179362	AY260451	AY331396

[a] Species used to establish the taxonomic criteria.
[b] NA, sequences not amplifiable.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA, *gltA*, *ompA*, *ompB*, and gene D genes of *R. heilongjiangensis* sp. nov. have been deposited in the GenBank database under accession numbers AF178037, AF178034, AF179362 (69), AY260451, and AY331396, respectively.

RESULTS

Nucleotide sequences. *R. peacockii*, the 21st validated *Rickettsia* species, was excluded from our analysis, as only incomplete sequences of *rrs*, *gltA*, and *ompA* and no *ompB* and gene D sequences were available in GenBank; and we did not have this species to determine the missing sequences. Following removal of deletions and insertions, nucleotide sequence fragments of 1,400 and 1,047 bp of the 16S rRNA and *gltA* genes, respectively, of the 20 valid *Rickettsia* species; one species of each of the genera *Orientia*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia*; and several unclassified *Rickettsia* strains were studied (Table 1). Nucleotide sequence fragments of 4,682 and 2,725 bp of the *ompB* gene and gene D, respectively,

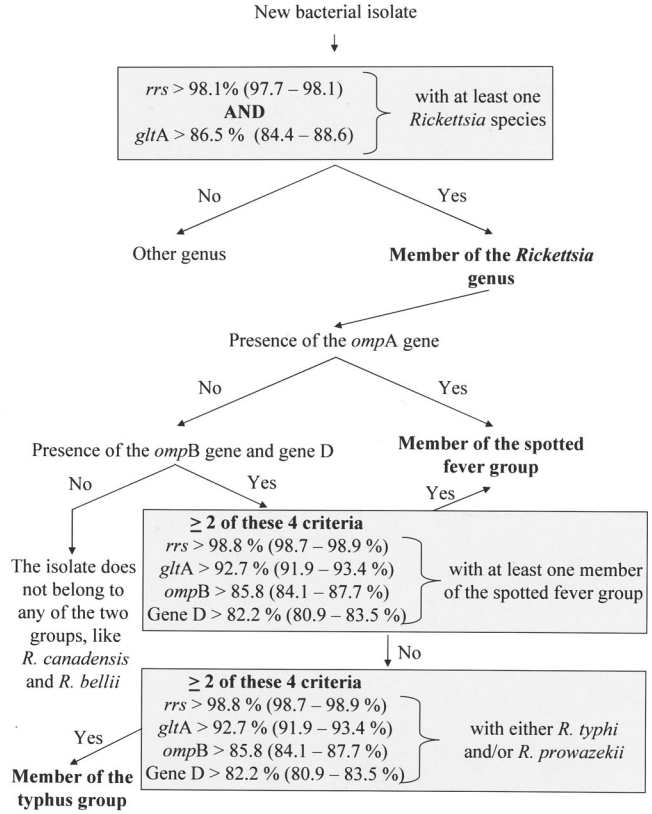

FIG. 1. Genotypic scheme for classification of the rickettsiae at the genus and group levels.

of 16 valid *Rickettsia* species of the spotted fever group, both valid species of the typhus group, and several unclassified *Rickettsia* strains were studied (Table 1). *ompB* and gene D sequences were not available for members of the ancestral group (45, 47). For *ompA*, we studied a fragment of 565 bp that was available for 14 valid *Rickettsia* species of the spotted fever group (Table 1). Although *R. akari* and *R. helvetica* are members of the spotted fever group, as well as members of the ancestral group, they were not included in the *ompA* sequence analysis, as this gene could not be identified in either species (16).

The variances of the differences between the average d_S and the average d_N values were 0.8, 0.2, 0.2, and 0.3 for the *gltA*, *ompA*, and *ompB* genes and gene D, respectively. The variances of the p distances were 0.048, 0.09, 0.08, 0.08, and 0.07 for the *rrs*, *gltA*, *ompA*, and *ompB* genes and gene D, respectively.

Determination of cutoff values at the genus level (Fig. 1). (i) *rrs*. Within the genus *Rickettsia*, the pairwise nucleotide sequence similarities ranged from 97.7% between *R. prowazekii* and *R. akari* to 99.8% between *R. sibirica* and *R. rickettsii* (Table 2). The mean level of nucleotide sequence similarity less 3 SDs among the 20 species was 97.9%. However, by using the omit test, *R. akari* was sufficiently different from the other species to be excluded from the complete analysis. Therefore, we recalculated the cutoff value as 98.1%. When this value was applied to the 20 *Rickettsia* species, it was validated for 168 of 180 similarity rates (sensitivity, 93.3%). None of the five outgroup species fulfilled this criterion with any of the 20 *Rickettsia* species (specificity, 100%) (Table 3).

TABLE 2. Pairwise nucleotide sequence similarities for each of the five genes studied among the three *Rickettsia* groups

Group and gene	% Similarity		
	Ancestral group	Typhus group	Spotted fever group
Ancestral			
rrs	98.9		
gltA	85.5		
Typhus			
rrs	97.8–98.4%	99.4	
gltA	85.7–89.9%	96.6	
ompB	NA[a]	92.5	
Gene D	NA	91.6	
Spotted fever			
rrs	97.6–99.3	97.7–98.8	97.9–99.8
gltA	85.9–92.2	90.3–93.2	93.3–99.9
ompA	NA	NA	55.4–98.8
ompB	NA	68.9–83.0	72.4–99.2
Gene D	NA	77.0–81.2	85.4–99.3

[a] NA, not applicable.

(ii) *gltA*. Within the genus *Rickettsia*, the pairwise nucleotide sequence similarities ranged from 85.5% between *R. canadensis* and *R. bellii* to 99.9% between *R. parkeri* and *R. sibirica* (Table 2). The mean level of nucleotide sequence similarity less 3 SDs among the 20 species was 86.5%. The omit test showed a high degree of homogeneity of the *gltA* sequences among the 20 species. When this cutoff was applied to the 20 *Rickettsia* species and the 4 outgroup species, it had a sensitivity of 173 of 180 (96.1%) and a specificity of 84 of 84 (100%) (Table 3).

Determination of cutoff values at the group level (Fig. 1). (i) Ancestral group. Members of the ancestral group exhibited degrees of pairwise nucleotide sequence similarity of 98.9 and 85.5% for the *rrs* and *gltA* genes, respectively (Table 2). These cutoffs had specificities of 25 of 36 (69.4%) and 0 of 36 (0%), respectively.

(ii) Typhus group. The typhus group was characterized by degrees of pairwise nucleotide sequence similarity of 99.4, 96.6, 92.4, and 91.6% for the *rrs*, *gltA*, and *ompB* genes and gene D, respectively. These cutoff values had specificities of 36 of 36 (100%), 36 of 36 (100%), 32 of 32 (100%), and 32 of 32 (100%) for the *rrs*, *gltA*, and *ompB* genes and gene D, respectively.

(iii) Spotted fever group. Within the spotted fever group, the degrees of pairwise nucleotide sequence similarity ranged from 97.9% between *R. akari* and *R. parkeri* to 99.8% between *R. conorii* and *R. sibirica* for the *rrs* gene; from 93.3% between *R. akari* and *R. honei* to 99.9% between *R. parkeri* and *R. sibirica* for the *gltA* gene; from 72.4% between *R. akari* and *R. helvetica* to 99.2% between *R. africae* and *R. sibirica*, *R. parkeri*, or *R. slovaca* for the *ompB* gene; and from 84.5% between *R. rickettsii* and *R. akari* to 99.3% between *R. conorii* and *R. slovaca* for gene D (Table 2). The mean level of *rrs* (without *R. akari*) nucleotide sequence similarity less 3 SDs among the 16 spotted fever group species was 98.8%. This cutoff had a sensitivity of

TABLE 3. Pairwise nucleotide sequence similarities of species of the genera *Orientia*, *Anaplasma*, *Ehrlichia*, *Wolbachia*, *Neorickettsia*, and strains of validated species not used to calculate the taxonomic criteria by comparison with validated *Rickettsia* species

Species	Strain	Gene	Range of % Pairwise nucleotide sequence similarity
O. tsutsugamushi	Gilliam	*rrs*	90.2–91.0
A. phagocytophilum	Webster	*rrs*	83.8–83.9
		gltA	56.6–58.1
E. chaffeensis	Arkansas	*rrs*	82.5–83.3
		gltA	60.6–63.1
W. pipientis	Isolate from *F. candida*	*rrs*	83.1–84.0
		gltA	62.1–63.4
N. sennetsu	Miyayama	*rrs*	82.5–83.1
		gltA	54.9–56.9
R. prowazekii	Madrid E	*gltA*	85.6–99.9
	Virginia	*ompB*	69.0–99.8
	Florida	*ompB*	69.1–99.9
R. conorii	Moroccan	*rrs*	98.3–100
		gltA	86.9–100
		ompA	57.0–100
		ompB	75.4–100
		Gene D	80.0–100
	Indian tick typhus rickettsia	*rrs*	98.3–100
		gltA	87.0–100
		ompA	57.0–99.8
		ompB	75.4–99.8
		Gene D	79.9–99.9
R. massiliae	GS	*ompA*	57.3–100
R. honei	RB	*ompB*	75.5–99.8
R. australis	PHS	*ompA*	55.4–99.9

95 of 112 (84.8%) and a specificity of 50 of 64 (78.1%). The mean level of *gltA* nucleotide sequence similarity less 3 SDs among the 16 spotted fever group species was 92.7%. This cutoff had a sensitivity of 112 of 112 (100%) and a specificity of 40 of 64 (68.7%). The mean level of *ompB* nucleotide sequence similarity less 3 SDs among the 16 spotted fever group species was 76.7%. However, by the omit test, *R. helvetica* was different enough from the other species to be excluded from the analysis. Therefore, we recalculated the cutoff value as 85.8%. All spotted fever group species except *R. helvetica* fulfilled this criterion (sensitivity, 97 of 112 [86.6%]), and the specificity was 32 of 32 (100%). The mean level of gene D nucleotide sequence similarity less 3 SDs among the 16 spotted fever group species was 82.2%. The omit test showed a high level of homogeneity of the gene D sequences among the 16 species. All spotted fever group species fulfilled this criterion (sensitivity, 112 of 112 [100%]), and the specificity was 32 of 32 (100%).

Application of genus and group criteria to valid species. When the combination of the genus and group criteria (Fig. 1) was applied to the 20 valid *Rickettsia* species and 5 outgroup species, all of them were correctly classified (sensitivity and specificity, 100%).

Determination of cutoff values at the species level. The highest pairwise nucleotide sequence similarity rates among the 20 validated species were 99.8, 99.9, 98.8, 99.2, and 99.3% for the *rrs*, *gltA*, *ompA*, and *ompB* genes and gene D, respectively (Table 2). All strains of validated species that were not used in the analysis exhibited levels of nucleotide sequence similarity to other strains of their respective species equal to or higher than these cutoffs (Table 3).

Application of the criteria to *R. akari*. As *R. akari* was excluded from the calculation of the *rrs* cutoff and was not included in the determination of the *ompA* cutoff, we applied our criteria to this species to estimate their validity for this species. The nucleotide sequences of the *rrs* and *gltA* genes of *R. akari* exhibited similarity rates ranging from 97.7 to 98.6% and 85.9 to 97.0%, respectively, to those of the other 19 valid *Rickettsia* species. These values classified *R. akari* within the genus *Rickettsia*. At the group level, this species was classified within the spotted fever group (Table 4), and at the species level it was considered a separate species.

Application of the taxonomic criteria to unvalidated species. By using the taxonomic criteria, all seven unvalidated species studied belonged to the genus *Rickettsia* and to a single group, the spotted fever group (Table 4). "*R. mongolotimonae*" and BJ-90 belonged to the species *R. sibirica* on the basis of four and five validated cutoffs, respectively. Strain S belonged to the species *R. africae* on the basis of two validated cutoffs. Israeli tick typhus rickettsia and Astrakhan fever rickettsia belonged to the species *R. conorii* each on the basis of two validated cutoffs. Strain Bar 29 belonged to the species *R. massiliae* on the basis of three validated cutoffs. "*R. heilongjiangii*" fulfilled only one cutoff and, thus, was classified as a separate species.

Comparison of sequence-based criteria and phylogeny. The phylogenetic classification at the genus level matched our findings perfectly (Fig. 2), as none of the rickettsiae studied clustered with any of the bacteria used as outgroups for the *rrs* and *gltA* genes. At the group level, we observed a discrepancy in the position of the ancestral group between the analyses of these two genes, since, by using *rrs*, the typhus group was the ancestor of the ancestral group, whereas the opposite result was observed by using *gltA* sequences. At the species level, all rickettsiae closely related to *R. conorii* formed a homogeneous cluster together in analyses with all genes except *rrs*, with which *R. parkeri* grouped with *R. conorii*, and *gltA*, with which the Astrakhan fever rickettsia was grouped with *R. honei*. This group was supported by high bootstrap values for all genes except the *rrs* and *gltA* genes. "*R. mongolotimonae*" and BJ-90 formed a reliable cluster with *R. sibirica* in analyses with all genes. Likewise, Bar 29 was reliably associated with *R. massiliae* in analyses with all five genes. "*R. heilongjiangii*" was grouped with *R. japonica* with high bootstrap values in analyses with all five genes. However, it was impossible to deduce from the phylogenetic analysis that "*R. heilongjiangii*" was a strain of a species distinct from *R. japonica* (Fig. 2). In addition, it was not clear from the phylogenetic analysis to which species strain S belonged, as it was grouped with *R. africae*, as expected from our results, in analyses with the *rrs* gene and gene D and with the *R. sibirica* group in analyses with the *gltA* and *ompA* genes.

TABLE 4. Pairwise nucleotide sequence similarities for various rickettsial isolates with each of the three *Rickettsia* groups and with the 20 validated species

Group and gene	% Similarity			% Pairwise similarity with phylogenetically closest validated species[a]
	Ancestral group	Typhus group	Spotted fever group	
R. akari				
rrs	97.6–98.3	97.7–97.9	98.1–98.6	98.6
gltA	85.9–89.3	90.9–91.2	93.3–97.0	97.0
ompB	NA[b]	80.0–80.3	86.1–94.2	94.2
Gene D	NA	77.6–77.8	84.5–94.9	94.9
"R. mongolotimonae"				
rrs	98.3–99.0	98.2–98.3	98.0–100	100
gltA	87.0–91.5	92.8–93.0	94.0–99.8	99.8
ompA	NA	NA	59.1–99.8	99.0
ompB	NA	82.1–82.4	76.9–99.6	99.6
Gene D	NA	80.5–80.6	84.0–99.4	99.4
BJ-90				
rrs	98.1–98.9	98.1–98.2	97.8–99.8	99.8
gltA	87.2–91.7	93.0–93.1	94.2–100	100
ompA	NA	NA	58.8–99.8	99.8
ompB	NA	82.1–82.4	86.1–99.7	99.7
Gene D	NA	80.8–81.6	84.9–99.9	99.9
Strain S				
rrs	98.2–99.0	98.2–98.3	98.1–99.8	99.8
gltA	87.1–91.6	92.7–93.0	94.0–99.8	99.4
ompA	NA	NA	59.1–99.5	99.5
ompB	NA	82.0–82.5	77.0–99.0	99.0
Gene D	NA	80.0–80.5	83.6–99.2	99.2
Israeli spotted fever rickettsia				
rrs	98.3–99.0	98.3–98.4	98.0–99.8	99.8
gltA	86.7–91.5	92.7–93.0	94.0–99.8	99.3
ompA	NA	NA	58.6–99.3	98.3
ompB	NA	81.8–82.1	76.7–98.6	98.6
Gene D	NA	80.2–80.6	84.0–99.3	99.3
Astrakhan fever rickettsia				
rrs	98.3–99.0	98.3–98.5	98.3–99.9	99.9
gltA	86.9–91.6	92.9–93.0	94.1–99.5	99.5
ompA	NA	NA	58.6–98.4	98.3
ompB	NA	81.9–82.3	76.8–98.7	98.7
Gene D	NA	80.3–80.8	84.0–99.4	99.4
Bar 29				
rrs	98.3–99.0	98.2–98.3	98.3–99.9	99.9
gltA	87.0–91.5	92.8–93.0	94.0–99.8	99.8
ompA	NA	NA	58.1–99.5	99.5
ompB	NA	82.5–82.8	77.3–99.4	99.4
Gene D	NA	79.8–80.3	84.0–98.7	98.7
"R. heilongjiangii"				
rrs	96.5–97.0	96.7	96.2–98.0	98.0
gltA	86.9–91.6	93.0	93.9–99.6	99.6
ompA	NA	NA	58.8–97.2	97.2
ompB	NA	82.4–82.7	87.2–98.8	98.8
Gene D	NA	81.0–81.3	85.1–99.4	99.4

[a] The species to which each of the seven unvalidated species studied belonged were *R. australis* for *R. akari*, *R. sibirica* for "*R. mongolotimonae*" and BJ-90, *R. africae* for strain S, *R. conorii* seven for Israeli spotted fever and Astrakhan fever rickettsiae, *R. massiliae* for Bar 29, and *R. japonica* for "*R. Leilongjiangii.*"

[b] NA, not applicable.

DISCUSSION

We propose gene sequence-based criteria for the taxonomic classification of rickettsial isolates at the genus, group, and species levels. To date, attempts to define a species among strictly intracellular bacteria have been very difficult. The application of the phenotypic characteristics used for extracellular bacteria to the order *Rickettsiales* is limited since few are expressed by these bacteria. Rickettsiologists have demonstrated that the 70% DNA-DNA homology criterion (61) does

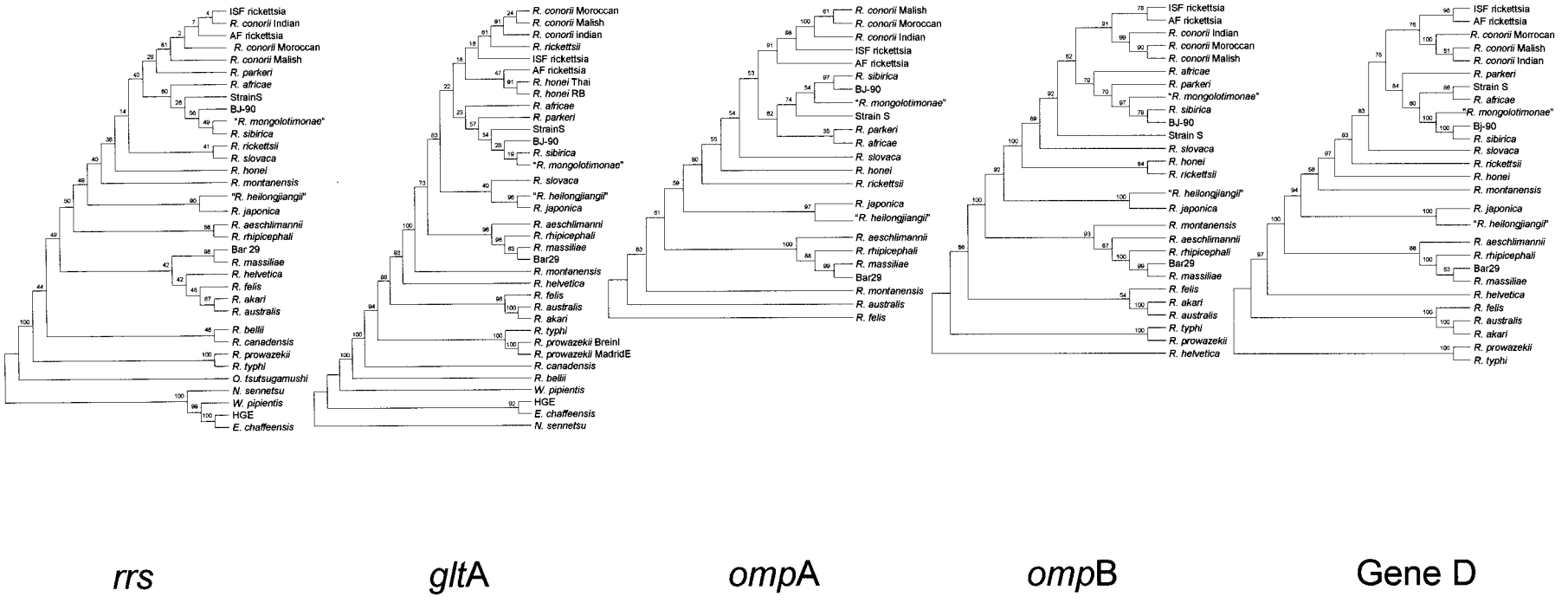

FIG. 2. Unrooted trees showing the phylogenetic relationships among the rickettsiae studied, as inferred from sequence analysis of each of the five genes by the maximum-parsimony method. Bootstrap values are indicated at the nodes. ISF rickettsia, Israeli spotted fever rickettsia; AF rickettsia, Astrakhan fever rickettsia; HGE, the former human granulocytic ehrlichiosis agent (*Anaplasma phagocytophilum*).

not adequately differentiate rickettsiae. Among the classical laboratory methods for the identification rickettsiae, cross immunity and vaccine protection tests with guinea pigs (13), complement fixation tests with washed specific particulate antigens (38), and mouse toxin neutralization tests (22) have largely been superceded by mouse serotyping assays for the spotted fever group rickettsiae (37). Although monoclonal antibodies are helpful (60, 65), they are limited by the lack of an accepted standardized panel directed to all known isolates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and pulsed-field gel electrophoresis (PFGE) have proven to be useful for differentiating rickettsiae, but their values are limited because of the variations in the molecular weights of rOmpA and rOmpB within species for SDS-PAGE and the absence of any database allowing the comparison of profiles for PFGE.

Recently, a number of genes, including those encoding 16S rRNA (*rrs*), citrate synthase (*gltA*), the 17-kDa common antigen, and surface-exposed, high-molecular-weight antigenic proteins of the *sca* family (*ompA*, *ompB*, gene D) (32), have been used to rapidly differentiate spotted fever group rickettsiae either by analysis of restriction fragment length polymorphisms of PCR products or by direct sequence determination (16, 45–47). The usefulness of these genes for taxonomic purposes was demonstrated by the valid description of two new, at that time, uncultured *Rickettsia* species, *R. felis* (6) and *R. peacockii* (31), mainly on the basis of genotypic criteria. The classification of these two rickettsiae was later confirmed by the study of isolates in vitro (23, 39, 49). Despite these examples and the demonstration that nucleotide sequence-based taxonomy was more discriminative than taxonomy based on phenotypic characteristics, no formal guidelines have been accepted among rickettsiologists for the use of this tool in classifying rickettsial isolates. Polyphasic taxonomy, which integrates phenotypic, genotypic, and phylogenetic data, has proven to be very useful for the taxonomic classification of several bacterial genera (57). For that objective, multilocus sequencing, which reduces the risk of error caused by lateral gene transfer, has been demonstrated to be suitable for the taxonomic classification of bacteria (26). Recently, La Scola et al. (24) have demonstrated that gene sequence-based criteria are useful for species definition within the genus *Bartonella*. However, multilocus sequencing must use genes that are undergoing neutral selection (50). In our study, the *Z* test demonstrated that the four protein-coding genes were undergoing stabilizing selection, thus confirming their suitability for our study. In addition, we estimated the normality of the distribution of similarity values used to infer our criteria for each gene. With the exception of the *rrs* gene, which is highly conserved among spotted fever group rickettsiae, the test showed that our data set was normally distributed and thus constituted a reliable basis on which to establish our criteria.

We propose sequence-based guidelines for the classification of new rickettsial isolates (Fig. 1). These criteria can be applied to a bacterium only if the sequences of the gene fragments used to establish the guidelines have been determined for this isolate. We are aware that these guidelines may later be updated by the introduction of additional genetic and/or phenotypic characteristics and of new *Rickettsia* species. Using the *rrs* gene, we determined that a rickettsia-like organism belongs to the *Rickettsia* genus if it exhibits a nucleotide sequence homology of ≥98.1% with any of the recognized species. The *rrs* gene has been proposed to be the best tool for the classification of prokaryotic taxa at the genus level (50). In addition, we also determined a cutoff value of ≥86.5% for the *gltA* gene, which is widely present among bacteria. Both the *rrs* and the *gltA* cutoffs were validated by using both reiterative omission tests and comparisons with five genera most closely related to *Rickettsia*. Both values were supported by elevated sensitivities and specificities. To belong to the genus *Rickettsia* a bacterium should exhibit degrees of *rrs* and *gltA* homology equal to or higher than our cutoffs with at least 1 of the 20 validated *Rickettsia* species.

Within the genus *Rickettsia*, we determined cutoff values to allow the classification of rickettsial isolates in the different groups. These taxonomic criteria for the classification at the group level were based on five genes for 14 of the 20 (70%) species, four genes for an additional 4 species (20%), and two genes for only 2 species (10%). To belong to the typhus group, an isolate should exhibit pairwise nucleotide sequence similarity with the sequence of either *R. prowazekii* or *R. typhi* with at least two of the following four cutoffs: ≥99.4, 96.6, 92.5, and 91.6% for *rrs*, *gltA*, *ompB*, and gene D, respectively. The presence of an *ompA* gene warrants classification of a rickettsial isolate into the spotted fever group (16). However, lack of detection of this gene does not exclude an isolate from the spotted fever group. Therefore, for *ompA*-negative isolates, a minimum of two of the following four cutoffs for homology with any member of the spotted fever group warrants inclusion in the spotted fever group: ≥98.8, 92.7, 85.8, and 82.2% for *rrs*, *gltA*, *ompB*, and gene D, respectively. The specificities obtained for the *rrs* and *gltA* genes were low, which may be because both genes are highly conserved (44, 46). In contrast, the sensitivities and specificities for the *ompB* and gene D cutoffs were elevated. For the ancestral group, both cutoff values exhibited low specificities, which may be due to either the high conservation of these genes or the heterogeneity of this group, which initially comprised two rickettsial species which could not be classified into either the typhus or the spotted fever group but which are also clearly different from each other (52). Therefore, we believe that *R. bellii* and *R. canadensis* do not belong to a single group but may be representatives of two distinct groups.

In order to classify a rickettsial isolate as a new species, we propose that candidates possess no more than one value above the following nucleotide sequence similarities by comparison with any validated *Rickettsia* species: ≥99.8 and 99.9% for the 16S rDNA and *gltA* genes, respectively, and, when available ≥98.8, 99.2, and 99.3% for the *ompA* and *ompB* genes and gene D, respectively. Our criteria should not be used for the official description of a rickettsia as a new species if no established isolate is available. By use of these criteria, eight strains of validated *Rickettsia* species were accurately classified.

When our taxonomic scheme was applied to *R. akari*, this species was correctly classified, despite its removal from a part of the analysis, thus strengthening our guidelines. In addition, the application of our criteria to *R. peacockii* by use of the similarity rates described by Niebylski et al. (31), i.e., 99.7 and 93.2% for *rrs* and *ompA*, respectively, with *R. rickettsii* would

classify this rickettsia into the spotted fever group as a separate species.

When our taxonomic scheme was applied to seven rickettsial strains not previously officially classified, all of them were correctly classified into the spotted fever group within the genus *Rickettsia*. "*R. mongolotimonae*" and BJ-90 belonged to the species *R. sibirica*, strain S belonged to the species *R. africae*, the Israeli tick typhus and Astrakhan fever rickettsiae belonged to the species *R. conorii*, and Bar 29 belonged to the species *R. massiliae*. Our results for all strains except strain S were consistent with those obtained from the phylogenetic analysis. The phylogenetic position of strain S varied depending on the gene. Moreover, although the phylogenetic approach was valuable in grouping closely related rickettsiae, often there was no possibility to determine the species status of some rickettsiae, as was shown for "*R. heilongjiangii*."

Our results confirm the previously established taxonomic classification of seven strains whose taxonomic status is not yet established. "*R. heilongjiangii*" exhibited low rates of similarity to *R. japonica*, which prevented its classification in this species. In 1982, "*R. heilongjiangii*" was first isolated as the Heilongjiang isolate or strain 054 from *Dermacentor silvarum* ticks collected in the city of Suifenhe in Heilongjiang Province of northeastern China (25). It was later isolated from patients with symptoms consistent with a spotted fever rickettsiosis in Suifenhe (64). On the basis of phenotypic and genotypic analyses, this strain was previously proposed to be a new species (64, 68). Our data demonstrate that this rickettsia belongs to a new species. Thus, we formally propose the creation of *Rickettsia heilongjiangensis* sp. nov., which contains strain heilongjiangii or strain 054 (type strain).

In conclusion, we propose objective, gene sequence-based guidelines for the taxonomic classification of new rickettsial isolates. These guidelines are designed mostly for use with sequenced amplified genes or gene fragments. Thus, absence of detection is not equivalent to absence of the gene, a concept accommodated by the criteria. Moreover, they are time-saving, do not require the availability of all known rickettsial strains for the classification of a new isolate, and are not subject to reproducibility problems such as those encountered with other methods, in particular, mouse serotyping assays. In addition, we propose the creation of one new species: *R. heilongjiangensis* sp. nov.

Description of *Rickettsia heilongjiangensis* sp. nov. *Rickettsia heilongjiangensis* (hei.long.iang.en′sis. N.L. gen. n. *heilongjiangensis*, from Heilongjiang, the Chinese province where the *D. silvarum* tick providing the first isolate was collected [25]), is an obligate gram-negative intracellular bacterium. It grows on Vero cells at 32°C in minimal essential medium supplemented with 2% fetal calf serum and 2 mg of L-glutamine per ml. It is nonmotile. Pathogenicity for humans has been demonstrated. It is transmitted to humans through the bite of *D. silvarum* ticks.

The type strain is strain 054, which was isolated from *D. silvarum* ticks in 1982 in the city of Suifenhe in the Chinese province of Heilongjiang (25). Type strain 054 has been deposited in the American Type Culture Collection under the reference designation ATCC VR-1524 and in the Collection of the World Health Organization Collaborative Center for Rickettsioses, Borrelioses and Tick-Borne Infections, Marseille, France.

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